Nrf2 in Host Defense:
Over the Rainbow

Guest Editors: Hye-Youn Cho, Mi-Kyoung Kwak, and Jingbo Pi
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Editorial
Nrf2 in Host Defense: Over the Rainbow

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Nuclear factor (erythroid-derived 2)-like 2 or NF-E2-related factor 2 (NRF2 or Nrf2 for rodents), was discovered about two decades ago and has been known as a key transcriptional regulator of antioxidant response element (ARE) bearing cytoprotective genes. Extensive investigation of NRF2 in host defense has been paralleled with increasing interests in oxidative stress that is a common occurrence in various critical disease conditions. A broad spectrum of NRF2 functions has been uncovered in clinical settings as well as in model diseases using gene-specific knockout mice or diverse cells and organs insulted by various oxidative stresses. Kelch-like ECH-associated protein 1 (KEAP1 or Keap1 for rodents) is a cytoplasmic suppressor of NRF2 and a critical modulator of NRF2 homeostasis and activity. Substantial discovery efforts into the molecular mechanisms of the KEAP1-NRF2 axis have found that stress-induced modifications of KEAP1 and NRF2 liberate NRF2 from the “hinge and latch”-like affinity binding of NRF2-KEAP1 [1], allowing nuclear accumulation and transactivation of NRF2. In normal physiologic conditions, however, NRF2 homeostasis is maintained by an ubiquitin ligase (Cullin 3-based E3 ligase) attached to the NRF2-bound KEAP1 homodimer, which tags NRF2 for proteasomal degradation. Interestingly, Nrf2 has been shown to induce specific catalytic subunits of the proteasome (e.g., murine Psmb5 and Psmb6 in the 20s proteasome) directly through ARE binding or indirectly [2]. While the lysosomal system is the principal mechanism for degrading proteins with long half-lives, the ubiquitin-proteasome system maintains protein quality by endoplasmic reticulum-associated degradation of misfolded or damaged proteins (e.g., oxidized proteins) as well as selective degradation of short-lived and regulatory proteins.

In conjunction with the evidence of ARE-mediated NRF2 autoregulation [3, 4], it indicates that NRF2 homeostasis is maintained and adjusted by a tight regulatory circuit according to redox and proteolytic demands.

NRF2 is known to subsidize host defense through involvement in complex pathways including thiol and other antioxidant activation as well as cell cycle and death, metabolism, immunity, selective protein degradation, development, and carcinogenesis. Potent phytochemical NRF2 agonists such as isothiocyanates including broccoli-originated sulforaphane have been widely applied to experimental systems to support the role for NRF2. In this special issue, fifteen research articles demonstrate molecular, cellular, and physiological aspects of NRF2- and ARE-mediated downstream mechanisms in various cells and tissues, many of which utilized gene knockout mice or their primary cells, NRF2 agonists or proteasome inhibitors, and NRF2 or KEAP1 gene silencing techniques. In addition, nine review articles highlight topics on recent advancements in NRF2 research, chemoprevention, immunity, metabolic disease, and genetic variation.

NRF2 is highly expressed in organs such as liver, kidney, and lung that undergo routine detoxification processes. A number of contributions in this issue deal with the protective role of NRF2 in chemical-induced liver toxicity. A “graded Nrf2 activation model” is tested in mice by C. D. Klaassen and colleagues and elucidates an Nrf2 dose-dependent protection against various hepatotoxins in mice. Y. Kumagai et al. summarize the findings of their own and by others on the S-mercuration of cellular proteins by methylmercury and the important protective role of the KEAP1/NRF2 pathway. S. I.
Gum and Cho demonstrate the herbal medicine component ginsenoside (Rg3) as a potent inducer of Nrf2 and ARE-responsive multidrug resistance-associated proteins in the acetaminophen-induced hepatotoxicity model. M. K. Liu et al. suggest a coordinated nuclear export of BACH1 and nuclear accumulation of Nrf2 in liver cells insulted by arsenic. A thorough review by Y. Shin et al. covers potential mechanisms and Nrf2 targets in various models of liver diseases including viral hepatitis and hepatocarcinomas in mice.

Diabetes is one of the major causes of chronic kidney disease worldwide. One therapeutic approach to diabetic nephropathy is the use of compounds inducing cytoprotective genes as oxidative stress known to be implicated. Three articles by L. Cai and colleagues demonstrate that treatment with low-dose radiation (25 milligray daily for 3 days), sulforaphane (3 month), or a peptide aldehyde proteasome inhibitor (MG132) is effective in protection against diabetic nephropathy in mice, which is, at least in part, attributed to its action through Nrf2-ARE responses. Supporting these, murine Nrf2 has been known to have an essential role in metabolic disorders by controlling the capacity of white adipose tissue expansion, insulin sensitivity, and glucose and lipid homeostasis [5]. It is also known that Nrf2 protects mice from high-fat-diet-induced obesity and insulin resistance. D. V. Chartoumpekis et al. profile hepatic transcriptome changes in high-fat-diet-induced obese mice. Their data ascertain that Nrf2 is involved in metabolic gene networks including bile acid synthesis from cholesterol, free fatty acid binding and transport, glucose metabolism, and glycerol transport in liver.

In murine heart, Nrf2 deletion significantly increased susceptibility to environmental pollutants (ozone, particle) through changes in cardiac functions as demonstrated in an original research article by R. Howden et al. A beneficial contribution of Nrf2 in the heart transplantation model is suggested by K. C. Wu et al.: Nrf2-deficient (Nrf2−/−) recipient mice did not support graft survival for longer than 7.5 days, while wild-type (Nrf2+/+) hosts showed prolonged graft survival especially with sulforaphane treatment. Evidence suggests that Nrf2 activity does not always lead to a positive outcome and may accelerate the pathogenesis of some cardiovascular diseases. For example, Nrf2 is found to promote atherosclerosis in animal models (e.g., apolipoprotein E mutant mice). Association of antioxidant defenses in cardiovascular diseases such as atherosclerosis, hypertension, heart failure, and ischemia-reperfusion injury is addressed in a review by R. Howden.

The detrimental effects of NRF2 due to its aberrant activation have also been highlighted in recent years. Investigation in Keap1-deficient mice demonstrated uncontrolled accumulation of cytoplasmic Nrf2, which caused constitutive transactivation of Nrf2 and overproduction of downstream target genes leading to lethality associated with esophageal and forestomach hyperkeratosis [6]. Importantly, considerable investigations on biopsies of non-small-cell lung cancers (squamous cell carcinoma, large cell carcinoma, and adenocarcinoma) have indicated that frequent somatic (missense) mutations in KEAP1 and/or NRF2 are associated with persistent transactivation of Nrf2 in metastatic cells. As a result, uncontrolled, overexpression of cytoprotective genes including drug efflux pumps is proposed to give selective growth advantage and chemoresistance of the metastatic cells. Comprehensive genome analyses also reported that the NRF2/KEAP1/CUL3 pathway is one of the four most frequently mutated ones in human lung squamous cell carcinomas [7]. As discussed in a review by A. K. Bauer et al., these critical findings are controversial as to whether activation, or alternatively inhibition, of NRF2 is a strategy for the prevention or treatment of cancer. It implies that KEAPI may be a potential “tumor suppressor” gene, while NRF2 may conversely be “oncogenic” in chemotherapy-resistant cancers, and effective personalized therapy may be warranted in patients with mutations. A research article by K.-A. Jung and M.-K. Kwak demonstrates a similar notion that KEAPI silencing enhances ARE-mediated aldo-keto reductase expressions and decreases cytotoxicity of colon cancer cells. A review by H.-Y. Cho profiles sequence variations including single nucleotide polymorphisms and somatic mutations discovered in human NRF2 and murine Nrf2 along with details on the genomic structure of Nrf2 and its homology to other vertebrate species. This review article compiles genetic and somatic mutations in association with disease risks including cancer metastases.

While genetic and somatic mutations of NRF2 are significantly relevant to the adverse consequences of neoplastic cells, preventative roles of Nrf2 toward experimentally-induced tumorigenesis have been demonstrated in diverse mouse tissues including colon, forestomach, gall bladder, and skin. E. Kobayashi et al. mention in a review that in the case of a tumor development model, Nrf2 controls phagocytosis, acute inflammation, and reactive oxygen species generation that are required for T cell suppression, whereby Nrf2 supports antitumor immunity and reduces tumor metastasis in myeloid-derived suppressor cells. The anticarcinogenic activity and specific targets of isothiocyanates are discussed by B. N. Das et al. in the current issue. Bioavailability of oral sulforaphane in various tissues has been determined recently [8], providing insights into its efficacy as a host-defense and chemopreventive agent. Increasing evidence indicates favorable roles for NRF2-ARE activation in neuronal disorders. C. Lee et al. demonstrate that β-amyloid peptide-induced brain cell apoptosis and toxicity is inhibited by sulforaphane, suggesting a potential intervention for Alzheimer’s disease. R. Zhao et al. demonstrate in their paper a protective effect of another phytochemical antioxidant curcumin in keratinocytes against the cytotoxicity caused by a carcinogen inorganic arsenite.

Airways are one of the most vulnerable tissues to oxidant injury. Protective roles for Nrf2 in nonmalignant lung disorders have been intensely studied [9], and two articles in this issue further support it: H. Y. Cho et al. demonstrate that Nrf2−/− mice have increased airway susceptibility to environmental ozone-induced inflammation and mucous cell metaplasia. H. R. Potteti et al. demonstrate that the Nrf2 pathway was not compromised but consistently functional during chronic hyperoxia exposure which induces airway
epithelial cell death. This issue also adds a review article by X. Li et al. on Nrf2 in defense of allergic asthma caused by diesel exhaust particles present in urban air pollution.

Novel information from advanced approaches of molecular genetics and computational modeling is reported in the current issue. M. R. Campbell et al. profile Nrf2 target genes in sulforaphane-treated human lymphoblastoid cells by chromatin immunoprecipitation sequencing. Key pathways included hematopoiesis, which was further tested in erythroleukemic cells by silencing Nrf2 or KEAPI to support the role for Nrf2 in heme metabolism and erythropoiesis. T. Korcsmáros and colleagues introduce “NRF2-ome (http://nrf2.elte.hu/)” an integrated online resource and discovery tool for protein interaction and regulatory networks of Nrf2. The authors extend the previously published Nrf2 interactome and regulome [10], which lead to a total of 7,777 manually curated, integrated, and predicted interaction data for Nrf2 along with its first neighbor interactors, target genes, regulating transcription factors, and microRNAs as well as Nrf2 signaling pathways.

We believe that the authors in the current issue add knowledge to the current understanding of Nrf2-mediated molecular, cellular, and physiological mechanisms. We hope that readers of this Nrf2 issue gain insights into potential therapeutic strategies of Nrf2 agonists or antagonists in host defense and disease pathogenesis.

Disclosure

The author Hye-Youn Cho's contribution to the work was done as part of the author's official duties as a National Institutes of Health (NIH) employee and is a work of the United States Government. Therefore, copyright may not be established in the USA.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Hye-Youn Cho
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References


Review Article

Genomic Structure and Variation of Nuclear Factor (Erythroid-Derived 2)-Like 2

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High-density mapping of mammalian genomes has enabled a wide range of genetic investigations including the mapping of polygenic traits, determination of quantitative trait loci, and phylogenetic comparison. Genome sequencing analysis of inbred mouse strains has identified high-density single nucleotide polymorphisms (SNPs) for investigation of complex traits, which has become a useful tool for biomedical research of human disease to alleviate ethical and practical problems of experimentation in humans. Nuclear factor (erythroid-derived 2)-like 2 (NRF2) encodes a key host defense transcription factor. This review describes genetic characteristics of human NRF2 and its homologs in other vertebrate species. NRF2 is evolutionally conserved and shares sequence homology among species. Compilation of publically available SNPs and other genetic mutations shows that human NRF2 is highly polymorphic with a mutagenic frequency of 1 per every 72 bp. Functional at-risk alleles and haplotypes have been demonstrated in various human disorders. In addition, other pathogenic alterations including somatic mutations and misregulated epigenetic processes in NRF2 have led to oncogenic cell survival. Comprehensive information from the current review addresses association of NRF2 variation and disease phenotypes and supports the new insights into therapeutic strategies.

1. Overview

The gene nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) or more commonly the used synonym nuclear factor erythroid 2- (NF-E2-) related factor 2 (NRF2) and its mouse homolog (Nfe2l2, Nrf2) encode a ubiquitous transcription factor belonging to the basic leucine zipper (bZIP) protein family [1, 2]. NRF2 modulates downstream genes by binding to their cis-regulatory module antioxidant response elements (AREs). NRF2 targets include ARE-bearing effector genes such as reactive oxygen species (ROS) scavenging enzymes (e.g., superoxide dismutases, SODs), phase-2 defense enzymes (e.g., glutathione-S-transferase, GST; heme oxygenase-1, HO-1), drug efflux pumps (e.g., multidrug resistance proteins, MRPs), and various interacting and indirectly modulated proteins [3–6]. The NRF2-ARE pathway has emerged in mechanisms of human diseases in which oxidative stress is implicated. Importantly, three lines of gene-targeted (knockout) mice were generated by Drs. M. Yamamoto (Nfe2l2ghtm1Mym), Y. W. Kan (Nfe2l2ghtm1YwK), and P. A. Ney (Nfe2l2ghtm1Ney) [7–9], and 124 gene-trapped or gene-targeted cell lines have been established (http://www.informatics.jax.org/searches/allele_report.cgi?markerID=MGI:108420). During the last decade or more, wide application of the knockout mice to human disease models has led to new insights into disease pathogenesis and therapeutic potential (Figure 1).

Kelch-like ECH-activating protein 1 (KEAPI for humans, Keap1 for mice, or iNrf2 for rats) is a cytoplasmic suppressor of NRF2 and is critical in NRF2 homeostasis and activity [10]. Substantial efforts have led to the discovery of the molecular mechanisms of KEAPI-mediated NRF2 regulation. In unstressed conditions, the NRF2-bound KEAPI homodimer is complexed to a ubiquitin ligase (Cullin 3-based E3 ligase), which polyubiquitinates NRF2 for proteasomal degradation and maintains NRF2 homeostasis (20 min half-life of cellular...
NRF2 [11]). However, modifications of KEAP1 (e.g., cysteine residues) and NRF2 (e.g., serine residues) under stressed conditions activate NRF2 by liberating it from a "hinge and latch" NRF2-KEAP1 affinity binding, allowing its nuclear translocation [12].

In the current review, I address genetic aspects of human NRF2 and its homologs in other vertebrate species. Sequence variations in human NRF2 and murine Nrf2 including single-nucleotide polymorphisms (SNPs) were collected from public databases and compiled. Mutations that have been associated with disease risks are defined. Nongenetic variations including somatic mutations and epigenetic modifications are also described. Although the current review does not deal with mutations in other species, recent characterization of nrf2 mutant zebrafish which were hypersensitive to environmental toxicants [13] also provides a useful investigational tool.

2. Sequence of NF-E2-Related Factor 2 and Cross-Species Homology

Homology scores of gene (coding DNA sequences, cds) and protein across 10 species were compared with human NRF2. The highest sequence homology (98%-99%) was with chimpanzee and rhesus monkey while the lowest similarity was found with zebrafish (Table 1). While there is approximately 83% homology in cds and protein sequences of humans and rodents, 5'-untranslated regions (5'-UTR, UTR-5) of these strains extend differentially (114 bp in human, 233 bp in mouse, and 82 bp in rat), and the human 5'-UTR does not share significant sequence homology with either rat or mouse (the rat is 94% homologous with the 3' portion of mouse 5'-UTR).

Human NRF2 is located in the cytogenetic band 2q31.2 of chromosome 2 spanning 178,095,031–178,129,859 bp as a complementary sequence (gene ID: 4780, Table 1). Murine Nrf2 maps as a complementary sequence to chromosome 2 C3 (44.75 centimorgan) and spans 75,675,319–75,704,641 bp (gene ID: 18024, Table 1). The complete cds of NRF2 is 2,859 bp, and there are 14 transcript variants reported (http://useast.ensembl.org/Homo_sapiens). Mouse Nrf2 mRNA spans 2,469 bp, and another variant has been reported (http://useast.ensembl.org/Mus_musculus). The human NRF2 protein (ID: NP_006155) contains 605 amino acid (aa) residues with molecular weight of 67.7 kDa (isofrom 1), and total 12 isoforms are published (the National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov/RefSeq/; eEnsemble, http://useast.ensembl.org/index.html; UniProt Consortium, http://www.uniprot.org/uniprot/Q16236, http://www.uniprot.org/uniprot/Q60795). Mouse Nrf2 protein (ID: NP_035032) comprises 597 aa at 66.8 kDa (Table 1). Structurally, there are 6 NRF2-ECH
## Table 1: Gene orthology of NF-E2-related factor 2 across the species.

<table>
<thead>
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<th>Species</th>
<th>Human (Homo sapiens)</th>
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<td>XP_00145876.2 (605/67686)</td>
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<td><strong>Variants</strong></td>
<td>14 transcripts, 12 isoforms</td>
<td>3 transcripts, 3 isoforms</td>
<td>5 transcripts, 5 isoforms</td>
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<td>89.3/91.4</td>
<td>99.9/99.9</td>
<td>89.3/88.8</td>
<td>90.5/89.1</td>
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<td>Official full name</td>
<td>Ancestor name</td>
<td>Gene synonyms</td>
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<td>Rhesus monkey</td>
<td>Macaca mulatta</td>
<td>nuclear factor (erythroid-derived 2)-like 2</td>
<td>NFE2L2, NRF2, Mmu.966</td>
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<td>Nfe2L2, Nrf2</td>
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</table>

homology (Neh) domains configuring the protein sequence of either species (Table 2), and the potential functions of each region, particularly the highly conserved KEAPI-binding Neh2 and DNA- (ARE-) binding Neh1 domains, have been intensively investigated [12, 14, 15].

3. Genetic Variation of NF-E2-Related Factor 2 in Human and Mouse

3.1. Evolution, Genome Sequence, and Polymorphism Discovery in Human and Mouse. While rare and monogenic Mendelian diseases are inheritable mutations in a single gene [16], many common diseases are complex traits, and the disease phenotypes are affected by variants in multiple genetic loci. Recent advancements in high-throughput technology have enabled sequencing of entire mammalian genomes [17–19], and information on DNA sequence and variation have enabled sequencing of entire mammalian genomes (NP

Table 2: Protein domains of NF-E2-related factor 2.

<table>
<thead>
<tr>
<th>Domains</th>
<th>Human (605 aa)</th>
<th>Mouse (597 aa)</th>
<th>Predicted functions</th>
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<td>Neh2</td>
<td>16–89</td>
<td>16–89</td>
<td>KEAP1 repression through DLG/ETGF motif-DC motif binding, fast, redox-sensitive proteasomal degradation.</td>
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<tr>
<td>Neh5</td>
<td>182–209</td>
<td>172–201</td>
<td>Degeron motif-associated constitutive turnover, slow, redox-insensitive (Keap1-independent).</td>
</tr>
<tr>
<td>Neh6</td>
<td>337–394 (or 338–388)</td>
<td>328–385</td>
<td>Dimerization for nuclear translocation, DNA binding through basic motif-leucine zipper.</td>
</tr>
<tr>
<td>Neh1</td>
<td>basic motif 503–518</td>
<td>basic motif 494–509</td>
<td>CHD6 binding, stability, or transactivation.</td>
</tr>
<tr>
<td>Neh3</td>
<td>leucine zipper 525–539</td>
<td>leucine zipper 517–531</td>
<td>CHD6 binding, stability, or transactivation.</td>
</tr>
</tbody>
</table>

* Varies slightly among publications.

fact, biomedical studies of human genes are complemented by experimental manipulation of corresponding mouse genes, and they have aided functional understanding of genes in human health. Following the 2003 completion of the Human Genome Project of approximately 3.1 giga base pairs (Gbp), the Mouse Genome Project assembled the complete genome sequence of one strain (C57BL/6; 2,716,965,481 bp) in 2011. Using this reference strain, whole genome sequencing data across 16 additional inbred strains were done (http://www.sanger.ac.uk/resources/mouse/genomes/,[21]). Discovery of high-density SNPs in the mouse genome supports evolutionary history of the strain and provides a tool to investigate models of human disease processes that cannot often be practically achieved through direct human studies.

3.2. Genetic Mutations in Human NRF2. Human NRF2 codes three major isoforms of protein (Figure 2). Transcript variant 2 (NM_001145412.2, 2746 bp) has an alternate promoter, 5’-UTR, and a downstream start codon, compared to variant 1 (NM_006164.4, 2859 bp). It encodes an isoform 2 missing N-terminal 16 aa (NP_001138884 or Q16236-2, 589 aa) relative to isoform 1 (NP_006155.2 or Q16236, 605 aa). Isoform 3 (NP_001138885.1 or Q16236-3, 582 aa) is encoded by transcript variant 3 (NM_001145413.2; 2,725 bp) and lacks an internal segment relative to isoform 2 due to an alternate intron splice site in the 3’ coding region. In public databases, more than 583 sequence mutations are reported in NRF2 (34,827 bp) and 7,000 bp upstream (Table S1; data acquired as of December, 2012) (See Table S1 in Supplementary Material available on line at http://dx.doi.org/10.1155/2013/286524). NRF2 locates on 178,130,354-178,129,304 bp of GRCh37.p10 Primary Assembly, and Figure 2 shows sequences of proximal promoter (~1 to ~500), partial mRNA variant 1 including 5’-UTR (exon 1, up to TSS), and protein isoform 1 (NP_006155, encoded by variant 1 NM_006164.4; 556–2,373 bp). Based on the current assembly and sequence update, previous promoter positions ~686 [22]/~653 [23] are identified as ~214; ~684 [22]/~651 [23] as ~212; and ~650 [22]/~617 [23] as ~178. Overall frequency of NRF2 SNPs and other mutations is about 1 per 72 bp. The genetic mutations include 37 in...
3.3. SNPs, Haplotypes, and Association with Disease Risk

The use of gene knockout mice in model systems has provided potential insights into the role of NRF2 in the pathogenesis of various human disorders (see Figure 1). Recent epidemiological and association studies have revealed significant associations of NRF2 sequence variations with disease risks, which further supports NRF2 as a susceptibility gene. Most of the phenotype-associated variants are in the promoter region and presumed to be involved in NRF2 gene regulation. Table 4 summarizes NRF2 SNP and/or haplotype alleles that have been associated with oxidant-related disease risks. Interestingly, there is no evidence for exon SNPs as at-risk alleles. For convenience and consistency, intronic and 3′ distal SNP alleles are presented as chromosome contig (HGVS) alleles while promoter and exon SNPs are presented as reversed contig alleles throughout the text.

Pulmonary Diseases. NRF2 SNPs in the promoter and intron 1 sequences have been investigated for their potential associations with risk of pulmonary critical disorders including acute lung injury (ALI), cigarette smoke-induced chronic obstructive pulmonary disease (COPD), and asthma. A heterozygous C/A SNP at −178 position (rs6721961T>C or T>G, previously ~617 or ~650) significantly increased the risk for developing ALI following major trauma in the literature [23]. Promoter activity of the A allele (C/C or C/T) was significantly lower than C/C allele at that locus in vitro [23] and in vivo [22].

Among exon SNPs, 26 are nonsynonymous (Cns) mutations.

Among exon SNPs, 26 are nonsynonymous (Cns) mutations.
<table>
<thead>
<tr>
<th>ID</th>
<th>Map on chromosome 2* (HGVS name)</th>
<th>Position in Nfe2l2 (HGVS name)</th>
<th>Regions (position from mRNA)*</th>
<th>Variation class and consequences (HGVS name)†</th>
<th>Minor allele frequency (MAF)/MA counts (cohort size)</th>
<th>MAF sources</th>
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Table 3: Continued.

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<th>Variation class and consequences (HGVS name)†</th>
<th>Minor allele frequency (MAF)/MA counts‡ (cohort size)</th>
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<td>Cns (p.Arg43Trp)</td>
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<td>1000 Genomes</td>
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<td>Cns (p.Arg43Gln)</td>
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<td>T‡</td>
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<td>g.178097185&gt;A&gt;C</td>
<td>c.529T&gt;G</td>
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<td>Cns (p.Leu177Val)</td>
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<tr>
<td>rs18151334</td>
<td>g.178096710&gt;G&gt;T</td>
<td>c.621G&gt;A</td>
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<td>c.656A&gt;G</td>
<td>Exon 5 (1211)</td>
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<td>rs139187151</td>
<td>g.178096634&gt;G&gt;A</td>
<td>c.697C&gt;T</td>
<td>Exon 5 (1252)</td>
<td>Cns (p.Pro233Ser)</td>
<td>A = 0.0005/1</td>
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<td>rs35557421</td>
<td>g.178096620delG</td>
<td>c.711delA</td>
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<td>Frame shift/deletion (p.Lys237 = fs)</td>
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<td>c.802G&gt;A</td>
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<td>c.925&gt;T</td>
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<td>g.178096237C&gt;A</td>
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<td>Exon 5 (1649) Cns (p.Ser365Ile)</td>
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<td>c.* 468G&gt;A</td>
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<th>Map on chromosome 2 (HGVS name)</th>
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<th>Disease association and references</th>
<th>Risk alleles and statistics</th>
<th>Ethnic group (number of case)</th>
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<td>G = 0.718/252 (^4) in haplotype OR = 0.90 or 0.40; CI = 0.60–1.40 or 0.3–0.6</td>
<td>Swedish/Polish Caucasian (357)</td>
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<td>−3306T&gt;C</td>
<td>Breast cancer survival (2012 [27])</td>
<td>T/T = 0.324 OR = 1.687; CI = 1.105–2.75</td>
<td>Finland KBCP (452)</td>
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<tr>
<td>rs35652124</td>
<td>g.178130073T&gt;C</td>
<td>−214A&gt;G</td>
<td>Ulcerative colitis (2008 [31])</td>
<td>G(^2) in haplotype for ulcer (OR = 2.52; CI = 1.19–5.45)</td>
<td>Japanese (159)</td>
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<td>(rs57695243)</td>
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<td>G in haplotype OR = 2.90; CI = 1.14–7.36 A in haplotype P = 0.022</td>
<td>Japanese (209)</td>
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<td>(previously −653 [23] or −686 [22])</td>
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<td>Lupus with nephritis in female (2010 [25])</td>
<td>A/G (OR = 0.45; CI = 0.22–0.93) G (OR = 2.57; CI = 1.01–6.60)</td>
<td>Japanese (89)</td>
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<td>Parkinson's disease (2010 [26])</td>
<td>A = 0.884/312 (^5) in haplotype OR = 0.9 or 0.4; CI = 0.60–1.40 or 0.30–0.60 G = 0.52(^6) hazard ratio = 0.95; CI = 0.91–0.99 (Haplotype)</td>
<td>Japanese (209)</td>
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<td>COPD (2010 [32])</td>
<td>G(^7) in haplotype OR = 2.52; CI = 1.19–5.45 G in haplotype OR = 2.90; CI = 1.14–7.36 G in haplotype, P = 0.022</td>
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<td>A/G (OR = 0.45; CI = 0.22–0.93) G (OR = 2.57; CI = 1.01–6.60)</td>
<td>Japanese (89)</td>
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<td>(previously −651 [23] or −684 [22])</td>
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<td>Lupus with nephritis in female (2010 [25])</td>
<td>A = 0.884/312 (^5) in haplotype OR = 0.9 or 0.4; CI = 0.60–1.40 or 0.30–0.60 G = 0.52(^6) hazard ratio = 0.95; CI = 0.91–0.99 (Haplotype)</td>
<td>Japanese (209)</td>
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<td>Maternal acetaminophen and asthma (2010 [33])</td>
<td>A = 0.232/1137 (^7) OR = 1.73; CI = 1.22, 2.45</td>
<td>UK ALSPAC (&gt;4000 mothers, &gt;5000 children)</td>
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<td>Disease association and references</td>
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<td>COPD (2010 [32])</td>
<td>G = 0.98&lt;sup&gt;2&lt;/sup&gt; in haplotype hazard ratio = 0.95; CI = 0.91–0.99</td>
<td>German (69)</td>
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<td>G = 0.972/343&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>rs6723961</td>
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<td>Acute lung injury following trauma (2007 [23])</td>
<td>C/A = 0.119&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Caucasian/African-American (164)</td>
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<td>Vitriligo (2008 [35])</td>
<td>OR = 0.9 or 0.4; CI = 0.60–1.40 or 0.3–0.6</td>
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<td>COPD (2010 [32])</td>
<td>G = 0.73&lt;sup&gt;2&lt;/sup&gt; in haplotype hazard ratio = 0.95; CI = 0.91–0.99</td>
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<td>Postmenopausal venous thromboembolism (2011 [36])</td>
<td>A = 0.333&lt;sup&gt;11&lt;/sup&gt;</td>
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<td>Breast cancer survival and NRF2 protein expression (2012 [27])</td>
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<td>Finland KBCP (452)</td>
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<td>Acute lung injury-related mortality following systemic inflammatory response syndrome (2012 [37])</td>
<td>OR = 4.656; CI = 1.35–16.06</td>
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<td>Infection-induced asthma (2012 [38])</td>
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<td>OR = 0.437; CI = 0.28–0.80</td>
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<td>GCC&lt;sub&gt;4&lt;/sub&gt; = 0.53&lt;sup&gt;3&lt;/sup&gt; in haplotype hazard ratio = 0.95; CI = 0.91–0.99</td>
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<td>T = 0.878/309&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>Basal and smoker FEV&lt;sub&gt;1&lt;/sub&gt; (2009 [39])</td>
<td>CI = −63.60–−1780, C = 0.525&lt;sup&gt;3&lt;/sup&gt; in haplotype</td>
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<td>Intron 1</td>
<td>Annual FEV&lt;sub&gt;1&lt;/sub&gt; decline (2011 [34])</td>
<td>A = 0.082&lt;sup&gt;11&lt;/sup&gt;</td>
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<td>Basal and smoker FEV&lt;sub&gt;1&lt;/sub&gt; (2009 [39])</td>
<td>G = 0.401/1578&lt;sup&gt;2&lt;/sup&gt; in haplotype</td>
<td>Netherland (2542)</td>
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<td>rs1806649 (rs58745895)</td>
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<td>Breast cancer [40]</td>
<td>T with NQO1/NOS3/HO1 risk alleles: OR = 1.56; CI = 0.97–2.51 T = 0.263±119.in haplotype CI = −87.30–(−1.70)</td>
<td>Caucasian and others (505)</td>
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<td>Parkinson's disease (2010 [26])</td>
<td>C with low vitamin C level (OR = 3.1; CI = 1.50–6.30)</td>
<td>Swedish/Polish Caucasian</td>
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<td>rs4243387 (rs60038464)</td>
<td>Intron 1</td>
<td>Basal and smoker FEV1 (2009 [39])</td>
<td>T = 0.09±425.in haplotype</td>
<td>Netherland (2542)</td>
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<td>Annual FEV1 decline (2011 [34])</td>
<td>A = 0.082 in haplotype</td>
<td>Japanese (915)</td>
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<td>Breast cancer NRF2 and ARE expression (2012 [27])</td>
<td>A (P = 0.036)</td>
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<td>OR = 0.9 or 0.4; CI = 0.60–1.40 or 0.30–0.60</td>
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−686 in reference [22] = −653 in reference [23] = currently −214; −684 in reference [22] = −651 in reference [23] = currently −212; −651 in reference [22] = −617 in reference [23] = currently −178. Chromosome contig (intron, 3' flanking) or reversed (5' flanking promoter) alleles in bold have been used in the text and Table. OR: odds ratio. CI: 95% confidence interval.
−212G/G (98%, rs67006649, previously −684/−651), −178C allele (73%), and GCC₄ (53%) was predicted to increase respiratory failure development (hazard ratio = 0.95, CI 0.91–0.99) in German COPD patients [32]. Significant interaction was also identified between an intronic SNP G allele (rs6726395, g17810329A>G, 88.4% frequency) and smoking status on FEV₁ decline, relative to the reference A/A allele, in the above Japanese cohort [34]. Siedlinski et al. [39] reported that the C/C genotype of another intronic SNP (rs2364723, g17812654G>C) was associated with a lower FEV₁ level compared to the wild-type genotype (G/G) in two Netherland cohorts (CI, −63.6–17.8, frequency = 0.525, and pooled cohort size = 2,542). This SNP alone or as a haplotype with 4 more intronic SNPs (rs13001694G/rs1806649T/rs4243387T/rs6726395G) was also associated with high FEV₁ levels in individuals that ever smoked [39]. In a Hungarian population of childhood asthma, SNPs at −178 (C/A) and 3′ flanking (rs2588882T/G) loci were inversely associated with infection-induced asthma (OR 0.47; CI 0.28–0.80, OR 0.290; CI 0.13–0.62, resp.), and these SNPs significantly influenced an asthma-environmental pollution interaction [38]. The intronic SNP rs1806649 (C>T) was associated but not significantly with an increased risk of hospitalization during high-level particulate matter (PM₁₀) periods in asthma or COPD patients (n = 209) of the United Kingdom (UK) [41]. Asthma and COPD admission rates were related to the increase in environmental PM₁₀ concentration. Importantly, effects of interaction between prenatal stress and NRF2 SNPs on descendant pulmonary health were investigated by the Avalon Longitudinal Study in the UK: maternal smoking during pregnancy was not associated with lung function change determined by maximum mild expiratory flow (FEF₂₅−₇₅) or with asthma incidence in school-aged children, and this relation was not modified by NRF2 SNP genotypes [42]. However, early gestation acetaminophen exposure significantly influenced the risk of asthma and wheezing at the age of 7 years in >4,000 mothers and >5,000 children [33]. When maternal copies of the −212A allele were present, association with asthma (1,137/4,891; OR 1.73, CI 1.06–2.20) was significantly increased [33].

Gastrointestinal Disorders. While there was no evidence in lung cancer cases, studies in Japanese populations suggested a potential association of NRF2 and downstream effectors in postmenopausal mammary cancer. In a study of a Finish population (Kuopio Breast Cancer Project, n = 452 patients, 370 controls), the −178A/A homozygous genotype (OR 4.656; CI = 1.35–16.06) and 3′ flanking rs2706110 (T/T; OR 2.079, CI 1.18–3.68) genotype were associated with increased risk of breast cancer, while the 5′ flanking −3,306T/T homozygous allele was significantly associated with lower survival (frequency = 71/219, OR 1.687, CI 1.105–2.75) [27], suggesting that NRF2 genetic polymorphisms affect susceptibility and outcome of the patients. The −178A allele carriers together with intronic rs1962142A allele carriers were associated with lowered tissue levels of NRF2 proteins [27]. In postmenopausal women, the −178A allele (OR 179; 95% CI 3.70–85.70) appeared to modify the risk of venous thromboembolism caused by oral estrogen therapy (A/A or A/C frequency = 33.3%) as demonstrated by the French ESTHER study (161 cases, 474 controls) [36]. An intronic rs1806649C>T SNP did not associate with breast cancer risk in postmenopausal women [40]. However, when this SNP and other at-risk alleles of ARE-responsive genes (NQOI, HO-1, NOS3) were combined, there was a significant gene-dose effect on the breast cancer risk [40]. Although coding region SNPs in NRF2 and KEAP1 were identified in the Japanese endometrial adenocarcinoma patients, no association of NRF2 SNPs with the disease was found [44].

Neurodegenerative Diseases. Oxidative stress is known to be involved in Parkinson’s disease (PD) presumably due to production of ROS from high-dopamine metabolism and low levels of antioxidants in the substantia nigra of the brain. Investigators found a protective NRF2 haplotype consisting of four 5′ flanking SNPs (−5238G/−214A/−212G/−178C) (OR 0.45, CI 0.22–0.93), and the −214G−212G genotypes were positively associated (chronic continuous phenotype; OR 2.57, CI 1.01–6.60) with ulcerative colitis (89 patients, 141 controls) in a Japanese population [31].

Autoimmune Disorders. Systemic lupus erythematosus (SLE) is a long-term autoimmune disease more frequently found in females than in males. It affects organs including skin, joints, kidneys, and brain, and nephritis is an aggressive characteristic in some patients. Genome-wide association studies in humans identified a suggestive quantitative trait locus near NFE2L2 [43]. A study of a Mexican Mestizo population (362 patients with childhood-onset SLE, 379 controls, and 212 nephritis diagnosed) determined that lupus with nephritis was significantly (OR 1.81, CI 1.04–3.12) associated with the −214G/A SNP in females [25]. The same SNPs were not closely associated with SLE risk in a Japanese cohort [22]. Vitiligo is a skin condition in which there is a loss of brown color (pigment) from areas of skin, resulting in irregular white patches. It is thought to be an autoimmune disease caused by loss of cells (melanocytes) that produce brown pigment. A study indicated that the −178A allele increased the risk of vitiligo dose-dependently (OR 1.724, 95% CI 1.35–2.21 for C/A; OR 2.902, CI 1.62–5.19 for A/A) [35].

Female Disorders. It is well known that estrogen metabolites (e.g., catechols) cause ROS formation suggesting correlation of NRF2 and downstream effectors in postmenopausal mammary cancer. In a study of a Finish population (Kuopio Breast Cancer Project, n = 452 patients, 370 controls), the −178A/A homozygous genotype (OR 4.656; CI = 1.35–16.06) and 3′ flanking rs2706110 (T/T; OR 2.079, CI 1.18–3.68) genotype were associated with increased risk of breast cancer, while the 5′ flanking −3,306T/T homozygous allele was significantly associated with lower survival (frequency = 71/219, OR 1.687, CI 1.105–2.75) [27], suggesting that NRF2 genetic polymorphisms affect susceptibility and outcome of the patients. The −178A allele carriers together with intronic rs1962142A allele carriers were associated with lowered tissue levels of NRF2 proteins [27]. In postmenopausal women, the −178A allele (OR 179; 95% CI 3.70–85.70) appeared to modify the risk of venous thromboembolism caused by oral estrogen therapy (A/A or A/C frequency = 33.3%) as demonstrated by the French ESTHER study (161 cases, 474 controls) [36]. An intronic rs1806649C>T SNP did not associate with breast cancer risk in postmenopausal women [40]. However, when this SNP and other at-risk alleles of ARE-responsive genes (NQOI, HO-1, NOS3) were combined, there was a significant gene-dose effect on the breast cancer risk [40]. Although coding region SNPs in NRF2 and KEAP1 were identified in the Japanese endometrial adenocarcinoma patients, no association of NRF2 SNPs with the disease was found [44].
3.4. Genetic Mutations in Mouse Nrf2. Tsang et al. [46] compiled 673 SNPs in 55 mouse strains and constructed their phylogenetic tree to correlate and clarify the origins of strains based on the assembled mouse genome sequence and SNP data [20, 47, 48]. Recently, using the complete genome sequence of C57BL/6J (B6) mouse as a reference, high-density SNP screening in other laboratory strains or in panels of strains has been published (see [17]). Although millions of mouse SNPs (>10,089,892 as of December 2012) and haplotype mappings from more than 120 strains have been published as valuable references for dissecting the genetic basis of complex traits [49–51], little attention has been paid to polymorphisms of Nrf2 and their correlation with disease phenotypes.

Figure 3 demonstrates the proximal promoter region (−1 to −950)/5′-UTR (exon 1, up to TSS) and protein sequence of mouse Nrf2 based on GRCm38.p1 Primary Assembly (75,704,641–75,675,513 bp), mRNA variant 1, and protein (NP_035032, encoded by NM_010902: 234–2,027 bp) sequences. Genetic variations in the Nrf2 genome of inbred strains collected from public databases are listed in Tables S2 and 5. (See Supplementary Table S2) Overall, 968 genetic mutations are compiled for Nrf2 gene and 5 kb upstream/2 kb downstream regions: 785 SNPs between B6 and another 16 strains were acquired from the Mouse Phenome Database (MPD, http://phenome.jax.org/db/q?rt=snp+ret1), and additional SNPs and other mutations were acquired from NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp/?term=mus+musculus%20nfe212). In total, 132 mutations are in the promoter (37 in proximal 1 kb), 49 in exons (38 in coding region, 19 Cns), 727 in introns, and 60 in the 3′ flanking region. Excluding mutations in the 5′ and 3′ flanking sequences, murine Nrf2 sequences appear to be more highly variable (1 variation per 37.5 bp) than much of the mouse genome which has an approximate frequency of one SNP per every 245 bp (http://www.informatics.jax.org/mgihome/homepages/stats/allstats.shtml#allsnps).

Nrf2 was found to be a susceptibility gene from genome-wide linkage analysis in a murine model of hyperoxia-induced ALI [52]. A promoter SNP −103T>C (previously published as −336T>C) in Nrf2 was found and predicted to add an additional Sp1 binding site in hyperoxia-susceptible B6 mice, but not in resistant C3H/HeJ mice [52]. Genotypes from the SNP and from simple-sequence length polymorphism markers of the Nrf2 locus (D2Mit248 and D2Mit94) cosegregated in the B6C3F2 mouse cohort [52], and Nrf2 deficient mice were significantly more susceptible to ALI sub-phenotypes caused by hyperoxia than similarly exposed wild-type mice, supporting Nrf2 as a contributor to the phenotypic traits [53]. Although no other functional analyses on Nrf2 SNPs or haplotype association studies have been conducted in inbred mice, strains bearing haplotypes such as multiple Cns in functional domains (e.g., F71L, L451V, H543Q, and L575M) may be useful to elucidate the role of Nrf2 in differential susceptibility to oxidative diseases.

4. Oncogenic Somatic Mutations in Human NF-E2-Related Factor 2

Somatic mutation is a change in the DNA of somatic cells that affects derived cells but is not inherited by offspring. Efforts to discover somatic mutations have provided insight into mutagenesis and cancer development. Lung cancer, particularly non-small cell lung cancer (NSCLC), is the leading cause of cancer death worldwide. Somatic mutations of Nrf2 and KEAP1 discovered in lung cancer patients have determined the oncogenic potential of Nrf2 [54, 55]. KEAP1 somatic mutations were associated with its reduced protein levels in lung cancer tissues and cells [56, 57]. Investigations of NSCLC in various ethnic populations as well as cancers in gastrointestinal, breast, and prostate have coordinately demonstrated that multiple Cns somatic mutations in KEAP1 cause dysfunction of the translated protein and in turn constitutive activation of Nrf2, increasing risk of neoplasia and chemoresistance [12, 55, 58, 59]. Somatic mutations of Nrf2 have been detected in various cancer tissues (largely squamous cell carcinomas) in Asian populations (Table 6). Nrf2 mutations were significantly associated with NSCLC cases (squamous cell lung carcinoma, adenocarcinoma) of the Japanese (10.7%, [54]), the Chinese (23%, [60]), and the Koreans (8%, [61]) as well as with lung cancer cell lines. Smoking history was also correlated with mutation occurrence in all of the studies [54, 60, 61]. In addition to lung cancers, laryngeal squamous carcinoma (13% in [61]), esophageal squamous cancer (ESC, 22% in [60], 11.4% in [61]), head and neck cancers (25% in [54]), skin (1/17 case in [61]), and oral cancer cell lines had somatic changes in Nrf2. In contrast to wide-spread KEAP1 mutations, mutations in Nrf2 were clustered in DLG/ETGE motifs of the Neh2 domain, which are critical in the “hinge and latch” model of KEAP1 binding [12]. Similar to KEAP1 somatic mutations, it has been postulated that Nrf2 mutations in cancer cells lead to Nrf2 accumulation by suppressing its ubiquitination or KEAP1 binding, which eventually confers malignant potential and resistance to chemotherapy.

Most variable sites in Nrf2 included aa residues 29 (Asp, D), 31 (Gly, G), 77 (Asp, D), and 79 (Glu, E) (Table 6). Residue 33 (Ser, S) in the Neh2 domain is mutated by either somatic mutations, it has been postulated that Nrf2 mutations in cancer cells lead to Nrf2 accumulation by suppressing its ubiquitination or KEAP1 binding, which eventually confers malignant potential and resistance to chemotherapy.

Most variable sites in Nrf2 included aa residues 29 (Asp, D), 31 (Gly, G), 77 (Asp, D), and 79 (Glu, E) (Table 6). Residue 33 (Ser, S) in the Neh2 domain is mutated by either genetic or somatic processes (Figure 4). Cns in the EDGF motif of Nrf2 was experimentally determined to impair recognition of KEAP1 [54]. Nrf2 mutations were significantly correlated with increased (2.5-fold) copy number (31% of mutants versus 3% wild types) in Japanese NSCLC cases [63]. Aberrant mutation of Nrf2 also led to increased expression of downstream effectors including RagD known to
Genomic DNA (reversed contig, GRCm38. p1 C57BL6/J/NM_010902.3)

−950 ccacaacgcc aclatatla aaggcccgc attacagca qgtgagaaag
−900 cgcctccta aaacacctt gaggctggct cctctgaag attatatctt
−850 aataatcttta atgttctgc gatctagcttg cagttacttac ggtttag
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Protein (NP_035032)

Genetic mutation (Table 3 and Figure 2)

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Table 6: NRF2 somatic mutations revealed in various human cancers.

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<tr>
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<td>NSCLC: non-small cell lung cancer, ESC: esophageal squamous cancer, and * errors in reference fixed. Number of cases: 82 NSCLC and 10 ESC in [62]; 125 NSCLC, 70 ESC, 23 larynx, and 17 skin in [61]; 103 NSCLC and 12 head and neck in [54]; 90 NSCLC in [63]; 103 NSCLC in [60].</td>
<td></td>
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</table>

be involved in squamous lung cancer cell proliferation [64], suggesting that the mutation is functional and overcomes KEAP1 inhibition. Singh et al. [65] determined in vitro that RNAi-mediated depletion of NRF2 in lung cancer cells enhanced ROS production and susceptibility to cell death by ionizing radiation. These studies support the concept that elevated NRF2 and ARE responsiveness provides cancer cells with proliferative advantage for malignant transformation and undue protection from anti-cancer therapy. Onco-
genic epidermal growth factor receptor (EGFR) signaling is recently found to be critical in NRF2-mediated proliferation of NSCLC cells [66].

Collectively, “gain of function” mutations in NRF2 that reduce KEAP1 recognition are suggested to be predictive markers for poor responsiveness to chemotherapy and radiation therapy. Although NRF2-mediated cellular defense
processes are essential in the initiation stage, enhanced NRF2-ARE activity in advanced stages of cancer development may create a favorable intracellular environment for tumor cell growth and survival [67, 68]. In this context, NRF2 may be a potential molecular target for the treatment of radio-resistance cancers, especially those that have “loss of function” mutations in EGFR, KRAS, or KEAP1 as well as “gain of function” mutations in NRF2.

5. Epigenetic Alterations of NF-E2-Related Factor 2

Epigenetic modifications are alterations of molecules interacting with genes without changes to the primary DNA sequence. They include post-translational modification of histones, DNA methylation events, chromatin conformational changes, and alterations to noncoding regulatory RNAs. Epigenetic alterations are stable and often inheritable but are reversible and may affect expression of the gene. Dysregulation or defects in epigenetic processes, particularly hypermethylation of tumor suppressor gene promoters (e.g., CpG islands) or histone modifications, are thought to be associated with carcinogenesis. Investigators have reported hypermethylation in CpG islands of KEAP1 which were associated with reduced KEAP1 expression in human cancers from lung, prostate, colon, and so forth, [69–72]. Similar to somatic mutations, epigenetic changes on KEAP1 impaired the function of its encoded protein leading to constitutive NRF2 activation.

Supporting the role of “pathogenic mutations” in NRF2, expression of Nrf2 and downstream Nqo1 was suppressed in prostate tumors of mice (transgenic adenocarcinoma of mouse prostate, TRAMP). Among 15 promoter CpG islands located between −942 and −654 (c,−1175,c,−1132 and c,−1059,c,−887, gap in c,−1131,c,−1060; see Figure 3), hypermethylation of the first 5 CpG islands (−942,−899 and c,−1175,c,−1132) was significantly associated with tumorigenesis [73]. Moreover, treatment with inhibitors for DNA methyltransferase and histone deacetylase restored Nrf2 expression in these tumor cells [73]. A dietary phytochemical curcumin known as a DNA hypomethylation agent restored epigenetically silent Nrf2 expression through CpG demethylation in carcinogen-induced mouse tumor cells [74].

The whole genome epigenetic datasets for 5 species are publicly accessible at NCBI Epigenomics [75, 76]. The human NRF2 epigenome of primary cells (breast, penis) and H1 stem cell line as well as mouse Nrf2 CpG island methylation data for sperm, blood, and cerebellum are currently available (http://www.ncbi.nlm.nih.gov/epigenomics). Although no direct evidence of disease-associated epigenetic modulation has been identified in human NRF2, various phytochemical NRF2 agonists such as sulforaphane and curcumin have shown their roles in DNA methylation and histone modification (see reviews by Lee and colleagues, e.g., [77]). Taken together, epigenetic modifications of the Nrf2/KEAP1 axle are predicted to cause dysregulation of ARE-mediated cellular defense leading to deleterious health effects, and phytochemical antioxidants as epigenetic modulators for NRF2 are suggested to be useful in cancer prevention.

6. Conclusions

NRF2 is evolutionally conserved with high-sequence homology in many species. However, it is a highly mutable gene, and numerous genetic variants have been discovered in human ethnic groups. Importantly, certain SNPs or haplotypes have been identified in various diseases as “at-risk” alleles and are related to functional alterations. In addition to genetic variations, multiple somatic mutations identified in the KEAP1 recognition domain of NRF2 in cancer cells have been found to be oncogenic due to dysregulation of NRF2 homeostasis by its excess “gain of function”. Epigenetic alteration of the NRF2 is under investigation and is predicted to have pathogenic influences as learned from mouse and phytochemical agonist studies. Continuous updates of Nrf2 allelic variants in inbred mouse strains will provide a useful tool for effective experimental designs for models of oxidative disorders to provide insight into the disease mechanisms and intervention strategies.

Abbreviations

AD: Alzheimer’s disease
ALI: acute lung injury
ARE: antioxidant response element
bZIP: basic leucine zipper
cds: coding DNA sequences
CI: confidence interval
COPD: chronic obstructive pulmonary disease
ESC: esophageal squamous cancer
FEV\textsubscript{1}: forced expiratory volume in one second
Gbp: giga base pairs
GST: glutathione S-transferase
GWAS: genome-wide association study
HGVS: Human Genome Variation Society
HO-1: heme oxygenase-1
H. pylori: Helicobacter pylori
KEAP1: Kelch-like ECH activating protein 1
MPD: Mouse Phenome Database
MRP: multidrug resistance protein
NCBI: National Center for Biotechnology Information
Neh: NRF2-ECH homology
Nfe2l2: nuclear factor (erythroid-derived 2)-like 2
Nrf2: NF-E2-related factor 2
NSCLC: nonsmall cell lung cancer
NQO1: NAD(P)H:quinone oxidoreductase 1
OR: odds ratio
PD: Parkinson’s disease
PM: particulate matter
ROS: reactive oxygen species
SLE: systemic lupus erythematosus
SNP: single nucleotide polymorphism
SOD: superoxide dismutase
URT: untranslated region.
Disclosure

Author's contribution to the Work was done as part of the Author's official duties as a NIH employee and is a Work of the United States Government. Therefore, copyright may not be established in the United States.

Conflict of Interests

The author declares that there is no conflict of interests.

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References


Review Article

The Role of the Keap1/Nrf2 Pathway in the Cellular Response to Methylmercury

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Methylmercury (MeHg) is an environmental electrophile that covalently modifies cellular proteins with reactive thiols, resulting in the formation of protein adducts. While such protein modifications, referred to as \( S \)-mercuration, are thought to be associated with the enzyme dysfunction and cellular damage caused by MeHg exposure, the current consensus is that (1) there is a cellular response to MeHg through the activation of NF-E2-related factor 2 (Nrf2) coupled to \( S \)-mercuration of its negative regulator, Kelch-like ECH-associated protein 1 (Keap1), and (2) the Keap1/Nrf2 pathway protects against MeHg toxicity. In this review, we introduce our findings and discuss the observations of other workers concerning the \( S \)-mercuration of cellular proteins by MeHg and the importance of the Keap1/Nrf2 pathway in protection against MeHg toxicity in cultured cells and mice.

1. Introduction

Methylmercury (MeHg) is strongly accumulated by fish and marine mammals and is found at the highest concentrations in large predatory species at the top of the aquatic food chain. MeHg readily penetrates the blood-brain barrier and affects the central nervous system. It is transferred into cells by passive diffusion and/or transport by the L-type large neutral amino acid transporter (as its L-cysteine complex) and reacts covalently with protein thiols because of its high affinity for nucleophilic groups, having a dissociation constant of \( 10^{-15} \) [1]. This covalent modification by MeHg is referred to as “\( S \)-mercuration.” Some MeHg, however, binds to glutathione (GSH) that is present in a variety of cell types at relatively high concentrations (>1 mM) to form a MeHg-GSH adduct that is transported into the extracellular space by multidrug resistance-associated proteins (MRPs) [2–4] (Figure 1). While the MeHg-mediated \( S \)-mercuration of cellular proteins is believed to be involved in the toxicological significance of MeHg [5], oxidative stress from the presence of MeHg in the body is also a critical factor associated with its toxicity [6]. Interestingly, antioxidant proteins such as heme oxygenase-1 (HO-1), glutamate-cysteine ligase (GCL; a rate-limiting enzyme for GSH synthesis), and MRPs are regulated by transcription factor NF-E2-related factor 2 (Nrf2) [7–12].

2. \( S \)-Mercuration of Proteins by MeHg

Although there is little doubt that MeHg is able to covalently modify cellular proteins, and it is thought that a protein-MeHg complex is, at least partly, involved in MeHg toxicity (Figure 1), there have been only a limited number of studies in which the \textit{in vivo} effects of MeHg have been investigated with regard to \( S \)-mercuration. This is probably because of the lack of appropriate methods for identifying \( S \)-mercuration. Here, we describe our efforts to investigate the effects of MeHg using chemical biology approaches (Table 1).

We have found that the subcutaneous administration of MeHg (10 mg/kg) to mice resulted in a time-dependent decrease in brain manganese-superoxide dismutase (Mn-SOD) activity, whereas MeHg exposure had no effect on cuprozinc-SOD (Cu,Zn-SOD) activity [13]. Although levels
In the body, MeHg accumulates to high concentrations in the liver [16, 17], but few molecular targets of MeHg have been identified in this tissue. We hypothesized that MeHg could interact with arginase I, an abundant Mn-binding protein in the liver, through covalent modification, resulting in alterations in not only its catalytic activity but also in hepatic Mn levels through in vivo MeHg exposure. After the subcutaneous administration of MeHg (10 mg kg$^{-1}$ day$^{-1}$ for 8 days) to rats, mercury levels in the liver were greater than those in the cerebrum or cerebellum [18]. A marked suppression of arginase I activity was also detected under these conditions. Using purified rat arginase I, we found that MeHg-induced S-mercuration of arginase I caused the protein to aggregate and substantial leakage of Mn ions from the arginase I active site. We speculate that the MeHg-mediated suppression of hepatic arginase I activity in vivo is, at least partly, attributable to the S-mercuration of this protein.

In the course of the study, we coincidentally detected another protein, with a 42 kDa subunit moleule, from the same hepatic preparation that readily underwent S-mercuration and subsequent aggregation. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate the protein, and it was identified by peptide mass fingerprinting using matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF/MS). This 42 kDa protein was identified as sorbitol dehydrogenase (SDH) [19]. Using recombinant rat SDH possessing 10 cysteine residues in a subunit [20], we found that MeHg was covalently bound to SDH through Cys44, Cys119, Cys129, and Cys164, resulting in the inhibition of its catalytic activity and the release of zinc ions, facilitating protein aggregation [19]. Subsequent mutation analysis confirmed that Cys44, which ligates the active site zinc atom [21], and Cys129 play a crucial role in the MeHg-mediated aggregation of SDH [19].

### 3. Cellular Response to MeHg via the Keap1/Nrf2 Pathway

As mentioned above, MeHg reacts readily with cellular nucleophiles and also causes oxidative stress, implying that it may lead to decreased GSH levels in tissues. As a result, MeHg undergoes GSH conjugation and is excreted into the extracellular space through the action of MRPs [2–4]. However, several studies have indicated that MeHg exposure also upregulates GCL [22–28]. This suggests that there is an initial response to MeHg in the cells to compensate for decreased GSH levels and to repress oxidative cell damage. On the other hand, Dr. Yamamoto and his associates reported that transcription factor Nrf2 cooperatively regulates antioxidant proteins such as GCL and HO-1, phase-II xenobiotic detoxifying enzymes, and phase-III xenobiotic transporters such as MRPs [10, 29]. They subsequently found that Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1) [30]. Interestingly, Keap1 has 27 and 25 cysteine residues in humans and mice, respectively [31, 32], and some thiols (e.g., Cys151, Cys273, and Cys288) that have been

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**Table 1: Molecular targets of MeHg.**

<table>
<thead>
<tr>
<th>Target protein</th>
<th>In vivo effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn-SOD</td>
<td>Reduction of catalytic activity in mouse brain</td>
<td>Shinya and colleagues [13]</td>
</tr>
<tr>
<td>nNOS</td>
<td>Induction of NOS in rat brain</td>
<td>Shinya and colleagues [15]</td>
</tr>
<tr>
<td>Arginase I</td>
<td>Reduction of activity in rat liver, Decreased Mn levels in tissues</td>
<td>Kanda and colleagues [18]</td>
</tr>
<tr>
<td>SDH</td>
<td>Not defined</td>
<td>Kanda and colleagues [19]</td>
</tr>
</tbody>
</table>

**Figure 1:** S-mercuration of cellular proteins by MeHg and the MeHg detoxification pathway.

of mRNA and protein synthesis of Mn-SOD were unaffected by MeHg administration. MeHg caused a facile reduction in the activity, and a drastic decrease in the native form, of Mn-SOD (but not of Cu,Zn-SOD) with purified enzyme preparations. It was subsequently shown that the S-mercuration of Mn-SOD, through Cys196, by MeHg results in a decrease in the enzyme activity in vivo [14]. Therefore, the selective inhibition of Mn-SOD activity caused by S-mercuration could play a critical role in MeHg-induced neurotoxicity because SOD protects the cell against oxidative stress by scavenging superoxide.

We also found that the subcutaneous administration of MeHg (10 mg kg$^{-1}$ day$^{-1}$ for 8 days) to rats caused significant increases in nitric oxide synthase (NOS) activities in the cerebrum and cerebellum with time [15]. The increase in NOS activity seems to be caused by an increase in neuronal NOS (nNOS) protein levels, but not an increase in inducible NOS. In contrast to the in vivo observations, however, the addition of MeHg to a cerebellar enzyme preparation in vitro caused a concentration-dependent decrease in nNOS activity, suggesting that MeHg modifies NOS through reactive thiol groups, thereby affecting its catalytic activity in vitro. Experiments with arylmercury column chromatography have confirmed that this is possible [15]. A reasonable explanation for our observations is that the increase in nNOS proteins observed from MeHg exposure in vivo may be one of the initial responses to counteract the metal-induced negative effects on the central nervous system.
Table 2: MeHg modification sites in Keap1.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Position</th>
<th>Peptide sequence</th>
<th>Calculated mass</th>
<th>Observed mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>484–494</td>
<td>LNSAECYYPER</td>
<td>1345.5</td>
<td>1345.1</td>
</tr>
<tr>
<td>2</td>
<td>363–380</td>
<td>SGLAGCVGGGLLYAVGGR</td>
<td>1649.9</td>
<td>1649.6</td>
</tr>
<tr>
<td>3</td>
<td>151–169</td>
<td>CVLHVMNGAVMYQIDSVVR</td>
<td>2135.6</td>
<td>2135.1</td>
</tr>
<tr>
<td>P-1</td>
<td>484–494</td>
<td>+MeHg LNSAECYYPER</td>
<td>+MeHg (214)</td>
<td>1560.5</td>
</tr>
<tr>
<td>P-2</td>
<td>363–380</td>
<td>+MeHg SGLAGCVGGGLLYAVGGR</td>
<td>+MeHg (216)</td>
<td>1863.8</td>
</tr>
<tr>
<td>P-3</td>
<td>151–169</td>
<td>+MeHg CVLHVMNGAVMYQIDSVVR</td>
<td>+MeHg (214)</td>
<td>2350.6</td>
</tr>
</tbody>
</table>

Figure 2: Covalent modification of Keap1 cysteine residues by electrophiles. The BTB, IVR, and DC domains are essential for the formation of homodimers, associated with cullin 3 and the binding of Nrf2. 1,2-NQ: 1,2-naphthoquinone; TBQ: tert-butyl-1,4-benzoquinone.

Established as being highly reactive [31–34], so the "cysteine code," which defines the preferential target cysteine(s) and distinct biological effects, was proposed [35–37]. These observations led us to assume that MeHg could modify Keap1 through S-mercuration and so activate the Nrf2-regulated gene expression of GCL, HO-1, and MRPs.

Figure 3 shows a summary of the Keap1 modification sites, consisting of NTR (N-terminal region), BTB (Board complex, Tramtrack, and Bric-à-brac), IVR (intervening region), and DC (DGR/CTR: double glycine repeat/C-terminal region) domains that can be modified by a variety of electrophiles [31, 33, 38, 39]. Among them, Cys151, Cys273, and Cys288 are well established as being essential for regulating Nrf2 function [31, 32, 40, 41], although other cysteine residues (e.g., Cys257, Cys297, and Cys613) in Keap1 can also be modified by dexamethasone 21-mesylate, biotinylated iodoacetamide, and 1,2-naphthoquinone [31, 33, 38]. Our MALDI-TOF/MS analysis revealed that Keap1 undergoes S-mercuration by MeHg at three cysteine residues, Cys151, Cys368, and Cys489 (see Figure 3 and Table 2), suggesting that the S-mercuration of Cys151 in Keap1 potentially causes a structural alteration, activating Nrf2, while Cys368 and Cys489 are also targets for 1,2-naphthoquinone and tert-butyl-1,4-benzoquinone [38, 39].

As expected, MeHg activated Nrf2 in SH-SY5Y cells in a concentration- and time-dependent manner and upregulated the downstream proteins such as the GCL catalytic subunit (GCLC) and the GCL modifier subunit (GCLM) (Figure 4) [42]. The same observation was seen in primary mouse hepatocytes and HepG2 cells (data not shown). Other researchers also reported that MeHg led to Nrf2 activation, its nuclear accumulation, and upregulation of Nrf2 downstream antioxidant genes such as HO-1 in a variety of cell types [43–45]. Interestingly, Ni et al. demonstrated different response kinetics in astrocytes and microglia upon MeHg treatment [46]. They concluded that these unique sensitivities appear to be dependent on the cellular thiol status of the particular cell type. This viewpoint is consistent with the evidence that...
reactive thiols of Keap1 undergo S-mercuration, resulting in Nrf2 activation. Pretreatment with Trolox, an antioxidant, blocked MeHg-mediated oxidative stress, determined by intracellular reactive oxygen species levels, to a significant degree but did not affect Nrf2 activation during the exposure of SH-SY5Y cells to MeHg (Toyama et al., unpublished data). We therefore speculate that S-mercuration, rather than oxidative stress, is involved in the MeHg-dependent activation of Nrf2.

4. Protection against MeHg Toxicity through the Keap1/Nrf2 Pathway

Several lines of evidence indicate that Nrf2-deficient mice are susceptible to the toxicity of a variety of chemicals, including acetaminophen, 7,12-dimethylbenz[a]anthracene, kainic acid, dextran sulfate sodium, and benzo[a]pyrene [47–52]. To examine the role of Nrf2 in protecting against MeHg, we used primary hepatocytes from Nrf2+/+ or Nrf2−/− mice. As shown in Figure 5(a), steady-state levels of Nrf2 downstream proteins, such as GCLC, GCLM, MRP1 [42], and MRP2 [42], were drastically decreased in Nrf2−/− cells. Under these conditions, the Nrf2−/− cells were highly sensitive to MeHg compared with the Nrf2+/+ cells (Figure 5(b)) [42]. In agreement with these observations, Ni et al. demonstrated that Nrf2 knockdown by the small hairpin RNA approach reduced the upregulation of its downstream genes such as HO-1 in primary astrocytes and microglia and decreased viability for both cells [45, 46]. The accumulation of mercury in the cerebellum, cerebrum, and liver after oral MeHg administration was significantly higher in the Nrf2−/− mice than in the Nrf2+/+ mice (Figure 5(c)) [33] because detoxifying enzymes associated with MeHg excretion were relatively low in the Nrf2−/− mice. Consistent with this result, the Nrf2−/− mice were also highly susceptible to MeHg in vivo (Figure 5(d)). MeHg-induced neuropathological changes in the cerebellum were evaluated in the Nrf2+/+ and Nrf2−/− mice (Figure 6), and the degeneration of Purkinje cells (Figure 6(a)) and vacuolar degeneration of the medulla (Figure 6(b)) were observed in the cerebellum of MeHg-treated mice. Nrf2 deletion clearly increased these types of MeHg-induced degeneration. These results suggest that Nrf2 is a crucial transcription factor for protecting against MeHg toxicity in vitro and in vivo.

5. Conclusions

We found that MeHg S-mercuration reactions on Mn-SOD and arginase I cause dysfunction in the activities of these catalysts and, therefore, possibly cause oxidative stress in mouse brain and the release of Mn from the active site, leading to a decrease in hepatic Mn levels (seen in rats). The evidence showed that MeHg also S-mercurates Keap1 at its reactive thiols, including Cys151, resulting in Nrf2 activation in a variety of cell types, and thereby upregulation of the downstream gene products such as antioxidant proteins, phase II xenobiotic-metabolizing enzymes, and phase III transporters (as shown in Figure 7). Because these Nrf2-regulated gene products contribute to the blocking of oxidative stress and the facilitation of the detoxification and excretion of MeHg, the findings indicate that the Keap1/Nrf2 system plays a critical role not only in the cellular response to MeHg, but also in the suppression of MeHg toxicity in vitro and in vivo.

Conflicts of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

The authors thank Dr. Masayuki Yamamoto, Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, for his contributions to the studies. This work was supported by a grant-in-aid (no. 23117703 to Yoshito Kuma-gai) for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.
Figure 5: Nrf2 confers protection against MeHg toxicity. (a) Total cell lysates (upper) or crude membrane fractions (lower) from primary hepatocytes from Nrf2^{+/+} or Nrf2^{−/−} mice were subjected to western blotting with the antibodies indicated. (b) Primary hepatocytes from Nrf2^{+/+} or Nrf2^{−/−} mice were exposed to MeHg (1, 5, 10, and 20 \(\mu\)M) for 24 h, and then the MTT assay was performed. Each value represents the mean ± SE of three independent experiments. (c) Nrf2^{+/+} or Nrf2^{−/−} mice were orally administrated MeHg (1 mg/kg). After 48 h, the total mercury contents of the cerebellum, cerebrum, and liver were determined by atomic absorption mercury detection. Each value represents the mean ± SE of five independent experiments. (d) Nrf2^{+/+} or Nrf2^{−/−} mice were orally administrated MeHg (5 mg kg^{-1} day^{-1}) for 12 days (\(n=4\)) and mortality was recorded. \(^* P < 0.05\) and \(^{**} P < 0.01\) for the Nrf2^{−/−} mice compared to the Nrf2^{+/+} mice. Partially reprinted from Toyama et al. [42, 53] with the permission of Biochemical Biophysical Research Communications and Environmental Health Perspectives.
References


Research Article

Attenuation of β-Amyloid-Induced Oxidative Cell Death by Sulforaphane via Activation of NF-E2-Related Factor 2

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2 Research Institute of Pharmaceutical Sciences, College of Pharmacy, Kyungpook National University, Daegu 702-701, Republic of Korea

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β-amyloid peptide (Aβ), a major component of senile plaques, plays important roles in neuropathology of Alzheimer’s disease (AD). An array of in vitro and in vivo data indicates that Aβ-induced neuronal death is mediated by oxidative stress. In this study, we aimed to investigate effects of sulforaphane (SUL), an isothiocyanate in cruciferous vegetables, on Aβ-induced oxidative cell death in SH-SY5Y cells. Cells treated with Aβ25–35 exhibited decreased cell viability and underwent apoptosis as determined by MTT assay and TUNEL, respectively. Aβ25–35-induced cytotoxicity and apoptotic characteristics such as activation of c-JNK, dissipation of mitochondrial membrane potential, altered expression of Bcl-2 family proteins, and DNA fragmentation were effectively attenuated by SUL pretreatment. The antiapoptotic activity of SUL seemed to be mediated by inhibition of intracellular accumulation of reactive oxygen species and oxidative damages. SUL exerted antioxidant potential by upregulating expression of antioxidant enzymes including γ-glutamylcysteine ligase, NAD(P)H:quinone oxidoreductase-1, and heme oxygenase-1 via activation of NF-E2-related factor 2 (Nrf2). The protective effect of SUL against Aβ25–35-induced apoptotic cell death was abolished by siRNA of Nrf2. Taken together, the results suggest that pharmacologic activation of Nrf2 signaling pathway by SUL might be a practical prevention and/or protective treatment for the management of AD.

1. Introduction

Alzheimer’s disease (AD) is one of the most common forms of senile dementia, characterized by progressive loss of memory and decline of cognitive functions due to neuronal death in the brain. There are two classical pathological hallmarks of AD [1]. One is extraneuronal accumulation of amyloid plaques composed of β-amyloid peptide (Aβ), which is produced by proteolytic cleavage from amyloid precursor protein (APP) with sequential actions of β- and γ-secretases. The other is intraneuronal deposits of neurofibrillary tangles (NFT) consisting of hyperphosphorylated tau protein generated by actions of upstream kinases such as glycogen synthase kinase-3β (GSK-3β) and cyclin-dependent kinase 5 (CDK5). Therefore, treatments for AD have been developed based on these two molecular approaches [2]. Aβ-based therapies include utilizing β- or γ-secretases inhibitors, Aβ aggregation blockers, and Aβ-catabolism inducers. Tau-based therapies make an advantage of upstream kinase inhibitors, microtubule stabilizers, and tau catabolism inducers.

However, pathogenesis of AD appears to be multifactorial events, whereby genetic as well as environmental factors, oxidative stress, depletion of endogenous antioxidants, altered ion levels, inflammation, disruption in neurotransmission, synaptic dysfunction, and neuronal cell death operate sequentially or in parallel [3]. Among them we have focused on Aβ-induced oxidative damages and neuronal cell death as one of the major causes of AD pathology. Oxidative stress has been proposed to be an important factor in the development and progression of AD and contributes to Aβ aggregation and NFT formation as well [4]. Reactive oxygen species (ROS) can be derived from diverse cellular sources, among which are enzymatic reactions, mitochondrial deterioration, and imbalance in redox transition metal ions. The excessive production and accumulation of ROS by
Aβ can cause functional and structural changes in critical macromolecules leading to lipid peroxidation, protein oxidation, and DNA cleavage and altered signal transduction [5]. The levels of molecular markers for lipid peroxidation (HNE, isoprostanes, etc.) and oxidation of proteins (carboxyls) and DNAs (8-OHdG) are reported to be elevated in the brains or cerebrospinal fluid of patients with AD [6].

Given the involvement of Aβ-induced oxidative stress in the etiology and pathology of AD, one of the promising approaches to preventive interventions for AD includes antioxidant therapy by inhibiting the detrimental effects of excess ROS through induction of endogenous antioxidant enzymes. Particularly, many studies highlighted natural phytochemicals derived from medicinal herbs and foods as potential candidates which can protect neurons against various toxic compounds and exert beneficial effects on neuronal cells [7, 8]. Sulforaphane (4-methylsulfinylbutyl isothiocyanate, SUL) is a naturally occurring isothiocyanate present in cruciferous vegetables, such as broccoli, cabbage, and cauliflower and has been shown to exhibit anticarcinogenic, anti-inflammatory, antioxidant, chemopreventive, and cytoprotective properties [9, 10]. Recently, it has been reported that SUL can penetrate blood brain barrier and exert inhibiting ROS production and oxidative damages.

In human neuroblastoma SH-SY5Y cells via augmentation of in vitro cell culture and in vivo animal models of neurological disorders [11, 12]. SUL has been reported to attenuate microglia-induced inflammation in hippocampus of LPS-treated mice and BV-2 microglia [13]. In addition, SUL protected against oxidative stress induced by hypoxia-ischemic injury [14], oxygen and glucose deprivation [15], 6-hydroxydopamine (6-OHDA) [16], superoxide [17], hydrogen peroxide (H₂O₂), and glutamate [18]. However, there has been no direct evidence demonstrating that the protective effect of SUL against Aβ-induced oxidative damage and cell death as yet.

Therefore, in this study we examined whether SUL can suppress Aβ25–35-induced oxidative damage and cell death in human neuroblastoma SH-SY5Y cells via augmentation of antioxidant defense capacity by activation of NF-E2-related factor 2 (Nrf2) and the subsequent expression of antioxidant and phase II detoxification enzymes which play key roles in inhibiting ROS production and oxidative damages.

2. Materials and Methods

2.1. Chemicals and Reagents. Aβ25–35 and SUL were purchased from American Peptide (Sunnyvale, CA, USA) and LKT Laboratories, Inc. (St. Paul, MN, USA), respectively. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin antibiotic were supplied from Gibco BRL (Grand Island, NY, USA). Tetramethylrhodamine ethyl ester (TMRE) and 2′,7′-dichlorofluorescin diacetate (DCF-DA) dyes were the products of Invitrogen Co. (Carlsbad, CA, USA). Anti-phospho-JNK (p-JNK), anti-JNK, anti-Bcl-2, anti-Bax, anti-γ-glutamylcysteine ligase (GCL), anti-NAD(P)H:quinone oxidoreductase-1 (NQO-1), and anti-Nrf2 antibodies were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Anti-heme oxygenase-1 (HO-1) antibody was provided by Stressgen (Ann Arbor, MI, USA). Anti-4-hydroxynonenal (4-HNE) and anti-phospho-Nrf2 antibodies were supplied from Abcam (Cambridge, MA, USA) and Epitomics, Inc. (Burlingame, CA, USA), respectively. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide], anti-actin antibody, and other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. SH-SY5Y Cell Culture. SH-SY5Y cells were maintained in DMEM media containing 10% FBS, penicillin (10000 U), and streptomycin (100 μg/mL) in a 5% CO₂ incubator at 37°C under a humidified atmosphere. The media were changed every other day. Cells were seeded at an appropriate density according to the experiment scale.

2.3. Cell Viability Assay (MTT Dye Reduction Assay). Cytotoxicity was determined by the conventional MTT dye reduction assay. Cells were seeded in 48-well plate at a density of 5 × 10⁴ cells/well and incubated with Aβ25–35 (15 μM) for 24 h with or without 30 min pretreatment of SUL (1, 2, and 5 μM) or N-acetylcysteine (NAC, 0.5 and 1 mM). After treatment, MTT solution (5 mg/mL) was added and further incubated for 2 h at 37°C. The formazan crystals formed in viable cells were extracted with 200 μL of dimethylsulfoxide (DMSO) and the absorbance was measured in a microplate reader at 570 nm (Emax, Molecular Device, CA, USA). Relative cytotoxicity was calculated as percentage of viable cells with respect to the optical density (OD) value of the living cells in the control as 100%.

2.4. Measurement of DNA Fragmentation (TUNEL). For detection of DNA fragmentation, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Roche diagnostics GmbH, Mannheim, Germany) was performed in SH-SY5Y cells (8 × 10⁴ cells/500 μL in 4-well chamber slide) exposed to 15 μM Aβ25–35 for 24 h in the presence or absence of SUL or NAC pretreatment. The slide was rinsed with phosphate-buffered saline (PBS) three times and fixed in 10% neutral buffered formalin solution for 30 min at room temperature (RT). After incubation with 0.3% H₂O₂ in methanol for 30 min at RT to inactivate endogenous peroxidase, the slide was further reacted with a permeabilizing solution (0.1% sodium citrate and 0.1% Triton X-100) for 2 min at 4°C. The cells were treated with TUNEL reaction mixture for 1 h at 37°C and then labeled with antidigoxigenin peroxidase for additional 30 min at 37°C. After rinsing with PBS three times, color development was performed with 3,3′-diaminobenzidine (Vector Laboratories, CA, USA). The stained images were examined under a light microscope (Leica Microsystems, Wetzlar, Germany).

2.5. Western Bolt Analysis. Cells extracts were prepared by washing cells with PBS and centrifugation at 7,000 g for 5 min. The collected cells were lysed with RIPA buffer (Pierce Biotechnology, Inc., Rockford, IL, USA) and protease inhibitor cocktail tablet (Roche Diagnostics) on ice for 30 min. Protein concentration was quantified by BCA Protein Assay (Pierce Biotechnology). Protein samples were boiled in
SDS sample buffer and separated on SDS-PAGE using 10%–12% acrylamide gels. Subsequently, protein samples were transferred onto polyvinylidene fluoride (PVDF, Roche Diagnostics) membranes by transblot electrophoretic transfer for 3 h at a constant current of 300 mA. The nonspecific binding of antibodies was blocked using 5% (w/v) nonfat milk in PBS containing 0.1% Tween-20 (PBST) for 1 h at RT. After blocking, the membranes were probed with the primary antibodies overnight at 4 °C. The membranes were washed in PBST three times for 10 min each. The corresponding secondary antibodies were diluted in PBST and reacted with the membranes for 1 h at RT. Finally, immunoreactive bands were visualized by chemiluminescence method (Pierce Biotechnology). The images and relative density of immunoreactive bands were analyzed by using ImageQuant LAS 4000 Multi-Gauge software (Fujifilm, Tokyo, Japan).

2.6. Measurement of Mitochondrial Membrane Potential (MMP). For detection of mitochondrial transmembrane potential in SH-SY5Y cells, TMRE probe was utilized. The cells were seeded at a density of 8 × 10^4 cells/500 μL in 4-well chamber slide and treated with 15 μM Aβ25-35 for 24 h in the absence or presence of SUL or NAC. After treatment, cells were washed with PBS and further incubated with TMRE solution (50 μM in PBS) for 15 min at 37 °C. The fluorescence images were recorded and quantified by using a fluorescence microscope (Leica Microsystems) with excitation at 540 nm and emission at 590 nm.

2.7. Measurement of Intracellular ROS Accumulation. To monitor the intracellular accumulation of ROS, the fluorescent probe DCF-DA was used. After treatment of SH-SY5Y cells (8 × 10^4 cells/500 μL in 4-well chamber slide) with Aβ25-35 (15 μM) in the presence or absence of SUL for 6 h, cells were incubated with DCF-DA solution (50 μM in PBS) at 37 °C for 15 min. The fluorescence signals inside cells were excited at 488 nm and emission was monitored at 535 nm. The images were recorded with a fluorescence microscope (Leica Microsystems).

2.8. Protein Oxidation. The levels of protein carbonyls were determined by using OxyBlot Protein Oxidation Detection Kit (Millipore, MA, USA) according to the manufacturer’s instruction. Briefly, the protein samples (15 μg) were denatured by SDS (6% final concentration) and then derivatized to 2,4-dinitrophenylhydrazone (DNPH-hydrazone) by incubation with 2,4-dinitrophenylhydrazine (DNPH) for 15 min at RT. After adding neutralization solution, the samples were electrophoresed on a 10% SDS-PAGE gel and transferred to PVDF membrane. The membrane was incubated with blocking buffer for 1 h to reduce nonspecific binding and then reacted with anti-DNP primary antibody for 1 h at RT. After two times washing with PBST, the membrane was further incubated with HRP-conjugated secondary anti-rabbit antibody for 1 h at RT. The carbonylation bands were detected by using chemiluminescence method (Pierce Biotechnology).

2.9. Nuclear Protein Extraction. Nuclear protein extracts were prepared by using the Nuclear Extraction Kit (Chemicon, Inc., MA, USA). After treatment, SH-SY5Y cells were washed with ice cold PBS and harvested by centrifugation. The harvested cells were resuspended in ice-cold cytoplasmic lysis buffer, incubated on ice for 15 min, and centrifuged at 8,000 g for 20 min at 4 °C. The pellet was resuspended in ice-cold nuclear extraction buffer, incubated on ice for 60 min using shaker, and centrifuged at 16,000 g for 7 min at 4 °C. The supernatant containing nuclear proteins were stored at −80 °C for western blot analysis and electrophoretic mobility shift assay (EMSA). Protein concentrations were determined by Bradford assay (BIO-RAD, CA, USA).

2.10. Electrophoretic Mobility Shift Assay (EMSA). The DNA binding activity of Nrf2 to antioxidant response element (ARE) was assessed by LightShift Chemiluminescent EMSA kit according to the procedure provided from Pierce Biotechnology. The isolated nuclear protein samples were combined with binding mixture (1 μg poly (dl-dc), 50% glycerol, 1% NP-40, 1 M KCl, 100 mM MgCl2, and 200 mM EDTA (Pierce Biotechnology)) and incubated on ice for 20 min. Subsequently, biotin-labeled oligonucleotide specific to Nrf2 (5'-TGGGGAACTGTGCTGACTGGAG-3', Panomics, CA, USA) was added to the reaction mixture and additionally incubated for 10 min at RT. The DNA-protein complexes were separated on the 6% nondenaturing polyacrylamide gel at 80 V for 1 h and then transferred to nylon membrane (Pall Co., MI, USA) at 380 mA for 45 min. The membrane was subjected to immediate cross-linking by transilluminator at 312 nm for 10 min. After blocking the membrane with blocking buffer for 15 min at RT, the membrane was incubated with stabilized streptavidin-HRP for 15 min at RT. After three times washing with wash buffer, the DNA-protein complex bands were detected by chemiluminescence method (Pierce Biotechnology).

2.11. Synthetic Small Interfering RNA (siRNA) Transfection. For the knockdown experiments of Nrf2, SH-SY5Y cells were transiently transfected with siRNA of Nrf2 (Nrf2-siRNA) using DOTAP transfection reagent (Roche Diagnostics GmbH) in accordance with the manufacturer’s protocol. The sequences of the sense and antisense strands of the human Nrf2-siRNA were as follows: 5'-AAG AGU AUG AGC UGG AAA AAC TT-3' (sense) and 5'-GUU UUU CCA GCU CAU ACU TT-3' (antisense) which were selected by siRNA Target Finder software provided by Invitrogen. After transfection of SH-SY5Y cells with Nrf2-siRNA, cells were further exposed to Aβ25-35 (15 μM) for 24 h in the presence or absence of SUL (5 μM) pretreatment and then cell viability and molecular markers for apoptotic cell death were examined.

2.12. Statistical Analysis. SPSS software 13.0 (SPSS, Inc, Chicago, IL, USA) was used for the statistical analysis. All data represent at least three independent experiments and are expressed as mean ± SD. Statistical comparisons between groups were made by one-way analysis of variance (ANOVA).
at value of $P$ for additional 24 h. Pretreatment of SUL protected against $A_{35}$-induced cytotoxicity and apoptosis (Figure 1(a)). SUL-treated cells exhibited significantly higher cell viability than $A_{25}$-treated group. However, pretreatment of SUL dramatically reduced $A_{35}$-induced cytotoxicity in a concentration-dependent manner (Figure 1(a)). SUL-treated cells exhibited significantly higher cell viability than $A_{35}$-treated group did. In addition, $A_{25}$-induced apoptotic cell death was effectively suppressed by the pretreatment with SUL as assessed by TUNEL, which detects DNA fragmentation in situ, a typical marker for apoptosis (Figure 1(b)). SUL significantly reduced the number of TUNEL-positive cells caused by $A_{25}$ treatment.

We also confirmed the protective effect of SUL against $A_{25}$-induced apoptotic cell death by examining pro- or antiapoptotic signals, such as activation of JNK, expression of Bcl-2 family proteins, and dissipation of mitochondrial membrane potential (MMP). $A_{35}$-induced apoptosis of SH-SY5Y cells was accompanied by activation of JNK via phosphorylation (Figure 2(a)) and a decreased Bcl-2 as well as an increased Bax protein levels (Figure 2(b)). However, pretreatment of SUL dramatically reduced $A_{35}$-elevated phosphorylation of JNK and expression of pro-apoptotic protein Bax. Moreover, anti-apoptotic protein Bcl-2 levels were effectively upregulated by SUL pretreatment. $A_{25}$-treatment also led to disruption of MMP as assessed by using TMRE cationic probe, which was shown as low fluorescence intensity compared with control group (Figure 2(c)). However, SUL pretreatment effectively restored $A_{25}$-decreased TMRE fluorescence intensity up to control levels representing recovery from the dissipation of MMP.

3. Results

3.1. Protective Effect of SUL against $A_{25}$-Induced Cytotoxicity and Apoptotic Cell Death. We have investigated the effect of SUL on $A_{25}$-induced cytotoxicity and apoptotic cell death in SH-SY5Y cells by MTT dye reduction assay and TUNEL staining, respectively. Cells were incubated with various concentrations of SUL (1 $\mu$M, 2 $\mu$M, and 5 $\mu$M) for 30 min followed by 15 $\mu$M $A_{25}$ treatment for additional 24 h. Pretreatment of SUL protected against $A_{25}$-induced cytotoxicity in a concentration-dependent manner (Figure 1(a)). SUL-treated cells exhibited significantly higher cell viability than $A_{25}$-treated group did. In addition, $A_{25}$-induced apoptotic cell death was effectively suppressed by the pretreatment with SUL as assessed by TUNEL, which detects DNA fragmentation in situ, a typical marker for apoptosis (Figure 1(b)). SUL significantly reduced the number of TUNEL-positive cells caused by $A_{25}$ treatment.

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3.2. Inhibitory Effect of SUL on $A_{25}$-Induced ROS Production and Subsequent Oxidative Damages. It has been reported that $A_{25}$-induced cytotoxicity and apoptotic cell death are mediated by oxidative stress. In another experiment, $A_{25}$-induced cytotoxicity (Figure 3(a)) and apoptotic cell death such as DNA fragmentation (Figure 3(b)) and impairment of MMP (Figure 3(c)) were effectively suppressed by pretreatment with NAC (0.5 mM and 1 mM), a glutathione (GSH) precursor with strong antioxidant activity. Based on the involvement of oxidative stress in $A_{25}$-induced apoptosis
**Figure 2:** Protective effect of SUL on Aβ25-35-induced pro-apoptotic signals. SH-SY5Y cells were exposed to 15 µM of Aβ25-35 in the presence or absence of SUL (2 µM and 5 µM) for 24 h. Activation of JNK (a) and expression of Bcl-2 family proteins (b) were assessed by western blot analysis using anti-phospho-JNK, anti-JNK, anti-Bcl-2, anti-Bax, and anti-actin antibodies. Actin levels were monitored to verify equal amount of protein loading. Relative expression levels of p-JNK/JNK and Bax/Bcl-2 were quantified from three independent experiments and are represented on the right panels. (c) Mitochondria membrane potential was measured by immunofluorescence staining using TMRE probe. The representative images of TMRE fluorescence were shown. (A) Vehicle-treated control; (B) Aβ25-35 alone (15 µM); (C) Aβ25-35 (15 µM) + SUL (5 µM).

In SH-SY5Y cells, in the next experiment we have examined the effect of SUL on Aβ25-35-induced ROS formation. Cells were pretreated with SUL for 30 min before incubation with Aβ25-35 (15 µM) for additional 6 h. Aβ25-35 treatment led to intracellular accumulation of ROS, which was attenuated by SUL pretreatment (Figure 4(a)) as assessed by relative fluorescence intensity of DCF-DA dye. The results indicated that SUL could inhibit Aβ25-35-induced ROS production in SH-SY5Y cells.

It is well known that ROS can cause oxidative stress to critical cellular macromolecules such as DNA, protein, and lipids. In the present study, treatment of Aβ25-35 (15 µM) caused oxidative damages to lipids (Figure 4(b)) and proteins in SH-SY5Y cells (Figure 4(c)), which were measured by formation of 4-HNE and protein carbonyls, respectively. 4-HNE and protein carbonyls are indicators of oxidative stress and key markers for oxidation of lipid and protein. Aβ25-35-induced lipid peroxidation (Figure 4(b)) and protein...
oxidation (Figure 4(c)) were substantially reduced by pretreatment of these cells with SUL.

3.3. Augmentation of Cellular Antioxidant Defense Capacity by SUL via Activation of Nrf2. To investigate molecular mechanisms of neuroprotection exerted by SUL against Aβ25-35-induced oxidative cell death, we have assessed expression levels of cellular antioxidant enzymes such as GCS, NQO-1, and HO-1. SH-SY5Y cells were treated with 5 μM SUL for the indicated time periods, and protein levels of GCS, NQO-1, and HO-1 were determined by western blot analysis using specific antibodies. As shown in Figure 4(d), the expression of GCS and NQO-1 was increased by SUL treatment in a time-dependent manner which peaked at 24 h. In addition, HO-1 protein levels increased from 3 h after SUL treatment and were maintained up to 12 h (Figure 4(d)). All these results indicated that SUL could induce the expression of antioxidant enzymes to protect cells from oxidative damages caused by Aβ25-35 in SH-SY5Y cells.

To elucidate upstream regulator for the SUL-induced up-regulation of the antioxidant enzymes, we have focused on the activation of redox-sensitive transcription factor Nrf2. When SH-SY5Y cells were treated with 5 μM SUL for the indicated times, nuclear translocation (Figure 5(a)), ARE-DNA binding (Figure 5(b)), and phosphorylation (Figure 5(c)) of Nrf2 were assessed by western blot analysis and EMSA. Treatment of SH-SY5Y cells with SUL increased nuclear levels of Nrf2 (Figure 5(a)) and Nrf2 binding to ARE promoter sequence (Figure 5(b)) with similar kinetic patterns. Moreover, SUL treatment increased phosphorylation of Nrf2 at Ser-40 residue as well (Figure 5(c)), which is known to facilitate the dissociation of Nrf2 from Keap1 rendering its translocation to nucleus.

To further verify the direct role of Nrf2 in mediating the cytoprotective effect of SUL against Aβ25-35-induced oxidative cell death, we have downregulated the Nrf2 expression by transient transfection of SH-SY5Y cells with Nrf2-siRNA. The cellular protection of SUL on Aβ25-35-induced cytotoxicity (Figure 6(a)) and DNA fragmentation (Figure 6(b)) were abolished by knockdown of Nrf2 gene with Nrf2-siRNA. Moreover, the protective effect of SUL on Aβ25-35-mediated proapoptotic signals such as decreased MMP (Figure 6(c)) and increased Bax/Bcl-2 ratio (Figure 6(d)) and subsequent oxidative damages to lipids determined by 4-HNE formation (data not shown) were substantially abrogated by Nrf2-siRNA.
transfection. These results suggest a critical role of Nrf2 in SUL-mediated protection against Aβ25-35-induced apoptotic cell death.

4. Discussion

In this study, we have examined the protective effect and molecular mechanism of SUL against Aβ25-induced oxidative and apoptotic cell death. The results from the MTT assay and apoptotic analysis (TUNEL) provided a direct evidence demonstrating that SUL could protect SH-SY5Y cells from Aβ25-35-induced toxicity through increasing cell viability as well as inhibiting the apoptotic cell death. We also have assessed the effect of SUL on the Aβ25-35-induced pro-apoptotic signals such as activation of JNK and increased ratio of Bax to Bcl-2. Pretreatment of SUL elevated the anti-apoptotic Bcl-2 protein levels, decreased the pro-apoptotic Bax protein expression, and attenuated JNK activation via inhibition of its phosphorylation.

It has been reported that Aβ25-35-induced cytotoxicity was mediated by oxidative stress. The excessive production of ROS by Aβ25-35 and exhaustion of the endogenous antioxidant defense system including GSH, catalase, superoxide dismutase, and glutathione metabolizing enzymes can cause oxidative damages to critical cellular macromolecules, mitochondrial dysfunction, and altered cellular signal transduction cascades. In the present study, Aβ25-35 treatment led to intracellular accumulation of ROS in SH-SY5Y cells, which was effectively inhibited by pretreatment with SUL. Moreover, SUL could alleviate Aβ25-35-induced oxidative damages including formation of 4-HNE and protein carbonyls through decreasing ROS production. Dissipation of MMP reflects the opening of the mitochondrial permeability transition pore due to the ROS release from mitochondria [19]. In this study, during the apoptotic cell death induced by Aβ25-35, MMP generated by the gradient of ion concentrations between two sides of the mitochondrial membrane was decreased, whereas SUL pretreatment restored the dissipation of MMP. In accordance with our finding, it has been reported that SUL increases the resistance of liver mitochondria to redox-regulated permeability transition pore opening and elevates expression of antioxidant proteins involved in mitochondrial defense against oxidative stress [20].

As the accumulation of ROS can trigger imbalance of redox state, neuronal cells have a set of antioxidant defense enzymes that maintain homeostasis between them. Therefore, one way to render neuronal cells more resistant to Aβ-induced oxidative cell death is to potentiate the endogenous antioxidant defense system, for instance, to up-regulate an array of antioxidant enzymes. In the present
Increasing evidence supports the role of quinones, preventing their redox cycling and eventually electron reduction of quinones to the redox-stable hydro-
critical causes. Because homeostasis of GSH and GSH-dependent enzymes are considered to be key determinants of antioxidant protection, dysregulation of GSH-related antioxidant network might bring about the initiation and progression of neurodegenerative diseases where oxidative stress is one of critical causes.

NQO-1 is a cytosolic flavoprotein that catalyzes the two-electron reduction of quinones to the redox-stable hydro-
quinoles, preventing their redox cycling and eventually generating the ROS. Increasing evidence supports the role of NQO-1 as a safety valve to sequester ROS and prevent severe oxidative damages in various neuronal disorders including AD, HO-1, known as heat shock protein 32, plays a crucial role in endogenous defense against oxidative stimuli-induced brain injuries by decomposing toxic heme into carbon monoxide, iron, and biliverdin. Biliverdin is subsequently converted into bilirubin through the action of biliverdin reductase and these two molecules serve as potent radical scavengers protecting cells from oxidative damages. The pharmacological up-regulation of HO-1 expression in brain regions showed promising therapeutic effects in the models of neurodegenerative diseases and brain infections.

To further elucidate the upstream regulators for the induction of endogenous antioxidant defense enzymes against oxidative stress, we have focused on the Nrf2-ARE signaling pathway. Recently, abundant evidence suggests the protective functions of Nrf2 and Nrf2-regulated gene products in diverse neuronal disorders. Considering that Nrf2 mediates general antioxidant responses, Nrf2 could be a potential therapeutic target for neurodegenerative diseases, where cells are suffering from chronic state of oxidative stress. Under normal quiescent state, Nrf2 is sequestered in the cytoplasm by a cytoskeletal associated specific negative regulator, Kelch-like ECH associating protein 1 (Keap1). Upon exposure to ROS or xenobiotics, Nrf2 is liberated from Keap1, translocates from the cytosol to the nucleus, heterodimerizes with accessory proteins such as small Maf protein family, and sequentially binds to antioxidant response element (ARE) promoter region. The binding of Nrf2 to ARE induces the production of diverse antioxidant enzyme and phase II detoxifying genes such as GCL, glutathione-S-transferase (GST), UDP-glycosyltransferases (UGTs), HO-1, and NQO1, which protect cells against oxidative stress as well as a wide range of other toxins.

In the present study, the cytoprotective effect of SUL against Aβ25-35-induced oxidative damage and cell death seemed to be mediated by up-regulation of antioxidant enzymes through Nrf2 activation. SUL has been considered as an indirect antioxidant because of its ability solely to induce many cytoprotective antioxidant enzymes through the Nrf2-ARE pathway. Induction of the Nrf2-ARE pathway by SUL has been reported to prevent cytotoxicity caused by oxygen and glucose deprivation, 6-OHDA, superoxide, H2O2 and glutamate, 5-S-cysteinyldopamine, or Aβ1-42 in neuronal cell lines as well as primary cultures. Furthermore, activation of the Nrf2-ARE pathway is able to protect against brain injuries in the animal models of neurodegenerative diseases, spinal cord injury, focal cerebral ischemia, hypoxia-ischemic injury, traumatic brain injury, subarachnoid hemorrhage, or epilepsy. According to in vivo studies, strategies to potentiate Nrf2-ARE pathway by SUL were proved to be useful in improving memory impairment and cognitive dysfunction caused by traumatic brain injury or Aβ.

Conversely, Nrf2 KO mice models of neurological disorders including Parkinson’s disease, spinal cord injury, traumatic brain injury, intracerebral hemorrhage, or epilepsy. According to in vivo studies, strategies to potentiate Nrf2-ARE pathway by SUL were proved to be useful in improving memory impairment and cognitive dysfunction caused by traumatic brain injury or Aβ.

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and epilepsy [43] exhibited increased susceptibility to neurological oxidative damages but did not maintain any benefits from the protective effects of SUL. In our experiment, the protective effect of SUL against Aβ25-35 caused apoptotic cell death was abolished by down-regulation of Nrf2 gene by transient transfection with Nrf2-siRNA.

Although the molecular milieu of SUL-induced Nrf2 activation in SH-SY5Y cells has not been elucidated, two possible mechanisms for the activation of Nrf2-ARE pathway by SUL have been proposed in other types of cells. One is structural change of Keap1 due to the modification of specific cysteine residues by binding of SUL [29, 45]. The other is the phosphorylation of Nrf2 at Ser-40 residue by mitogen-activated protein kinases [46], protein kinase C [46], and phosphatidylinositol 3-kinase/Akt activated by SUL [31, 47, 48]. Nrf2 phosphorylation by aforementioned kinases triggers the release of Nrf2 from inhibitory Keap1, thereby facilitating the Nrf2 translocation to nucleus. However, phosphorylation of Nrf2 at Tyr-568 residue by GSK-3β can promote its nuclear exclusion or proteolysis [46, 49]. Nevertheless, the molecular signaling pathways activating Nrf2 appears to be pleiotropic and dependent on cell types as well as stimuli.

5. Conclusions
In conclusion, a phytochemical SUL attenuates Aβ25-35 induced oxidative stress and pro-apoptotic signals such as activation of JNK, an increase in pro-apoptotic Bax, and a decrease in anti-apoptotic Bcl-2, thereby inhibiting apoptotic neuronal cell death in SH-SY5Y cells. Moreover, SUL induced the activation of Nrf2-ARE signaling pathway, which
consequently results in up-regulation of Nrf2-dependent antioxidant capacity, leading to reduction in the Aβ25–35- induced oxidative damages (Figure 7). Taken together, the results in the present study suggest pharmacologic activation of the Nrf2 signaling pathway by SUL might be a practical preventative and therapeutic strategy for AD patients. However, further studies are required to obtain more insights into the molecular mechanisms of SUL-induced Nrf2 activation and clinical application of SUL.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors’ Contribution

Chan Lee and Gyu Hwan Park contributed equally to this work.

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References


Roles Nrf2 Plays in Myeloid Cells and Related Disorders

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The Keap1-Nrf2 system protects animals from oxidative and electrophilic stresses. Nrf2 is a transcription factor that induces the expression of genes essential for detoxifying reactive oxygen species (ROS) and cytotoxic electrophiles. Keap1 is a stress sensor protein that binds to and ubiquitinates Nrf2 under unstressed conditions, leading to the rapid proteasomal degradation of Nrf2. Upon exposure to stress, Keap1 is modified and inactivated, which allows Nrf2 to accumulate and activate the transcription of a battery of cytoprotective genes. Antioxidative and detoxification activities are important for many types of cells to avoid DNA damage and cell death. Accumulating lines of recent evidence suggest that Nrf2 is also required for the primary functions of myeloid cells, which include phagocytosis, inflammation regulation, and ROS generation for bactericidal activities. In fact, results from several mouse models have shown that Nrf2 expression in myeloid cells is required for the proper regulation of inflammation, antitumor immunity, and atherosclerosis. Moreover, several molecules generated upon inflammation activate Nrf2. Although ROS detoxification mediated by Nrf2 is assumed to be required for anti-inflammation, the entire picture of the Nrf2-mediated regulation of myeloid cell primary functions has yet to be elucidated. In this review, we describe the Nrf2 inducers characteristic of myeloid cells and the contributions of Nrf2 to diseases.

1. Introduction

NF-E2-related factor like-2 (Nrf2) is a transcription factor that activates a battery of genes that protect cells from reactive oxygen species (ROS) or toxic electrophiles [1, 2] (Figure 1). Nrf2 activity is strictly regulated through the stress sensor protein Keap1 (Kelch-like ECH-associated protein 1). Under unstressed conditions, Nrf2 is captured by Keap1 in the cytosol and is constitutively ubiquitinated and degraded by the proteasome [3–5]. By contrast, under stressed conditions, Nrf2 is captured by Keap1 in the cytosol and is constitutively ubiquitinated and degraded by the proteasome [3–5]. By contrast, under stressed conditions, Keap1 senses stress or environmental insults and stops the degradation of Nrf2, resulting in the accumulation and nuclear translocation of the Nrf2 protein [6]. In the nucleus, Nrf2 dimerizes with small Maf proteins (sMaf), and the Nrf2-sMaf heterodimer binds to antioxidant/electrophile responsive elements (AREs/EpREs) to activate the expression of target genes [7, 8].

The chemicals that activate Nrf2 and Nrf2 inducers are structurally diverse but share a common electrophilic nature [9]. Of note, these inducers interact with certain reactive cysteine residues of Keap1 [10], which contains 25 cysteine residues [11]. This electrophilic modification results in the inhibition of the ubiquitin ligase activity of Keap1 [5, 12]. Typical Nrf2 inducers include diethyl maleate (DEM), tert-butyldihydroquinone (tBHQ), sulforaphane (SFN), and 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) derivatives [13]. In addition, upon the development of inflammation, several Nrf2-activating molecules accumulate, including 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) [14], nitric oxide (NO), and NO-derived products [15–20]. In the following chapter, we will focus on how the Keap1-Nrf2 system responds to inflammatory signals in myeloid cells.

In initial analyses, Nrf2 was found to regulate the expression of many antioxidant and detoxifying enzymes and proteins [1, 21, 22]. For example, genes encoding glutathione S-transferases (GSTs) and NAD(P)H:quinone oxidoreductase 1 (Nqo1) are the prime targets of Nrf2 regulation [23]. Glutathione peroxidase 2 (Gpx2), glutamate cysteine lyase catalytic and regulatory subunits, and heme oxygenase-1 (Hoe-1) are also target genes of Nrf2 [24–26]. This list of Nrf2 target genes reveals that Nrf2 is critical for the maintenance of redox homeostasis within cells. In fact, Nrf2 deficiency in mice leads to oxidative stress conditions that cause DNA damage and cell death [27, 28].
Additionally, recent analyses have revealed that Nrf2 also regulates genes that are essential for cellular metabolism, cell proliferation, selective protein degradation, and immune response [29–32]. Regarding primary myeloid cell functions, inflammatory regulation and phagocytosis are also associated with the Keap1-Nrf2 pathway.

2. Nrf2 Activation Mediated by Inflammation-Related Molecules

Two prevalent inflammatory signaling cascades, that is, the cyclooxygenase (COX)-2 pathway and the NO synthesis pathway, generate Nrf2-activating molecules (Figure 2). COX-2 catalyzes arachidonic acids and produces various bioactive prostaglandins. One of the COX-2 pathway products is 15d-PGJ2. Importantly, 15d-PGJ2 binds directly to cysteine residues of Keap1. 15d-PGJ2 is primarily produced by macrophages for inflammation resolution; thus, abrogating 15d-PGJ2 production with COX-2 inhibitors causes persistent neutrophil infiltration in carrageenan-induced pleurisy [14].

Although one type of Nrf2 inducers, including DEM, tBHQ, and SFN, modify cysteine residue 151 (Cys151) of Keap1, 15d-PGJ2 interacts with cysteine residues 273/288 (Cys273/288) of Keap1 [33, 34]. PGA2, another prostaglandin, also activates Nrf2 by binding to Cys273/288 [33]. In addition to these prostaglandins, COX-2 produces electrophilic ω3-fatty acid derivatives from eicosapentaenoic acids and docosahexaenoic acids, which induces Nrf2 and expression of its target genes in macrophages [35].

Although it is well accepted that NO activates Nrf2 by modifying Keap1, it remains to be clarified whether NO modifies Keap1 directly or through the generation of reactive nitrogen oxide species (RNOS). Consistent with the direct modification hypothesis, NO S-nitrosylates Cys151 of Keap1 [15, 16]. By contrast, the indirect hypothesis is supported by the observation that NO generates RNOS that nitrosylate cGMP and produce 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP) [17]. S-guanylation of Keap1 at Cys434 by 8-nitro-cGMP abrogates the Keap1-mediated inhibition of Nrf2 [18]. Alternatively, nitro fatty acids (OA-NO2) are produced by RNOS through the nitration of unsaturated fatty acids. OA-NO2 modifies Keap1 cysteines, primarily Cys273/288 [19, 20]. Thus, the Keap1-Nrf2 system appears to respond to multi-way signaling mechanisms utilizing NO.

3. Inflammatory Regulation by Nrf2 in Myeloid Cells

Nrf2 deficiency in myeloid cells provokes ROS accumulation, as is the case for other cell lineages. Excessive ROS affect inflammatory regulation in myeloid cells (Figure 3), as ROS activate various inflammatory signaling pathways. One such pathway is the NFκB (nuclear factor kappa B) pathway, the most potent activator of innate immunity, which induces the expression of various proinflammatory cytokines [36, 37]. ROS also enhance the translocation of TLRs (Toll-like receptors) to lipid rafts, in which signaling molecules cluster to effectively activate downstream signals [38, 39]. TLR accumulation in lipid rafts enhances inflammatory signals through the NFκB and IRF3 (interferon regulatory transcription factor 3) pathways [39]. Consistently, macrophages from Nrf2-deficient mice show an increase in the LPS-induced activation of TLRs and NFκB signaling, leading to an elevated expression of proinflammatory cytokines [40].

Intriguingly, the origins of ROS appear to differ between myeloid cells and other cell lineages. In macrophages and neutrophils, ROS are generated by the NADPH oxidase...
4. Nrf2 Regulates Phagocytosis

Phagocytosis is one of the myeloid-specific functions regulated by Nrf2. Nrf2-deficient myeloid cells show a decrease in phagocytosis and bactericidal activity, whereas Keap1-deficient myeloid cells in which Nrf2 is activated show an increase in these functions [41]. The decrease in phagocytosis in Nrf2-deficient mice is attributable to the absence of the LPS-induced expression of the scavenger receptor Marco, whose expression is also regulated by Nrf2 [42].

In this regard, it is interesting to note that Nrf2 regulates the differentiation of various types of cells. In 3T3-L1 cells, Nrf2 induces Cebpβ gene expression by binding to an ARE in its upstream promoter region and activating adipogenesis [43]. Because C/EBPβ also regulates the differentiation of myeloid cells [44], we hypothesize that the Nrf2-C/EBPβ axis may contribute to myeloid lineage differentiation.

5. Nrf2 and Acute Inflammation

Nrf2 expression in myeloid cells is tightly associated with a wide range of inflammation-related diseases. Of note, the Nrf2 contribution to myeloid cells is well known in a number of acute inflammation models, in which Nrf2 suppresses inflammation. For example, in lung inflammation models, Nrf2-deficient mice display more severe lung inflammation induced by cigarette smoke [45] and hyperoxia [46, 47] than wild-type mice, resulting in delayed recovery from emphysema. Nrf2-deficient mice also show worsened pneumonia caused by Staphylococcus aureus infection [48]. The antigen-specific immune response induced by sensitization to ovalbumin in a well-recognized asthma model is also aggravated by Nrf2 deficiency [49].

In addition to lung injury models, experimental sepsis has been exploited for the study of the Nrf2 contribution to acute inflammation. In Nrf2-deficient mice, sepsis caused by cecal ligation and puncture (CLP) gives rise to increased mortality compared with wild-type mice [50]. Endotoxin shock induced by the injection of a lethal dose of LPS leads to similar results, supporting the hypothesis that increased mortality in Nrf2-deficient mice is due to a hyper-activated inflammatory response but not a deficiency in bacterial killing ability. Because pretreatment with an antioxidant, N-acetylcysteine (NAC), improves the survival of Nrf2-deficient and wild-type mice after LPS-induced sepsis, the exacerbated inflammation in Nrf2-deficient mice appears to be attributable to excess ROS [50].

In these acute inflammation models, Nrf2 expression in myeloid cells is required for anti-inflammation. In bone marrow transplantation assays, recipient mice transplanted with Nrf2-deficient bone marrow cells display exacerbated porcine pancreatic elastase-induced emphysema similar to conventional Nrf2-deficient mice, although they have wild-type epithelial cells [51]. Similarly, the myeloid-specific deletion of Nrf2 leads to increased sepsis severity, whereas the myeloid-specific activation of Nrf2 through Keap1 conditional deletion leads to alleviated septic inflammation [41]. These observations clearly show that Nrf2 is an important regulator of acute inflammation in myeloid cells. Regarding the molecular basis, the Nrf2-mediated elimination of ROS seems to contribute to this process.

6. Antitumor Immunity and Nrf2

As shown in Figure 4, one of the most intriguing findings in recent Nrf2 analyses is that Nrf2 supports antitumor immunity [52]. In the absence of Nrf2, tumor-supporting Gr1+CD11b+ cells, designated as myeloid-derived suppressor
cells (MDSCs) [53], show a higher activity to attenuate the T cells involved in antitumor immunity than in the presence of Nrf2. Therefore, Nrf2 suppresses tumor cell development in the microenvironment. This function of Nrf2 in myeloid cells is in contrast to the phenomena in tumor cells, in which Nrf2-activation has been widely recognized to support tumor cell survival.

In chemical carcinogenesis experiments, Nrf2 has been considered to encourage cancer chemoprevention, and Nrf2 appears to be the key tumor-preventing transcription factor. In fact, Nrf2 detoxifies ROS and cytotoxic electrophiles that cause DNA damage. In some chemical carcinogenesis models, Nrf2 deficiency has been shown to increase the frequency of tumor occurrence [54–56].

However, somatic mutations in Keap1 and Nrf2 that interrupt Keap1-Nrf2 association and lead to constitutive Nrf2 activation are frequently detected in human cancers [57–59]. The latter observation indicates that Nrf2 activation is beneficial to the selfish growth of cancer cells. An important recent discovery is that this tumor-supporting effect of Nrf2 is mediated by not only the enhancement of cellular protection from stress but also the redirection of metabolic pathways to nucleotide synthesis in support of rapid cellular proliferation [29]. Taken together, Nrf2 protects normal cells from tumorigenesis but also helps the growth and survival of already developed tumors.

By contrast, Nrf2 expression in myeloid cells is required for repressing tumors. In myeloid cells surrounding tumors, Nrf2 eliminates ROS in MDSCs that attenuate antitumor immunity [52]. Metastasis experiments using the intravenous injection of Lewis lung carcinoma (3LL) cells clearly indicate that Nrf2 deficiency increases the metastasis of 3LL cells to the lung. Consistent with this observation, Nrf2 activation by Keap1 knockdown results in a reduction in the metastasis of 3LL cells; this observation was reproducible in a second cell line, melanoma-derived B16-F10. Bone marrow transplantation has revealed that the proper expression and function of Nrf2 are required in myeloid cells to suppress the metastasis of 3LL cells.

In tumor-bearing Nrf2-deficient mice, both the number and the ROS levels of MDSCs are increased. The putative mechanisms for the tumor suppressive activity of MDSCs depend on diverse mediators including NO, peroxynitrite produced from NO and superoxide anion, and ROS [53]. As intracellular ROS levels are elevated in Nrf2-deficient MDSCs, we hypothesize that the Nrf2 deficiency activates MDSCs possibly through an increase in ROS accumulation, resulting in the repression of antitumor immunity and an enhancement of metastasis. The demonstration of this link remains to be established.

7. Atherosclerosis and Nrf2

In contrast to many other inflammatory disorders alleviated by Nrf2, atherosclerosis is exacerbated by Nrf2. However, the molecular basis of this phenomenon has yet to be elucidated. In apolipoprotein E- (ApoE-) deficient atherosclerotic mice, multiple investigators have revealed that Nrf2 deficiency reduces atherosclerotic lesions in both high-fat diet (HFD)- and normal chow-fed ApoE-deficient mice [60–63] (Figure 4). No apparent change is found in the blood glucose levels, plasma lipid levels, or body weights of these mice, except that HFD-fed ApoE-deficient mice show increases in serum triglycerides and glucose caused by the Nrf2 deficiency [60]. Similar to the conventional genetic deletion of Nrf2, the transplantation of Nrf2-deficient bone marrow cells reduces atherosclerotic lesions in ApoE-deficient recipient mice, indicating that Nrf2 induction in myeloid cells is proatherogenic [63].

The Nrf2-mediated exacerbation of atherosclerosis may be attributable to the upregulation of CD36 expression by Nrf2 [64]. CD36 is a scavenger receptor required for the uptake of oxidized lipids by macrophages; thus, the increase in CD36 expression is expected to promote foam cell transformation and atherosclerotic plaque formation. Nrf2 activates CD36 gene expression by binding to an ARE located upstream of exon 1A1 [65]. In peritoneal macrophages, CD36 expression is activated in response to oxidized low-density lipoprotein (LDL) in an Nrf2-dependent manner [64]. In ApoE and Nrf2 double-knockout mice, CD36 expression is downregulated under both HFD- and normal chow-fed conditions. Consequently, these mice are less atherosclerotic than ApoE single-knockout mice [60, 61]. These observations suggest that CD36 downregulation by Nrf2 deficiency prevents atherosclerosis in these mice.

Consistent with this hypothesis, the reduced uptake of oxidized LDL has been observed in Nrf2-deficient peritoneal macrophages compared with wild-type macrophages [61, 64]. However, this point is controversial, as similar levels of oxidized LDL uptake were observed between Nrf2-deficient
and wild-type macrophages in a different study [62]. Furthermore, in another study, an increased uptake of modified LDLs was observed in Nrf2-deficient macrophages [66]. Thus, the mechanisms underlying the association between CD36 expression and modified LDL uptake by macrophages remain unknown.

An alternative mechanism has been suggested for the proatherogenic function of Nrf2. Cholesterol crystals induce the production of the proinflammatory cytokines IL-1α and IL-1β in the presence of Nrf2 but not in Nrf2-deficient macrophages [62]. Importantly, the improvement of atherosclerosis in Nrf2-deficient mice disappeared when IL-1α and IL-1β were neutralized by antibodies. These results support the contention that these cytokines are important for mediating the proatherogenic function of Nrf2.

It is interesting to note that in clear contrast to the situation in ApoE-deficient mice, LDL receptor (Ldlr) and Nrf2 double-deficient mice have exacerbated atherosclerotic phenotypes compared with Ldlr single-knockout mice. The bone marrow transplantation of Nrf2-deficient cells into HFD-fed Ldlr-deficient mice exacerbates atherosclerosis compared with the transplantation of wild-type cells [66, 67]. These observations indicate that Nrf2 is anti-atherogenic in myeloid-derived cells of Ldlr-deficient mice.

Nrf2 deficiency in peritoneal macrophages upregulates the expression of two receptors, scavenger receptor A and lectin-like oxidized LDL receptor-1, that contribute to the uptake of modified lipids in response to oxidized lipids [66]. Similarly, lipid uptake and Il6 expression are also upregulated in Nrf2-deficient peritoneal macrophages compared with wild-type macrophages [66]. Collectively, Nrf2 appears to play anti-atherogenic roles in Ldlr-deficient myeloid cells. However, because of the complexity of the atherogenic processes, which involves neutrophil infiltration, lipid uptake, inflammation and death of macrophages, a mechanistic understanding of how Nrf2 affects atherogenic processes remains elusive. Nonetheless, currently available data demonstrate that Nrf2 is definitely involved in the control of atherosclerosis.

8. Concluding Remarks

The Keap1-Nrf2 system is essential for protecting animals from environmental stresses. In myeloid-derived cells, phagocytosis and inflammatory regulation appear to be regulated by the Keap1-Nrf2 system. Nrf2 deficiency influences various functions of myeloid cells, which protect animals through anti-inflammatory and antitumor immunity activities. The functions of Nrf2 in both the progression of and protection against atherosclerosis remain elusive, and further studies are important.

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Mechanisms of Nrf2/Keap1-Dependent Phase II Cytoprotective and Detoxifying Gene Expression and Potential Cellular Targets of Chemopreventive Isothiocyanates

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Isothiocyanates (ITCs) are abundantly found in cruciferous vegetables. Epidemiological studies suggest that chronic consumption of cruciferous vegetables can lower the overall risk of cancer. Natural ITCs are key chemopreventive ingredients of cruciferous vegetables, and one of the prime chemopreventive mechanisms of natural isothiocyanates is the induction of Nrf2/ARE-dependent gene expression that plays a critical role in cellular defense against electrophiles and reactive oxygen species. In the present review, we first discuss the underlying mechanisms how natural ITCs affect the intracellular signaling kinase cascades to regulate the Keap1/Nrf2 activities, thereby inducing phase II cytoprotective and detoxifying enzymes. We also discuss the potential cellular protein targets to which natural ITCs are directly conjugated and how these events aid in the chemopreventive effects of natural ITCs. Finally, we discuss the posttranslational modifications of Keap1 and nucleocytoplasmic trafficking of Nrf2 in response to electrophiles and oxidants.

1. Regulation of Nrf2-Dependent Gene Expression by Natural Isothiocyanates

Natural Isothiocyanates (ITCs) are abundantly found in cruciferous vegetables such as broccoli, watercress, Brussels sprouts, cabbage, and cauliflower [1]. Epidemiological studies have shown that consumption of cruciferous vegetables is inversely associated with the risk of many types of cancer [2]. Anticarcinogenic properties of cruciferous vegetables might be attributed to their high content of glucosinolates and the composition of the glucosinolates among cruciferous vegetables differs, depending on the plant species, climates, and other agricultural conditions [3]. Glucosinolates in cruciferous vegetables exist as N-hydroxysulfate with sulfur-linked β-glucose together with various side chains [4]. Naturally occurring glucosinolates are converted into isothiocyanates (ITCs) with a physical stress, such as chopping or chewing of cruciferous vegetables, which in turn leads the plant cell wall to rupture and release the plant-specific enzyme myrosinase, converting the natural glucosinolates into ITCs [5]. Naturally occurring ITCs, including phenethyl ITC (PEITC), allyl ITC (AITC), benzyl ITC (BITC), and sulforaphane are effective cancer chemopreventive compounds in humans (Figure 1) [6]. While many dietary chemopreventive compounds (e.g., curcumin, resveratrol, and epigallocatechin gallate (EGCG)) possess polyphenolic moiety, chemopreventive ITCs are structurally distinct in that they are characterized by –N=C=S functional group [7].

The anticarcinogenic mechanisms of ITCs include a variety of biochemical mechanisms, such as cell cycle arrest, apoptosis induction, activation of anti-inflammatory programs, inhibition of cytochrome P450s for carcinogen activation, and modulation of the activities of various transcriptional factors, including NF-E2-related factor 2 (Nrf2) [8]. Nrf2 is a member of cap‘n’collar (CNC) family of basic leucine zipper (bZIP) transcription factor that allows cells to mediate a collective activation of phase II cytoprotective and detoxifying enzymes [9]. Phase II cytoprotective and detoxifying enzymes are implicated in the generation of cellular reduced glutathione (GSH), detoxification of reactive
Figure 1: Chemical structure of selected natural isothiocyanates (ITCs).

Figure 2: Nrf2 and Keap1 protein domains.

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Figure 1: Chemical structure of selected natural isothiocyanates (ITCs).

Figure 2: Nrf2 and Keap1 protein domains.

oxygen species (ROS), and drug metabolism in response to environmental electrophiles and oxidants [10]. Under normal condition, Nrf2 is constantly polyubiquitinated and degraded by proteasome through Cullin-3- (Cul3-) dependent E3 ubiquitin ligase enzyme. Exposure of electrophiles and oxidants inactivates Cul3-dependent E3 ubiquitin ligase enzyme in the cytosol by poorly characterized biochemical mechanisms and stabilizes Nrf2 protein, leading to its nuclear translocation and transcriptional activation by binding to the antioxidant response element (ARE), a cis-acting enhancer sequence TGA(G/C)NNNGC in the genome through heterodimerization with small Maf proteins [11]. Nrf2 activity is tightly regulated in the cytosol by Kelch-like ECH associating protein 1 (Keap1) as a scaffolding protein for Nrf2 as well as an adaptor protein for Cul3-dependent E3 ubiquitin ligase enzyme [12]. Analysis of Keap1-deficient mice has proven that Keap1 plays a central role in the repression of Nrf2 activity in vivo [13]. In addition, loss of Keap1 activity as a result of somatic mutations has been reported in a significant proportion of cancer patients, implying that constitutive activation of Nrf2 may have an important role in the elevated cytoprotective activity of human malignancy [14].

Nrf2 possesses 6 conserved Neh2-ECH homology (Neh) domains (Figure 2(a)). The Neh1 domain contains a basic leucine-zipper (bZIP) structure, required for DNA binding in association with small Maf proteins in the nucleus. The Neh2 domain is located in the most N-terminal region and exerts a negative effect on the ARE-dependent gene expression by binding to Keap1 protein. The Neh4 and Neh5 domains constitute transactivation domains that contribute to ARE-dependent gene activation by binding to coactivators, such as CBP and p300, and are essential for Nrf2 transactivation [15]. The Neh3 domain, located in the most C-terminal region, is known to play a permissive role in Nrf2 transactivation for the Neh4 and Neh5 domains. The Neh6 domain, located between the transactivation domain (the Neh4 and Neh5 domains) and the DNA binding domain (the Neh1 domain), is known to be necessary for the degradation of Nrf2 protein [16]. Keap1 is a negative regulator of Nrf2 protein by binding to the Neh2 domain of Nrf2 and was initially identified by a yeast two-hybrid assay [17]. Keap1 protein is a cytosolic protein and comprises 5 different domains: an amino-terminal region (NTR), a Broad complex, Traumtrack and Bric a bric (BTB) domain, an intervening region (IVR), six Kelch/double glycine repeats (DGRs), and a carboxy-terminal region (CTR) (Figure 2(b)) [18]. Structural analysis has shown that Keap1 proteins heterodimerize each other through the BTB domain, and the overall heterodimers resemble a "cherry-bob" structure [19]. Covalent modification of cysteine residues in Keap1 protein is believed to constitute a stress-sensing mechanism for electrophiles and oxidants, and the covalent binding of several electrophiles and thiol group(s) in Keap1 protein has been observed in vitro, including sulforaphane [20]. Structural observations and biophysical experiments have led to the conclusion that (1) the ratio between Keap1 and Nrf2 binding is 2:1 and
(2) the regulatory mechanism of Nrf2 and Keap1 system conforms to the so-called "hinge and latch" model, in which two distinct binding sites in the Neh2 domain of Nrf2 protein mediates high-affinity (the ETGE motif) and low-affinity (the DLG motif) interactions with a single Keap1 protein, respectively [21].

2. Indirect and Direct Protein Targets of Natural ITCs

Until now, the exact biochemical mechanisms by which ITCs activate Nrf2-dependent gene expression are largely unclear. However, there is an increasing number of evidence, showing that ARE-dependent transcriptional gene activation by ITCs is mediated, at least in part, by the activation of various intracellular signaling cascades, including the mitogen-activated protein kinase (MAPK) [22]. MAPK is one of the major signaling systems, which transmits various extracellular signals into the nucleus through a cascade of serial intracellular protein phosphorylation and is known to be responsible for the activation of ARE-dependent gene expression [23]. MAPK consists of three family members: extracellular signal-related kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAPK. MAPK is phosphorylated and activated by upstream signaling kinase modules, for example, MAPK kinase (MAPKK or MEK) and MAPKKK kinase (MAPKKK or MEKK). Upon activation, MAPK is phosphorylated in both threonine (T) and tyrosine (Y), existing in the TXY motif of activation loop and the central amino acid (X) is a defining amino acid motif for individual MAPKs: glutamic acid (E) for ERK, proline (P) for JNK, and glycine (G) for p38 MAPK [24]. Earlier studies have demonstrated that overexpression of wild-type ERK2 and JNK1 significantly elicited ARE-dependent luciferase activation and the addition of natural ITCs, including PEITC and sulforaphane, could potentiate the ARE-dependent gene expression, implying that upregulation of Nrf2/ARE-dependent gene expression by natural ITCs is mediated by MAPK pathway [25, 26]. While the positive regulation of Nrf2/ARE-dependent gene expression by ERK and JNK has been unequivocally supported by follow-up studies [27, 28], the exact role of p38 MAPK pathway in the ARE-dependent gene expression is still controversial, although a direct binding and phosphorylation residue(s) of Nrf2 protein by p38 MAPK has been demonstrated [29, 30]. In addition, the experimental evidence showing the direct phosphorylation and the exact residue(s) of Nrf2 or Keap1 protein by activated MAPK is still lacking. Therefore, it seems likely that the modulation of Nrf2/ARE-dependent gene expression by MAPKs is indirect.

Phosphatidylinositol 3-kinase (PI3K) is another intracellular signaling kinase that is implicated in the regulation of Nrf2/ARE-dependent gene expression. Earlier studies have demonstrated that PI3K and its downstream Ser/Thr kinase, Akt can positively regulate ARE-dependent gene expression. While there was a lack of evidence whether PI3K and Akt can directly phosphorylate Keap1 or Nrf2 protein and modulate the activity of ARE-dependent gene expression, Cuadrado and colleagues have demonstrated that active glycogen synthase kinase-3β (GSK3β) can directly phosphorylate and suppress the activity of Nrf2 protein by causing its nuclear exclusion [31]. GSK3β, a direct downstream target of Akt, is activated in response to growth factors and external oxidants such as H₂O₂ [32]. Because GSK3β activity is negatively regulated by Akt-mediated phosphorylation at Ser-9, it is possible to assume that PI3K-mediated Akt activation might cause a phosphorylation and inactivation of GSK3β, thereby promoting Nrf2 nuclear translocation and activation by relieving GSK3β-mediated negative regulation of Nrf2 activity. In addition, a novel phosphodegron motif, existing in the Neh6 domain of Nrf2 (DSGIS residues 334 to 338) was identified in the subsequent study, in which Nrf2 protein is destabilized as a consequence of its phosphorylation by GSK3β and subsequent recognition and polyubiquitination by Cul3/Skp1/β-TrCP E3 ubiquitin ligase enzyme, but not by Cul3/Keap1 E3 ubiquitin ligase enzyme [33]. In addition, Jaiswal and colleagues have identified that Fyn kinase can directly phosphorylate Nrf2 protein at Tyr-568 and promote its nuclear exclusion and degradation, thereby contributing to the suppression of ARE-mediated gene expression [34]. They also showed that GSK3β acts as an upstream kinase of Fyn that contributes to phosphorylation of Nrf2 protein at Tyr-568 [35]. Therefore, it seems likely that the PI3K-Akt-GSK3β axis regulates Nrf2-mediated ARE-dependent gene activation both in direct and indirect manners: GSK3β directly phosphorylates the phosphodegron motif existing in the Neh6 domain of Nrf2 protein and it leads to Keap1-independent, but β-TrCP-dependent proteasomal degradation of Nrf2 protein or GSK3β phosphorylates and activates Fyn kinase, leading to phosphorylation and an indirect nuclear exclusion of Nrf2 protein. At present, whether and, if it is so, how natural ITCs modulate GSK3β or Fyn kinases to Nrf2-dependent ARE activation is currently unknown. In addition to MAPK and PI3K/Akt/GSK3β/Fyn cascades, protein kinase C (PKC) and PKR-like endoplasmic reticulum kinase (PERK) are the other intracellular kinases to directly phosphorylate Nrf2 protein and modulate ARE-dependent gene expression. PKC directly phosphorylates Nrf2 protein at Ser-40 [36] and upregulates the Nrf2-mediated ARE activation by perturbing the interaction between Nrf2 and Keap1 proteins [37]. PERK can directly phosphorylate Nrf2 protein following the accumulation of unfolded proteins of endoplasmic reticulum, although the exact phosphorylation residue(s) were unidentified [38]. While it is largely unclear how Nrf2 phosphorylation contributes to ARE-dependent gene expression, Apopa et al. have provided interesting results, showing that treatment of tert-butyhydroquinone (tBHQ) elicited casein kinase 2-(CK2-) mediated phosphorylation of Nrf2 protein, thereby facilitating its nuclear translocation and activation of ARE-dependent gene expression [39]. This fact implies that Nrf2 phosphorylation might be closely associated, at least in part, with the nucleocytoplasmic trafficking of Nrf2 in cells.

As mentioned earlier, the chemopreventive mechanisms of ITCs are diverse and it is likely due to the fact that ITCs readily react with the nucleophilic amino acid residues. Based on this conjecture, Chung and colleagues have attempted to find out whether ITCs can directly react with cellular DNA, RNA, and proteins. To this end, they have exposed
14C-PEITC and 14C-sulforaphane in cultured cells and purified nucleotides or target proteins, using phenol/chloroform extraction or two-dimensional electrophoresis (2D-GE) followed by matrix-assisted laser desorption-ionization mass-spectrometry (MALDI-MS) [40]. As a result, they found that no discernable DNA or RNA was bound to radiolabeled ITCs, suggesting that nucleotides are unlikely direct targets for ITCs [41]. In contrast, several putative protein targets to which ITCs can be directly conjugated were identified. They include cellular reduced glutathione (GSH), tubulin, transient receptor potential channel, phosphatases (M3/6 and cdc25c), MEKK1 kinase, and transcriptional factors, such as activator protein-1 (AP-1), signal transducer and activator of transcription factor 3 (STAT3), and mutant p53 [42]. It is known that ITCs can be directly conjugated to thiol group-containing cysteine, amine group-containing lysines, arginines, proline, serines, threonine, and tyrosine. Among them are cysteines which are the most likely binding sites for ITCs and cysteine residues in the above-mentioned proteins are the possible conjugation candidates [43]. In addition, finding out the direct binding proteins for ITCs has been attempted in an alternative manner by taking advantage of affinity chromatography technique. To this end, HeLa cell lysates were incubated with biotin-labeled ITCs, separated with streptavidin-sepharose beads, and sent for mass spectrometry analysis. This approach was useful in revealing the direct conjugation of ITCs with a number of novel proteins, including macrophage-inhibitory factor (MIF) [44]. However, this approach has its weakness in that ITCs are strong electrophiles, and a false-positive binding of ITCs with nontarget protein(s) might likely occur.

3. Direct or Indirect Modulation of Keap1/Nrf2 Proteins by ITCs

ITCs are strong chemical inducers of ARE-dependent gene expression. Therefore, it is possible to assume that ITCs might be able to induce ARE-dependent gene expression by altering the interaction between Keap1 and Nrf2 proteins through a direct conjugation with cysteine residues in Keap1 or Nrf2 protein. In particular, Keap1 is a cysteine-rich protein (27 for human and 25 for mouse) with 4.3% of all residues being cysteines that exceed the average percentage of cysteine residues in proteins [45]. Because cysteines generally constitute the functional and redox-sensitive domains of proteins in response to the changes in the local environment [46], cysteine residues in Keap1 protein were proposed to be the prime mechanism, by which they selectively respond to a variety of electrophiles and oxidants. This hypothesis was supported by the observation that universal ARE inducers can react with cysteine thiol groups of Keap1 at rates that correlated with their potency of ARE-dependent gene activation, irrespective of their chemical structures [47]. It was shown that reactive cysteines were mostly located in the linker region, located between the BTB domain and the Kelch-repeats in Keap1 protein. The selective modification of cysteine residues in Keap1 protein by structurally similar Nrf2 chemical inducers led to the so-called hypothesis “cysteine code” or “multiple-sensor mechanism” [48]. Unlike Keap1, however, Nrf2 protein was excluded as a sensor for electrophiles or oxidant in this model because it contained no cysteines in the Neh2 domain. Nonetheless, it should be noted that observing a direct binding between Nrf2 chemical inducers and Keap1 protein was made in the test tube, using a recombinant protein. In addition, the experimental evidence that natural ITCs could be directly conjugated to any of cysteine residues in cellular Keap1 and Nrf2 proteins is still lacking. In this sense, Takaya et al. have recently observed that a point mutation of cysteine 151 resulted in a reduced Nrf2 activation in response to several Nrf2 inducers, including sulforaphane, but not to other inducers such as CDDO-Im and cadmium chloride [49]. This fact suggests a potential role for cysteine 151 of Nrf2 protein in sulforaphane-mediated ARE activation, although it is unclear yet whether this residue serves as a direct binding site for sulforaphane.

By now, significant attention has been focused on the modification of cysteine residues in Keap1. However, it is also possible to envisage that Nrf2 cysteine modification can serve as another potential mechanism for ARE-dependent gene regulation. To this end, He and Ma have demonstrated that selected evolutionary conserved cysteine residues in Nrf2 can be directly modified by arsenic or phenylarsine oxide (PAO), and these residues are important for its binding to ARE-dependent gene expression. This raises an interesting possibility that direct modification of Nrf2 amino acid residue(s) by ARE inducers constitutes an alternative mechanism for ARE activation [50]. In another study, Li et al. have identified a potential nuclear export sequence (NES) motif in the Neh5 transactivation domain of Nrf2 protein and observed that mutating cysteine residue at 183 position into alanine (C183A) abrogated the NES function of Nrf2, rendering a nuclear accumulation of Nrf2 [51]. In addition, several potential NES sequence motifs together with putative nuclear localization sequence (NLS) motifs were identified in the Nrf2 protein sequence [52]. Interestingly, Li et al. showed that EGFP-tagged Nrf2 segment (amino acids 162–295), in which a putative NES exists exhibited a cytosolic pattern and that an exposure of oxidants or electrophiles, including sulforaphane could alter subcellular localization of EGFP-tagged Nrf2 segment. This result suggests that this NES sequence is redox sensitive [51]. In contrast, they have conducted analogous experiments and demonstrated that some NES/NLS motifs might be redox insensitive; the subcellular localization of these NES/NLS is unaltered by treatment of many electrophiles and oxidants [53]. Collectively, these studies show that multiple NES/NLS motifs play an important role in the nucleocytoplasmic localization of Nrf2 protein and suggest that Nrf2 protein by itself might be able to behave as a Keap1-independent sensor. However, it is still uncertain whether these residues are direct targets of natural ITCs. Therefore, whether these cellular cysteine residues in Keap1 and/or Nrf2 serve as direct targets of ITCs requires experimental validations. More importantly, whether and, if so, how cysteine modifications of Keap1 and Nrf2 by ITCs are linked to phosphorylation-mediated regulation of Keap1 and/or Nrf2 activities needs to be further clarified.
4. Concluding Remark

To adapt to their aerobic lifestyle, mammals have developed an elaborate in vivo defense and metabolizing enzyme system. As mentioned earlier, Keap1/Nrf2-regulated gene expression of phase II cytoprotective and detoxifying enzymes is one of such prime cytoprotective mechanisms, and we are already aware that natural ITCs exploit this pathway to exert chemopreventive effects in humans. In addition, we have provided an overview of current knowledge regarding the direct and/or indirect cellular targets for ITCs. As mentioned earlier, whether cellular Nrf2 and/or Keap1 proteins are direct targets of ITCs is currently unknown, and there is a great deal of research conducted to fill this knowledge gaps, to the best of our knowledge. Recent analysis of Nrf2 interactome and regulome also highlights an enormous array of potential targets of natural ITCs and suggests that chemopreventive mechanisms, exerted by chemopreventive ITCs, might be much more complex than initially imagined [54].

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Research Article

Novel Hematopoietic Target Genes in the NRF2-Mediated Transcriptional Pathway

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Nuclear factor- (erythroid-derived 2) like 2 (NFE2L2, NRF2) is a key transcriptional activator of the antioxidant response pathway and is closely related to erythroid transcription factor NFE2. Under oxidative stress, NRF2 heterodimerizes with small Maf proteins and binds cis-acting enhancer sequences found near oxidative stress response genes. Using the dietary isothiocyanate sulforaphane (SFN) to activate NRF2, chromatin immunoprecipitation sequencing (ChIP-seq) identified several hundred novel NRF2-mediated targets beyond its role in oxidative stress. Activated NRF2 bound the antioxidant response element (ARE) in promoters of several known and novel target genes involved in iron homeostasis and heme metabolism, including known targets FTL and FTH1, as well as novel binding in the globin locus control region. Five novel NRF2 target genes were chosen for followup: AMBP, ABCB6, FECH, HRG-1 (SLC48A1), and TBXAS1. SFN-induced gene expression in erythroid K562 and lymphoid cells were compared for each target gene. NRF2 silencing showed reduced expression in lymphoid, lung, and hepatic cells. Furthermore, stable knockdown of NRF2 negative regulator KEAP1 in K562 cells resulted in increased NQO1, AMBP, and TBXAS1 expression. NFE2 binding sites in K562 cells revealed similar binding profiles as lymphoid NRF2 sites in all potential NRF2 candidates supporting a role for NRF2 in heme metabolism and erythropoiesis.

1. Introduction

NRF2 (encoded by nuclear factor-erythroid p45-related factor 2, NFE2L2) is the master regulator of antioxidant and phase II detoxification genes that collectively resist cellular damage due to electrophilic and oxidative stress [1, 2]. Oxidative stress can be caused by an imbalance between the production of reactive oxygen and the system’s ability to detoxify reactive intermediates or repair any damage. Under non-stressed conditions, NRF2, a member of the cap’n’collar family of basic leucine zipper transcription factors, is repressed in the cytosol by Kelch-like ECH-associated protein 1 (KEAP1) [3–6]. In the event of oxidative stress or other stimuli, NRF2 dissociates from KEAP1, translocates into the nucleus, forms heterodimers with small Maf and other bZIP proteins, and binds the NRF2 antioxidant response element (ARE; TGAntcaGC) in promoter regions of downstream detoxifying enzymes. Known examples of NRF2 gene targets include hemeoxygenase-1 (HMOX1), NAD(P)H: quinone oxidoreductase-1 (NQO1), glutathione S-transferase (GST) [7–10]. Several studies have indicated that NRF2 potentially regulates genes in other pathways such as protein transport, phosphorylation, cell cycle, and growth [11–15]. In addition, chromatin immunoprecipitation (ChiP) deep sequencing of DNA (ChiP-seq) performed in mouse embryonic fibroblasts [16] and human lymphoblastoid cells [17] revealed several hundred novel NRF2-mediated genes that are involved in cellular processes other than oxidative stress such as adipogenesis [17]. In our study, NRF2 was activated in lymphoblastoid cell lines with
sulforaphane treatment, a dietary isothiocyanate that acts as a phase II enzyme inducer through the NRF2 pathway [12]. We identified 849 NRF2 occupied peaks, 242 of which were considered high confidence with 96% of those containing one or more AREs [17]. Putative and novel NRF2-regulated genes were characterized by integrating bioinformatics, expression microarray, additional independent ChIP real-time quantitative polymerase chain reaction (qPCR), and NRF2 short interfering RNA (siRNA) silencing in multiple cell lines for selection of potential candidates for followup. Pathway analysis revealed genes involved in apoptosis, immune response, retinoid signaling, heme metabolism, and iron homeostasis. The potential role of NRF2 in heme metabolism and iron homeostasis was intriguing since NRF2 is structurally related to nuclear factor-erythroid derived 2 (NFE2), a transcription factor that is essential for erythroid differentiation and is specifically expressed in hematopoietic progenitors and lineages [18]. NFE2 dimerization partners overlap with NRF2 such as small Maf proteins, and NFE2 and NRF2 bind the same APl-like core motif consensus sequence [17,19]. In addition, we reported previously [17] that 112 of 242 (~46%) high confidence peaks colocated with ENCODE erythroleukemic cell line K562 NRF2 bound regions, [20] further supporting a possible role of NRF2 in heme metabolism and erythropoiesis.

Mature erythrocytes or red blood cells (RBCs) are packed with hemoglobin (Hb) which transport oxygen from the lungs to peripheral cells and tissues. Made of four subunits as a tetramer, adult Hb consists of two alpha-globin and two beta-globin chains, each chain consisting of an iron-containing heme group that can bind one oxygen molecule [21–23]. During RBC hemolysis in both normal and disease conditions, Hb can escape resulting in free heme and iron [24, 25]. Excess heme and iron have the ability to damage lipids, proteins, and DNA through oxidation and the production of reactive oxygen species (ROS) which can create a positive hemolytic feedback loop [23]. In addition to the well-studied NRF2 target gene HMOX1, which plays a large role in the heme degradation pathway, several known NRF2-regulated genes are involved in iron homeostasis, such as ferritin (FTH1 and FTL). In our ChIP-seq study and in the ENCODE ChIP database, the promoter regions of these genes showed high confidence NRF2-bound peaks with NFE2 peak overlap in K562 cells [17, 26, 27]. Moreover, we saw novel NRF2/NFE2 colocated binding site occupation in the globin locus control region near the β-locus for epsilon globin (HBEI) and gamma globin (HBG) indicating that NRF2 may be involved in erythroid differentiation (Figure 2(b)).

In order to further assess the relationship between NRF2 and NFE2 in erythroid cells, we selected five novel NRF2 candidate target genes identified by overlap between NRF2 ChIP-seq in lymphoid cells and NFE2 ChIP-seq in K562 cells. These genes are known to be involved in heme metabolism and iron homeostasis, and we evaluated their protein and expression levels under a variety of conditions. Candidate NRF2 target genes included α,-microglobulin/bikunin precursor (AMBPI), ATP-binding cassette (ABC) transporter 6 (ABCB6), ferrochelatase (FECH), solute carrier family 48, member 1 (SLC48A1) more commonly known in the literature as heme responsive gene 1 (HRG1), and thromboxane A synthase (TBXAS1). Using our SFN-induced NRF2 ChIP-seq results, we compared ENCODE NFE2 binding peaks, chromatin state (ChromHMM), RNA-seq data, and conservation tracks in order to assess each potential NRF2-regulated candidate. In addition, we activated NRF2 by sulforaphane treatment in K562 cells and compared candidate gene expression in cells of lymphoid or erythroid origin treated with SFN or with NRF2/KEAP1 gene silencing. Candidate genes were also assessed by protein analysis in K562 SFN treated cells as well as in two different stable KEAP1-silenced cell lines.

2. Materials and Methods

2.1. Cell Culture. Human lymphoblastoid cells (LCLs; Coriell) were grown in RPMI with 15% fetal bovine serum (FBS; Gemini) and 1X antibiotics/antimycotics (Life Technologies) in 5% CO2 at 37°C. Following ATCC recommendations, airway epithelial BEAS-2B (ATCC CRL-9609) and erythroleukemic K562 (ATCC CCL-243) cells were cultured in 10% FBS with 1X antibiotics/antimycotics. HepG2 cells (ATCC HB-8065) were grown in MEM plus 10% FBS, 2.5 mM L-glutamine (Life Technologies), and 1X antibiotics/antimycotics, and A549 cells (ATCC CCL-185) were cultured in Ham’s F12K supplemented with 10% FBS and 1X antibiotics/antimycotics. Cells were treated with 0.1% DMSO or 10 μM sulforaphane (SFN) for either 8 (NRF2 siRNA experiments) or 24 hours at 37°C before isolating RNA.

2.2. Chromatin Immunoprecipitation (ChIP) and ChIP-Seq. ChIP, sequencing results, and data analysis were previously described [17].

2.3. Real-Time Quantitative Polymerase Chain Reaction (qPCR). RNA was isolated from cells using Qiagen’s RNeasy kit per manufacturer’s instructions including the on-column DNA digestion step. Quantification of RNA via Qubit (Invitrogen) was followed by cDNA synthesis using the SuperScript First-Strand Synthesis System (Invitrogen). Target amplification was performed in at least triplicate using Applied Biosystems (ABI) primer/probe mixes or custom designed SYBR primers and Universal PCR Master Mix (ABI). Initial fluorescence (Ro value) was calculated using a method of Peirson et al. [28]. Several primers have been previously described [17]. In addition, TaqMan gene expression assays were used for HRG1 (SLC48A1; Hs00215236), BACT (Hs99999903), and I8S (Hs99999901). Custom exon junction spanning SYBR primers were designed for beta-globin (Fwd5'-GGTGTTCTACCCCTTGGGACCC-3', Rev5'-GATACTTGTGGCCA-GGGCA-3'), NFE2 specific primers (Fwd5'-CAGAGCAGG AACGGGGTAT-3', Rev5'-TGGAGTCCAGGGTGAG-3'), and TBXAS1 (Fwd5'-CTCCCTACTGGGTGCA-GC-3', Rev5'-ATAGCCACGATGAGGAAGA-3'). All measurements were reported as target values normalized to BACT or I8S as average values ± standard error of the mean.

2.4. NRF2 Gene Silencing (NRF2KD). NRF2 was silenced transiently in BEAS-2B, HepG2, and A549 cells with a
reverse transfection protocol using Ambion siRNAs. Non-specific scramble control (control; AM4643) and three NRF2 siRNA (ID no. 115764) were tested for NRF2 knockdown by transient transfection using Xtreme Gene (Roche) for HEAS-2B or Lipofectamine 2000 (Invitrogen) reagent for HepG2 and A549 cells following manufacturer’s instructions as previously described [17]; ID no. 115764 silencing was most effective and used for further analysis.

2.5. shRNA KEAP1 Knockdown (KEAPIKD). Mission shRNA lentiviral particles (Sigma) were used to stably silence KEAP1 (SHVRS-NM_012289) in K562 cells following the manufacturer’s protocol. Briefly, K562 cells (10⁵ cells/well) were plated in six-well plates the day before transduction. Cells were transduced with either five different shRNA clones targeting KEAP1 or a scrambled non-target control (Scr, SHC002V) in several multiplicity of infections (MOI) to get maximum transduction efficiency in the presence of hexadimethrine bromide (8 𝜇g/mL) and incubated overnight. Cells containing viral particles were replaced with fresh complete media the next day and allowed to grow overnight. To select the shRNAi transduced cells, media were removed and replaced with complete media containing 2 𝜇g/mL puromycin (Invitrogen). Puromycin-resistant cells containing transduced clones were grown for several passages and tested for KEAPI expression by qPCR to determine maximum silencing efficiency (data not shown). The most effective clone (C1) was then selected and maintained in complete media supplemented with puromycin to perform downstream qPCR experiments.

2.6. Western Blot. K562 cells were grown in 150 mm dishes and treated with 0.1% DMSO or 10 𝜇M SFN for 5 hours. In addition, K562 shRNA KEAP1-silenced cells from two different clones (C1 and C2) as well as the non-target shRNA control (Control) were grown and collected for protein. Total protein was isolated by incubating cell pellets in RIPA buffer with 5% milk (1 hour, room temp). After the final washes, membrane was incubated for one minute in ECL solution (GE Healthcare) and exposed to film. All membranes were incubated with β-actin as a loading control.

3. Results

3.1. SFN Induces NRF2 but not NFE2 in Erythroid Cells. In order to establish whether NRF2 mRNA was inducible by SFN in erythroid cells, K562 and lymphoid GM12878 cells were treated with 10 𝜇M SFN for 24 hours, total RNA was extracted, and qPCR was carried out to measure NRF2, the NRF2 target gene NQO1, and NFE2. Figure 1(a) shows that NRF2 expression was induced by treatment in both lines, and NQO1 displayed significant 6-fold (K562) and 8.3-fold (GM12878) increases over the respective untreated control (Figure 1(b)). Interestingly, we saw no SFN-induced NFE2 expression changes in K562 cells and, as expected, little to no NFE2 expression in lymphoid cells (Figure 1(c)). NFE2 is known to be exclusively expressed in hematopoietic progenitors and lineages [18]. Although SFN did not induce NFE2 expression in undifferentiated K562 cells, studies have used dimethyl sulfoxide (DMSO) in murine erythroleukemia (MEL) cells to significantly increase NF-E2 activity which resulted in erythroid differentiation [29]. Testing SFN or other NRF2 inducers in differentiated erythroid cells would provide additional insight into the inducibility of NFE2.

3.2. Lymphoid NRF2 ChIP-seq Peak Regions Colocate with ENCODE K562 NFE2 ChIP-seq Peaks. We examined SFN-induced NRF2 binding locations in the HMOXI gene using our lymphoid NRF2 ChIP-seq and ENCODE data tracks displayed in UCSC genome browser (Figure 2(a)). In addition to SFN-induced lymphoid NRF2 and untreated K562 NFE2 ChIP-seq tracks, we display ENCODE/Broad tracks showing chromatin state segmentation using the Hidden Markov Model (ChromHMM), RNA-seq profiles, as well as an evolutionary conservation track using the Genomic Evolutionary Rate Profile (GERP) [30] (Figure 2). ChromHMM profiles for GM12878 (lymphoid), K562 (erythroid), and HepG2 (hepatic) are based on the 15 chromatin state annotations determined by multiple histone ChIP-seq experiments and indicate the relationship between chromatin state, DNA function (e.g., active promoter, enhancer, repressed), and gene expression [27]. All three cells lines displayed open active promoters (red), strong enhancers (orange) under the 5′ proximal peak, and active transcription (green/lt green) in the gene body. The HMOXI genomic region revealed two occupied sites with colocated ChIP-seq peaks (blue boxes) for lymphoid NRF2 and erythroid NFE2, strongly suggesting that these related transcription factors share identical binding sites and binding motifs (Figure 2(a)). In addition, the conservation track showed strong conservation and the response element displays perfect human/rodent homology. RNA-seq tracks indicated slightly more expression in HepG2 cells as reflected by the transcription association state (green) compared to the weak transcription (light green) seen in both the ChromHMM GM12878 and K562 track. Display of the lymphoid NRF2 occupancy and ENCODE tracks allowed
**Figure 1:** NRF2, NQO1, and NFE2 gene expression in K562 and lymphoid GM12878 cells. (a) NRF2 mRNA from K562 and GM12878 cells treated for 24 hours with 10 μM SFN increased compared to control. (b) Known NRF2-regulated gene NQO1 expression significantly increased in both K562 and GM12878 SFN-treated cells. (c) NFE2-specific gene expression indicated that NFE2 was specific to erythroblastic K562 cells but not inducible upon SFN treatment. Values were normalized to IBS, and fold change (FC) over nontreated (NT) cells was calculated for (b) and (c). Bars display the average of 3 independent experiments ± SEM; *P < 0.05, **P < 0.01.

**Figure 2:** SFN-induced lymphoblastoid NRF2 ChIP-seq, ENCODE chromatin state annotation (ChromHMM), conservation, and ENCODE K562 NFE2 ChIP-seq tracks of NRF2-regulated known and novel target genes. ChromHMM legend shows 15 chromatin state annotations representing the relationship between chromatin state, function, and gene expression. Blue boxes display NRF2, NFE2, and conservation peaks. (a) Known NRF2-regulated HMOX1. (b–g) Novel NRF2-bound peak regions colocated with highly conserved ARE sequences and NFE2 ChIP-seq peaks in the (b) β-globin locus, (c) AMBP, (d) ABCB6, (e) FECH, (f) HRG1 (SLC48A1), and (g) TBXAS1 genes.
the adult therapeutic target for modifying sickle cell disease, in which expression region enhancer by NRF2 in lymphoid cells that did not enhancer regions. High occupancy of this globin control (orange) with some active promoter (red) and poised (yellow) while the K562 ChromHMM track indicated enhancers heterochromatin (grey) and insulator (CTCF, blue) regions completely devoid of expression and displayed repressive expression. Specifically, GM12878 and HepG2 cells were differences were seen in chromatin states and RNA-seq globin. Among lymphoid, erythroid, and hepatic cells, vast in a region adjacent to fetal (HBG1) and embryonic (HBE1) globin. Among lymphoid, erythroid, and hepatic cells, vast differences were seen in chromatin states and RNA-seq expression. Specifically, GM12878 and HepG2 cells were completely devoid of expression and displayed repressive heterochromatin (grey) and insulator (CTCF, blue) regions while the K562 ChromHMM track indicated enhancers (orange) with some active promoter (red) and poised (yellow) enhancer regions. High occupancy of this globin control region enhancer by NRF2 in lymphoid cells that did not express NFE2 suggested a possible role for NRF2 in erythroid cells. NRF2 activation and inducible binding has been suggested as a mechanism to increase expression of fetal γ-globin genes [37], and therefore NRF2 is being assessed as a potential therapeutic target for modifying sickle cell disease, in which the adult β-globin protein is mutant and undergoes hemolysis [37, 38].

a comprehensive visual assessment of known and novel NRF2-occupied regions.

3.3. NRF2 Binds the Globin Locus. It has been well established that NFE2 is essential in regulating the beta-globin (β-globin) locus control region resulting in activation of β-globin gene expression and erythroid differentiation [18, 31, 32]. In erythroleukemic NFE2-null CB3 cells, erythroid differentiation results in low adult globin gene expression [33]. Additionally, a majority of p45 NFE2 knockout mice die from hemorrhage with surviving adults displaying a mild anemia, including lower hemoglobin levels [18, 34] and sensitivity to oxidative stress [35]. Interestingly, Nrf2-deficient mice from various backgrounds also display a hemolytic anemia phenotype [9, 36]. We observed binding of NRF2 in the highly conserved globin locus control region in lymphoblast cells, a novel finding (Figure 2(b)). SFN-induced NRF2 occupancy in lymphoid cells and NFE2 in K562 cells were colocalized (blue box) in a region adjacent to fetal (HBG1) and embryonic (HBE1) globin. Among lymphoid, erythroid, and hepatic cells, vast differences were seen in chromatin states and RNA-seq expression. Specifically, GM12878 and HepG2 cells were completely devoid of expression and displayed repressive heterochromatin (grey) and insulator (CTCF, blue) regions while the K562 ChromHMM track indicated enhancers (orange) with some active promoter (red) and poised (yellow) enhancer regions. High occupancy of this globin control region enhancer by NRF2 in lymphoid cells that did not express NFE2 suggested a possible role for NRF2 in erythroid cells. NRF2 activation and inducible binding has been suggested as a mechanism to increase expression of fetal γ-globin genes [37], and therefore NRF2 is being assessed as a potential therapeutic target for modifying sickle cell disease, in which the adult β-globin protein is mutant and undergoes hemolysis [37, 38].

3.4. Validation Methods for Candidate Genes. Based on our previous lymphoid NRF2 ChIP-seq data [17], five potential NRF2-regulated genes involved in heme metabolism and iron homeostasis were selected and evaluated in erythroid K562 cells. In order to determine the impact of NRF2 binding in these novel hematopoietic genes, NRF2’s cytosolic repressor KEAP1 was stably silenced in K562 cells using shRNA (see Section 2). Inhibiting expression of NRF2’s repressor increases the amount of NRF2 available to translocate into the nucleus and increase induction of downstream targets. Figure 3(a) displayed KEAPI gene expression levels in vehicle control and in KEAPI knockdown (KEAPIKD) K562 cells. We effectively silenced KEAPI expression 82.7% compared to control K562 cells in three independent experiments. In order to determine the impact of silencing KEAP1 in erythroid cells, we ran qPCR, as well as western blot, for the NRF2-mediated gene NQO1. Figure 3(b) indicated that NQO1 expression in K562 KEAPIKD cells increased 3.2-fold over control. In addition, Figure 3(c) western analysis of K562 KEAPI silencing revealed an increase in NQO1 protein from two different KEAPI-silenced clone cells lines indicating that NRF2 downstream gene targets were induced in KEAPI-silenced cells allowing us to examine NRF2-mediated regulation of hematopoietic gene targets in erythroid cells.

3.5. AMBP. AMBP is a gene that encodes two different proteins, α1 -microglobulin (A1M) and bikunin upon peptide cleavage in hepatocytes, which are then secreted into the plasma [39, 40]. A1M is a 26 kDA glycoprotein belonging to the Lipocalin family with immunoregulatory characteristics and is involved in heme catabolism [41]. Bikunin is a Kunitz-type serine protease inhibitor of the inter-α-inhibitor family and a structural component of the extracellular matrix [23, 42]. A1M acts as a reductase and radical scavenger, binding heme in plasma, extravascular fluids, and cells protecting the cell from oxidative damage caused by hemoglobin and heme.
Figure 2(c) displays colocating peaks in SFN-treated induced by heme, hydrogen peroxide, and hydroxyl radicals [23]. In K562 cells, it has been shown to prevent oxidative and the upregulation of HMOX1 [23, 43, 44]. In contrast, HepG2 chromatin indicated open active promoters (red), enhancers (orange), and active transcription (green) which was supported by the RNA-seq data for this region of the AMBP gene. In order to confirm that NRF2 was regulating AMBP transcription, we transiently silenced NRF2 in airway (BEAS-2B and A549) and liver (HepG2) cell lines. These lines are common in vitro models used to examine cellular response to oxidative stress exposure. The level of SFN-treated, NRF2-silenced (NRF2KD) AMBP mRNA was significantly reduced when compared to the SFN-treated control by 78.6% (BEAS-2B), 33.9% (A549), and 74.9% (HepG2), indicating that AMBP transcription was regulated by NRF2 (Figure 4(a)). Next we compared AMBP gene expression in untreated and SFN-treated K562 and lymphoblastoid GM12878 cell lines. K562 cells showed a modest 2-fold AMBP induction compared to the 56-fold increase over no treatment in GM12878 cells, indicating that SFN was a stronger inducer of AMBP in lymphoblastoid cells (Figure 4(b)). Additionally, we created stable silencing of NRF2’s cytosolic repressor KEAP1 in erythroid K562 cells and assessed them for AMBP inducibility. A 2.75-fold increase over control indicated NRF2 may be binding and affecting the regulation of AMBP (Figure 4(c)). Other findings [23] suggest that AMBP may be an antioxidant that binds and degrades heme in erythroid lines, and the present study suggests that this function may be under the regulation of NRF2.

3.6. ABCB6. Lymphoid NRF2 binding was seen in ABCB6, a putative NRF2 target gene first identified in microarray analysis of human small airway epithelium cells from healthy smokers [45]. ABCB6 is a porphyrin transporter located at the outer mitochondrial membrane in RBCs and has been suggested to translocate CPgenIII from the cytoplasm to the mitochondria, an important step in the sixth enzymatic reaction in the heme biosynthesis pathway [46]. It is highly expressed during erythroid differentiation and controls the translocation of heme and heme precursors between the cytoplasm and mitochondria [25, 44]. The ABCB6 gene has also been shown to encode the Lan blood group antigen which causes severe hemolytic transfusion reactions [47]. In K562 cells, exogenous expression of ABCB6 resulted in cell surface detection of Lan antigen [46, 48]. It has recently been located not only on the plasma membrane on RBCs but also at the plasma membrane of HepG2 hepatocellular carcinoma (HCC) cells which may play a role in drug resistance in HCC and other cancer cells [47]. NRF2 occupancy was colocated with NFE2 peaks (Figure 2(d), blue box) in the ABCB6 gene with almost perfect human to mouse homology of the ARE sequence. ChromHMM tracks under the peaks displayed active open promoters (red) and active transcription (green), as supported by RNA-seq data, in lymphoid GM12878, erythroid K562, and hepatic HepG2 cell lines. In NRF2-silenced BEAS-2B, A549, and HepG2 cells, SFN-induced ABCB6 expression was significantly reduced by 68.2%, 68.1%, and 71.7% of control mRNA (Figure 5(a)). Comparing ABCB6 expression in K562 and GM12878 cells treated with SFN for 24 hours showed no SFN–induced changes in K562 cells and a 5.8-fold increase over control in GM12878 cells (Figure 5(b)). Interestingly, five-hour SFN treatment of K562 cells increased ABCB6 protein compared to untreated cells. Silencing KEAP1 in K562 cells did not have a significant effect on ABCB6 mRNA expression, although K562 cells had a very high baseline expression level in untreated and non-target shRNA conditions (Figures 5(b) and 5(c)).

Examining ABCB6 protein levels in K562 KEAP1KD from two different silenced cell lines and their respective controls, we observed that both C1 and C2 clones showed
an increase in ABCB6 compared to the control (Figure 5(d)).

Thus, ABCB6 responded to SFN treatment, and ABCB6 protein appears higher in the KEAP1KD cells. In summary, NRF2 may be regulating ABCB6 in lymphoid cells as well as in erythroid cells. Using a different model system, such as primary differentiating hematopoietic cells, would present an additional method to examine the impact of NRF2 binding in the ABCB6 gene.

3.7. FECH. Ferrochelatase (FECH) is an enzyme localized in the mitochondria where it is responsible for the last step of heme biosynthesis, catalyzing the insertion of the ferrous form of iron to protoporphyrin IX [25, 49]. It was proposed previously, studies have shown that FECH forms a macromolecular complex with MFRN1 and ABCB10 during erythroid differentiation [50–52]. FECH gene expression has been associated with erythropoietic protoporphyrin (EPP) in humans [53]. In human trials following dietary ingestion of SFN, NRF2-mediated genes such as NQO1 and HMOX1 displayed induced gene expression (200%–300% increase in epithelial tissues) [54]. Therefore, it was of interest to further examine FECH as an NRF2 regulated gene. NRF2 and NFE2 ChIP-seq peaks colocated (blue box) in the conserved ARE consensus sequence of the FECH gene (Figure 2(e)). Chromatin status indicated open promoters (red) with relatively weak transcription (lt. green) in GM12878 and HepG2 compared to stronger transcription levels (green) of FECH in the K562 cells. FECH mRNA levels were relatively low in A549 and BEAS-2B cells (Figure 6(a)). However, FECH mRNA expression was ∼100-fold higher in HepG2 cells compared with A549 or BEAS-2B cells, and it was induced 3-fold by SFN in LCLs (Figure 6(b)). NRF2 silencing reduced SFN-induced levels of FECH expression significantly by 42.2% (A549), 29.3% (BEAS-2B) and 61.9% (HepG2) compared to cell line specific controls (Figure 6(a)). However, FECH expression in K562 cells did not change with SFN treatment (either at mRNA or protein level) but increased 3.1-fold in lymphoid cells (Figure 6(b)). The very high baseline level of FECH expression in K562 may preclude inducibility, even if NRF2 is upregulated as in K562 KEAP1KD cells (Figure 6(c)). FECH protein levels did not change in the K562 KEAP1KD clones (Figure 6(d)). Previously, studies have shown that FECH is highly upregulated during erythroid differentiation [50] and in heme-stimulated differentiating K562 cells [55]. A model system that examined NRF2 mediation of FECH expression under differentiation conditions may allow further insight into this relationship.

3.8. HRG1 (SLC48A1). Initially discovered in C. elegans, heme responsive gene 1 (HRG1) was the first heme importer identified and is the only member of the solute carrier 48 (SLC48) family [48, 49, 56]. Located in the plasma and lysosomal membrane, HRG1 transports heme from the lysosome into the cytoplasm and is essential for normal development [25]. In addition, knockdown of HRG1 in zebrafish resulted in severe anemia and hydrocephaly [48]. As with the other genes we examined, NRF2 and NFE2 co-occupied the same HRG1 genomic region (blue box) in lymphoblastoid and K562 cells suggesting that they may have an overlapping regulatory role in the expression of HRG1 (Figure 2(f)). Comparisons of ChromHMM and RNA-seq tracks for the three cell lines indicate that HRG1 was highly expressed in K562 cells and shows an active promoter (red), strong enhancer (orange), and transcriptional elongation (green) chromatin
Figure 6: NRF2-silenced cell lines, erythroid, lymphoid, and KEAP1-silenced protein and mRNA expression of FECH. (a) FECH was significantly decreased in SFN-induced NRF2-silenced mRNA from airway and hepatic cell lines. (b) K562 cells did not respond to chemical treatment at mRNA (10 \( \mu M \) SFN, 24 hours) or protein level (5 hours with 10 \( \mu M \) SFN), unlike the SFN-mediated increase of FECH in lymphoid GM12878 cells. (c) K562 KEAP1KD FECH mRNA was unchanged compared to control. Gene expression values were normalized to BACT or 18S. Bars represent an average of 3–6 independent experiments ± SEM; *\( P < 0.05 \), **\( P < 0.01 \). (d) FECH protein did not increase in K562 KEAP1-silenced cells. Protein markers confirmed FECH monomers at ~40 kDA, and the membrane was reprobed with \( \beta \)-actin as a loading control.

Figure 7: HRG1 (SLC48A1) gene expression and protein analysis in NRF2KD, K562, GM12878, and K562 KEAP1KD cells. (a) Transient silencing of NRF2 in three cell lines (A549, BEAS-2B, and HepG2) reduced expression of HRG1. Most significant HRG1 reduction was observed in HepG2 followed by A549. (b) Chemical activation of NRF2 using SFN (10 \( \mu M \), 24 hours) induced HRG1 expression in GM12878, but high HRG1 baseline levels remained unchanged in SFN-induced K562 cells. K562 cells treated with 5 hours of 10 \( \mu M \) SFNs showed an increase in HRG1 protein indicating a response to chemical activation at the shorter timepoint. (c) Genetic activation of NRF2 by stably silencing its negative regulator KEAP1 induced HRG1 in K562 cells at mRNA and protein level. (d) Two different shRNA clones showed an increase in HRG1 as compared to non-target shRNA control. mRNA values were normalized to BACT or 18S. Bars represent an average of 3–6 independent experiments ± SEM; *\( P < 0.05 \), **\( P < 0.01 \). A 30 kDA HRG1 band was confirmed by protein markers, and the membrane was stripped and reprobed with \( \beta \)-actin as a loading control.

states (Figure 2(f)). Unlike K562 cells, expression of HRG1 in GM12878 was very low, and this was consistent with observed ChromHMM states of weak promoters (light red), weak poised enhancers (yellow), and a large insulator region (blue; Figure 2(f)). The expression of HRG1 was reduced in all three cell lines in which NRF2 was silenced transiently but was most strongly reduced in HepG2 (52.5%) suggesting cell-type specific regulation of HRG1 by NRF2 (Figure 7(a)). Upregulation of mRNA expression of HRG1 was observed both in response to SFN treatment (6.5-fold induction) and genetic activation in K562 (1.8-fold induction) (Figures 7(b) and 7(c)). Protein levels for HRG1 showed small increases for both chemical and genetic NRF2 activations (Figures 7(b) and 7(d)).

3.9. TBXAS1. TBXAS1 (cytochrome P450, family 5, subfamily A, Cyp5A1) is considered a member of the cytochrome P450 enzyme family based on sequence homology but is functionally very different from this group of monooxygenases and quite different from the other candidate genes. TBXAS1
resides in the endoplasmic reticulum membrane and functions as a catalyzer for the conversion of prostaglandin H2 to thromboxane A2, which is a potent vasoconstrictor, bronchoconstrictor, and inducer of platelet aggregation [57, 58]. In addition to its role in several pathophysiological processes including hemostasis, recent research suggested a role for TBXAS1 in several cancers as well as preeclampsia [59–61]. Because of its biological significance and its occupancy by NRF2 in lymphoblastoid cell lines, we further explored NRF2-dependent expression. Occupied binding sites were seen in NRF2 and NFE2 ChIP-seq experiments in GM12878 and K562 cells, respectively, indicating potential regulation by these transcription factors (Figure 2(g), blue box). Large differences in ChromHMM tracks were seen among the three cell lines reflecting the RNA-seq expression patterns shown (Figure 2(g)). HepG2 cells showed a repressed state while GM12878 and K562 cells showed strong enhancers (orange) under NRF2 and NFE2 binding sites. K562 cells, however, showed increased transcription (green) compared to lt green (weak transcription) and grey (repression) states in GM12878 cells resulting in higher expression of the TBXAS1 gene in K562 cells. Unlike the expression pattern of HRG1, both chemical and genetic activations of NRF2 led to the upregulation of TBXAS1 in K562 cells (Figures 8(a)–8(c)). The genetic activation of NRF2 in K562 cells by silencing KEAP1 resulted in a 3.9-fold induction of TBXAS1 compared to 2.2-fold induction with SFN treatment (Figures 8(b) and 8(c)). Transient silencing of NRF2 in all three cell lines reduced the expression of TBXAS1 with varying degree (35%–87%) suggesting that TBXAS1 was a potential direct target of NRF2 (Figure 8(a)). In aggregate, for each of these candidate genes we see evidence of NRF2 occupancy in enhancer regions that is colocated with NFE2 occupancy in K562 cells, as well as NRF2-dependent gene expression in one or more of the experimental systems tested.

4. Discussion

It is well known that NRF2 mediates the regulation of several genes involved in iron metabolism (FTL, FTH1) and the heme metabolic pathway (HMOX1) [10, 17]. NRF2 also plays a critical role in the survival of RBCs by regulating intracellular ROS levels when selenoproteins, enzymes with antioxidant properties, are depleted [9]. Here, we selected five novel NRF2-regulated hematopoietic genes based on NRF2 ChIP-seq in lymphoid cells and investigated their NRF2-dependent regulation using silencing techniques and activation treatments. In order to address NRF2-mediated expression of these hematopoietic genes in an erythroid line, K562 cells were treated with SFN to activate NRF2 and compared with lymphoid cells. Gene expression of candidate NRF2-mediated targets increased upon SFN treatment in lymphoid cells consistent with SFN-induced NRF2 occupancy detected by ChIP-seq. In addition, AMBP and TBXAS1 expression were increased both upon chemical induction by SFN and in KEAP1-silenced K562 cells. ABCB6, FECH, and HRG1 expression showed high baseline values in K562 and were not inducible by SFN at the 24 hr SFN timepoint. However, both ABCB6 and HRG1 protein levels increased in a five-hour SFN exposure as well as in the KEAP1-silenced condition. ABCB6 expression was not induced in SFN-treated A549 or BEAS2-B cells but did increase in HepG2 cells (data not shown). In addition, NRF2 silencing in untreated cells showed a reduction (50%–73%) in all three cell lines [17] suggesting NRF2 regulation at both a basal and inducible level for ABCB6, FECH expression showed a similar pattern with 24-hour SFN treatment, but A549 and BEAS-2B did not change with NRF2 silencing in untreated cells [17], unlike all three silenced SFN-treated cell lines (Figure 6(a)) suggesting the possibility of cell type-specific regulation of FECH by NRF2. HRG1 expression patterns were unchanged in all three untreated NRF2-silenced cell lines compared to control, yet
SFN treatment varied among the cell lines (data not shown) suggesting the possibility of inducible regulation by NRF2. Using primary erythroid differentiating cells may be a better model to investigate NRF2 regulation of ABCB6, FECH, and HRG1 since all three genes are developmentally essential and are highly upregulated during erythroid differentiation [25, 49, 62].

One novel finding was lymphoid SFN-induced NRF2 peaks colocalizing at the same ARE consensus sequence as erythroid NFE2 in all of the hematopoietic candidates as well as the β-globin control region locus. Lymphoid cells do not express NFE2 suggesting a possible interaction between NRF2 and NFE2 or a response to oxidative stress in erythroid cells at NFE2 enhancer sites. NRF2 and NFE2 bind the same consensus sequence and heterodimerize the same small Maf proteins (MaF, MaG, MaFk), possibly allowing either transcription factor to regulate the hematopoietic genes depending on cellular context, differentiation state, or redox homeostasis. In differentiating MEL cells, for example, MaFk, a corepressor and binding partner of Bach1, shifted dimerization partners to become a coactivator with Nfe2 and bind at the β-globin locus control region [63]. Studies suggest that Bach1 heterodimerizes the same small Maf and other bZIP proteins as NRF2 and NFE2, indicating an interactive competition for small Maf proteins [64]. A study by Sun et al. [65] using multiple ChIP assays in preadipocyte mouse 3T3 cells demonstrated that increasing heme levels caused displacement of Bach1 from MafK heterodimers at the Hmox1 enhancer, allowing Nrf2 to heterodimerize with MafK in the same response element. Therefore, NRF2 may be responding to cellular stress in erythroid cells by heterodimerizing with Maf proteins and binding NFE2 enhancer sites in the promoters of genes that can modify the response to oxidative damage.

NRF2 mediation of heme metabolism and iron homeostasis genes in response to oxidative stress would be beneficial to cell survival. In K562 cells, for example, AMBP is induced by heme, hemoglobin, and ROS, and this change is accompanied by upregulation of HMOX1 leading to prevention of intracellular oxidative damage [23]. Under conditions of phenylhydrazine-induced stress, ABCB6 was found to be essential to survival in ABCB6 knock-out mice as the sole ATP-dependent porphyrin importer [62], and it seems likely that NRF2-dependent upregulation of porphyrin transport under oxidative stress conditions would be protective as well. Similarly, upregulation of FECH under oxidative stress conditions would increase production of functional heme and to support higher levels of oxygen transport. HRG1 was proposed to be responsible for transporting endocytosed heme out of the phagosome and into the cytosol for subsequent degradation by HMOX1 [66], and coordinated regulation of these two genes seems likely. While TBXAS1 is functionally quite different from the other candidates, changes in TBXAS1 expression in lung cancer cell lines have been associated with increased ROS production and induction of apoptosis, a finding that was negated when treated with antioxidant treatment [60]. Taken together, NRF2 mediation of heme metabolic and hemostatic genes AMBP, ABCB6, FECH, HRG1, and TBXAS1 provides additional insight into the vast functional diversity of NRF2 transcriptional control.

**Conflict of Interests**

The authors declare no conflict of interests.

**Authors’ Contribution**

Michelle R. Campbell and Mehmet Karaca contributed equally to this work.

**Disclosure**

As stated in NIH Publishing Agreement “Author's contribution to the Work was done as part of the Author's official duties as a NIH employee and is a Work of the United States Government. Therefore, copyright may not be established in the United States.”

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Research Article

NRF2 Protection against Liver Injury Produced by Various Hepatotoxicants

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To investigate the role of NRF2 as a master defense against the hepatotoxicity produced by various chemicals, NRF2-null, wild-type, Keap1-knock down (Keap1-Kd) and Keap1-hepatocyte knockout (Keap1-HKO) mice were used as a “graded NRF2 activation” model. Mice were treated with 14 hepatotoxicants at appropriate doses, and blood and liver samples were collected thereafter (6 h to 7 days depending on the hepatotoxicant). Graded activation of NRF2 offered a NRF2-dependent protection against the hepatotoxicity produced by carbon tetrachloride, acetaminophen, microcystin, phalloidin, furosemide, cadmium, and lithocholic acid, as evidenced by serum alanine aminotransferase (ALT) activities and by histopathology. NRF2 activation also offered moderate protection against liver injury produced by ethanol, arsenic, bromobenzene, and allyl alcohol but had no effects on the hepatotoxicity produced by D-galactosamine/endotoxin and the Fas ligand antibody Jo-2. Graded NRF2 activation reduced the expression of inflammatory genes (MIP-2, mKC, IL-1β, IL-6, and TNFα), oxidative stress genes (Ho-1, Egr1), ER stress genes (Gadd45 and Gadd153), and genes encoding cell death (Noxa, Bax, Bad, and caspase3). Thus, this study demonstrates that NRF2 prevents the liver from many, but not all, hepatotoxicants. The NRF2-mediated protection is accompanied by induction of antioxidant genes, suppression of inflammatory responses, and attenuation of oxidative stress.

1. Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that promotes transcription of a battery of cytoprotective genes in response to oxidative/electrophilic stress [1]. Under basal conditions, Nrf2 is sequestered by kelch-like ECH associating protein 1 (Keap1) in the cytosol. In response to oxidative stress, Nrf2 is released from Keap1, translocates into the nucleus, and induces an array of cytoprotective genes as adaptive responses [1, 2]. Nrf2 target genes include NAD(P)H quinone oxidoreductase 1 (Nqo1), GSH synthesis (Gclc and Gclm), GSH conjugation (Gsts), and many other oxidized protein repair genes [2, 3]. A “graded Nrf2 activation” animal model, consisting of Nrf2-null mice, wild-type mice, Keap1-knockdown (Keap1-KD) mice with enhanced Nrf2 activation, and Keap1-hepatocyte knockout (Keap1-HKO) mice with maximum Nrf2 activation, has been used to study the functions of Nrf2 in the liver [3, 4]. Transcription profiling of the “graded Nrf2 activation” animal model by microarray analysis showed that many cytoprotective genes are constitutively expressed in a “gene dose-response” manner [3–5].

Using the “graded Nrf2 activation” animal model, this laboratory has reported that Nrf2 protects against the hepatotoxicity produced by cadmium [5], ethanol [6], and diquat [7]. In addition to the “graded Nrf2 activation,” Nrf2-null mice are more sensitive to the hepatotoxicity produced by acetaminophen [8–10], 1-bromopropane [11], and the chronic hepatotoxicity produced by carbon tetrachloride [12], arsenic [13], and fatty liver from feeding a methionine- and choline-deficient (MCD) diet [14]. However, graded Nrf2 activation did not confer protection against the steatosis from feeding a high fat diet for 6 months [15].

The purpose of the present study was to use this unique “graded Nrf2 activation” animal model to determine whether various levels of basal expression of Nrf2 protect against acute...
liver injury produced by 14 hepatotoxins. Each of these hepatotoxins produces liver injury by various mechanisms, which will provide further insight into the mechanism of how Nrf2 protects against hepatotoxins. This study focuses on inflammatory responses and oxidative stress as potential mechanisms of Nrf2-mediated hepatoprotection.

2. Results

2.1. Graded Nrf2 Activation in a Genetic Animal Model. To verify the graded Nrf2 activation, the expression of Nrf2 and Nrf2-targeted genes was quantified in 4 genotypes of mice. Table 1 shows that the higher basal expression of the cytoprotective genes in Keap1-KD and Keap1-HKO mice, for example, the expression of Nqo1 (0.037 for Nrf2, 0.293 for WT, 0.624 for Keap1-KD, and 3.17 for Keap1-HKO, % of G3PDH) and Gclc (3.84 for Nrf2, 7.02 for WT, 9.06 for Keap1-KD, and 21.4 for Keap1-HKO, % of G3PDH) was markedly higher in Keap1-HKO mice as compared to WT mice. Similarly, the expression of GSH homeostasis and conjugation genes such as glutathione reductase (Gsr), glutathione S-transferase (GSTmu, GSTal, GSTa4), and glutathione peroxidase (Gpx2) was also increased in a "gene-dose" manner. In addition, the basal expression of heme oxygenase-1 (Ho-1) and metallothionein (Mt-1), two other cytoprotective mechanisms [16, 17], was also higher in Keap1-HKO mice, confirming prior publications [4, 5].

2.2. Effects of Nrf2 in Protection against 14 Hepatotoxins. Table 2 shows the degree of liver injury produced by various hepatotoxins, based on increased serum enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) on histopathology. Nrf2-null mice were highly susceptible to the hepatotoxicity produced by CCl4, acetaminophen, microcystin, phalloidin, furosemide, and cadmium [5], and lithococholic acid was mild, and the hepatotoxicity produced by bromobenzene and allyl alcohol was moderate, and the Nrf2-targeted genes was quantified in 4 genotypes of mice. Table 2 shows the degree of liver injury produced by various hepatotoxins, based on increased serum enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) on histopathology. Nrf2-null mice were highly susceptible to the hepatotoxicity produced by CCl4, acetaminophen, microcystin, phalloidin, furosemide, and cadmium [5], and lithococholic acid was mild, and the hepatotoxicity produced by bromobenzene and allyl alcohol was moderate, and the Nrf2-targeted genes was quantified in 4 genotypes (data not shown). After hepatotoxicant challenge (microcystin, phalloidin, and lithococholic acid), mRNA levels of these inflammatory cytokines in Nrf2-null mice were more susceptible than Keap1-KD or Keap1-HKO mice, indicating that Nrf2-overexpression attenuated toxicant-induced inflammatory response in a "graded Nrf2 activation" manner.

2.3. Effects of Nrf2 Activation on Inflammation and Stress Gene Expression

2.3.1. Expression of Chemokine Genes. The mRNA expression of neutrophil-specific chemokine macrophage inflammatory protein 2 (MIP-2) and mouse keratinocyte-derived chemokine (mKC) is shown in Table 3. There was no difference in basal expression of MIP-2 (around 0.045% of G3PDH) and mKC (around 2% of G3PDH) among the four genotypes (data not shown). After hepatotoxicant challenge (microcystin, phalloidin, and lithococholic acid), mRNA levels of MIP-2 (20–30 fold) and mKC (5–15 fold) were increased in Nrf2-null mice, which were greatly attenuated in Keap1-HKO mice, indicating that Nrf2-overexpression attenuated toxicant-induced inflammatory response in a "graded Nrf2 activation" manner.

2.3.2. Expression of Inflammation Genes. mRNA levels of proinflammatory genes interleukin-1β (IL-1β), IL-6, and TNFα showed a similar pattern (Table 3). There was no difference in basal expression of IL-1β (around 0.026% of G3PDH), IL-6 (around 0.002% of G3PDH), and TNFα (around 0.004% of G3PDH) among the four genotypes (data not shown). Toxicants (microcystin, phalloidin, acetaminophen, CCl4, and lithococholic acid) administration markedly increased mRNA levels of these inflammatory cytokines in Nrf2-null mice, but only mild increases were seen in Keap1-HKO mice, indicating that Nrf2-overexpression attenuated toxicant-induced hepatic inflammation.

Table 1: The basal levels of the Nrf2 and Nrf2-targeted genes in the “graded Nrf2 activation” model.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nrf2-null</th>
<th>Wild-type</th>
<th>Keap1-Kd</th>
<th>Keap1-HKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nqo1</td>
<td>0.037 ± 0.007*</td>
<td>0.29 ± 0.02</td>
<td>0.74 ± 0.12*</td>
<td>3.17 ± 0.36*</td>
</tr>
<tr>
<td>Nrf2</td>
<td>0.002 ± 0.001*</td>
<td>0.07 ± 0.02</td>
<td>1.46 ± 0.05*</td>
<td>2.10 ± 0.24*</td>
</tr>
<tr>
<td>Gclc</td>
<td>3.84 ± 0.86*</td>
<td>7.01 ± 1.11</td>
<td>10.1 ± 1.37*</td>
<td>27.2 ± 2.49*</td>
</tr>
</tbody>
</table>

GSH-related

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nrf2-null</th>
<th>Wild-type</th>
<th>Keap1-Kd</th>
<th>Keap1-HKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gsr</td>
<td>0.11 ± 0.01*</td>
<td>0.21 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.06 ± 0.01*</td>
</tr>
<tr>
<td>Gsta4</td>
<td>1.48 ± 0.39*</td>
<td>2.97 ± 1.14</td>
<td>4.63 ± 1.02</td>
<td>7.93 ± 1.55*</td>
</tr>
<tr>
<td>Gstmu</td>
<td>20.7 ± 1.43*</td>
<td>107 ± 28.3</td>
<td>544 ± 98.2*</td>
<td>1547 ± 350*</td>
</tr>
<tr>
<td>Gstpi</td>
<td>101 ± 22.2</td>
<td>7.01 ± 1.11</td>
<td>10.1 ± 1.37*</td>
<td>27.2 ± 2.49*</td>
</tr>
<tr>
<td>Gpx2</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.03</td>
<td>0.06 ± 0.01</td>
<td>2.15 ± 0.81*</td>
</tr>
</tbody>
</table>

Acute-phase

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nrf2-null</th>
<th>Wild-type</th>
<th>Keap1-Kd</th>
<th>Keap1-HKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt-1</td>
<td>4.22 ± 0.93</td>
<td>3.48 ± 1.11</td>
<td>20.1 ± 5.43*</td>
<td>27.4 ± 2.49*</td>
</tr>
<tr>
<td>Ho-1</td>
<td>0.46 ± 0.06</td>
<td>0.64 ± 0.03</td>
<td>0.87 ± 0.15*</td>
<td>1.23 ± 0.37*</td>
</tr>
</tbody>
</table>

Data are % of the housekeeping gene G3PDH and represent mean ± SEM of serum ALT values (n = 6–10), * significantly different from wild-type mice, P < 0.05.
2.3.3. Expression of Acute Phase Protein Genes. mRNA levels of acute phase protein genes heme oxygenase-1 (Ho-1) and early growth response gene-1 (Egr-1) showed a similar pattern (Table 3). The basal levels of Ho-1 were higher in Keap1-HKO mice (Table 1) [6], and after hepatotoxicant insults, more increases in Ho-1 were observed in Nrf2-null mice than in Keap1-HKO mice; a similar pattern holds true for Egr-1, but the basal levels of Egr-1 were lower in Keap1-HKO mice. Acetaminophen, CCl₄, microcystin, phalloidin, and lithocholic acid are all effective inducers of the mRNA of two acute phase protein genes Ho-1 and Egr-1, implying higher levels of stress in the Nrf2-null mouse livers.

2.4. Effects of Nrf2 Activation on ER Stress and Cell-Death Genes

2.4.1. Expression of Genes Involved in ER Stress. The mRNA levels of two proteins involved in endoplasmic reticulum (ER) stress are shown in Table 4. After the challenge by CCl₄, acetaminophen, microcystin, and phalloidin, higher expression of Gadd45 and Gadd153 was observed in Nrf2-null mice, moderate in wild-type mice and lower expressed in Keap1-KD and Keap1-HKO mice, respectively, indicating the “graded Nrf2 activation” dose dependently protected against toxicant-induced ER stress.
The “graded Nrf2 activation” model [3–6] is a unique animal model to evaluate the role of genetic activation of Nrf2 as a host master defense against various hepatotoxins. The present study indicates that the genetic activation of Nrf2 results in mice more resistant to the acute hepatotoxicity produced by CCl₄, acetaminophen, microcystin, phalloidin, cadmium, furosemide, and lithocholic acid in a “Nrf2 gene-dose” manner. Nrf2 activation also offered moderate protection against the hepatotoxicity produced by ethanol [5] and arsenic. However, overexpression of Nrf2 had limited effects on the hepatotoxicity of D-galactosamine/endotoxin and the Fas ligand antibody Jo-2. Thus, activation of Nrf2 offered protection against many, but not all, hepatotoxins.

The hepatotoxins used in the present study produce liver injury through different mechanisms. Some hepatotoxins require metabolic activation to produce reactive intermediates such as the reactive intermediate N-acetyl-p-benzoquinoneimine (NAPQI) produced by acetaminophen [18] and trichloromethyl radical (CCl₃)⁺ produced by carbon tetrachloride [19]. The hepatotoxicity of bromobenzene derives from its reactive metabolites (epoxides and quinones), which arylate cellular proteins [20]. Cadmium...
and arsenic are metallic toxicants not requiring bioactivation to produce reactive intermediate, rather by producing oxidative stress in their acute toxicity [16, 21, 22]. Redox-cycling metabolism of the bipyridilium herbicide, and diquat generates oxidative stress which results in cytotoxicity and liver injury [23]. Acute microcystin poisoning is characterized by inhibition of serine/threonine phosphatases and subsequent hyperphosphorylation of cytoskeletal proteins, leading to disruption of hepatocyte architecture [24]. Phalloidin binds to F-actin, preventing trafficking along the cytoskeleton and “freezing” hepatocytes [25]. D-galactosamine/LPS and the Fas ligand Jo2 antibody treatment are well-established models of liver injury mediated by innate immunity [26, 27]. “Graded Nrf2 activation” seems to offer a generalized hepatoprotection against most hepatotoxicants under investigation except for D-galactosamine/LPS and the Fas ligand Jo2 antibody. Thus, the expression of genes related to hepatotoxicity was performed to look into the generalized mechanism.

The neutrophil-specific chemokine macrophage inflammatory protein 2 (MIP-2, CXC2R) and mouse keratinocyte-derived chemokine (mKC) are important mediators of inflammation in acute tissue injury [28]. In liver inflammation, recruitment of circulating polymorphonuclear leukocytes is essential for host defense and initiates specific immune responses. One pathological hallmark of acute liver injury is the uncontrolled transmigration of neutrophils into the liver. The extravasation of leukocytes from the vascular system into the liver is induced by chemokines that are released from the site of inflammation. In the present study, toxicant-elevated MIP-2 and mKC were significantly reduced in Keap1-HKO mice, indicating that activation of Nrf2 might reduce toxicant-induced liver injury which is mediated in part by reducing liver inflammation, or that with less toxicity there was less detection of the inflammatory cytokines. Proinflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin Ibeta (IL-1β), and interleukin 6 (IL-6) play important roles in acute liver damage [29]. Thus, these proinflammatory cytokine increases are implicated in toxicant-induced liver injury and can be detected at the molecular level. Again, Keap1-HKO mice had much lower expression of these cytokines, indicating that activation of Nrf2 reduces toxicant-induced liver injury in part by reducing liver proinflammatory cytokine release.

Inflammation is often associated with overproduction of reactive oxygen species (ROS) that play an important role in toxicant-induced acute liver injury. The increased lipid peroxidation is a sensitive biomarker for the hepatotoxicity produced by CCl₄ [30], acetaminophen [31], microcystin [32], cadmium [6, 16], ethanol [5], and diquat [7]. In combatting increased oxidative stress, the GSH synthesis and conjugating enzyme genes are increased as a Nrf2-targeted host defense against oxidative stress in Keap1-HKO mice in the protection against the hepatotoxicity produced by cadmium [5, 6], microcystin [33], and acetaminophen [31]. Thus, the reduction of oxidative stress, probably through the enhancement of the GSH system is one of the important mechanisms for Nrf2-mediated protection against hepatotoxicity.

Acute phase proteins are important adaptive mechanism in response to acute stress. For example, early growth response (Egr)-1, a transcription factor that regulates expression of inflammatory genes, plays a pathological role in many animal models of acute and chronic inflammatory disease [34]. Heme oxygenase-1 (HO-1) is an essential enzyme which degrades heme into carbon monoxide, biliverdin, and free iron. Induction of HO-1 in rodent models of acute and chronic hepatic inflammation results in improvement of liver damage and downregulation of proinflammatory cytokines [17]. Ho-1 is a Nrf2-targeted gene, and it is higher in Keap1-HKO mice under basal conditions. The higher the oxidative stress, the higher expression of Ho-1, and thus Ho-1 is also a biomarker for oxidative stress [5, 17]. Metallothioneins are important for cadmium detoxication [16]. Higher induction of these acute phase protein genes was observed in Nrf2-null mice, as compared to Keap1-HKO mice, implying higher generation of ROS and stress from toxicants in mice deficient in Nrf2.

Endoplasmic reticulum (ER) is the site of synthesis and folding of proteins. Perturbations of ER homeostasis affect protein folding and cause ER stress. ER stress is implicated in chemical-induced hepatotoxicity. One of the components of the ER stress-mediated apoptosis pathway is C/EBP homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153 (GADD153) [35]. The Gadd45 stands at the crossroad of cell fate by controlling the balance between cellular DNA repair, eliminating (apoptosis) or preventing the expansion of potentially dangerous cells [36]. As a biomarker of ER stress and DNA damage, both Gadd153 and Gadd45 were markedly increased in Nrf2-null mice as compared to graded Nrf2 activation in the “graded Nrf2 activation” models (Table 4), implying that Nrf2-deficiency makes animals susceptible to ER stress.

Lesions to DNA trigger the DNA-damage response, a complex, multibranched cell-intrinsic process targeted to DNA repair or elimination of damaged cells by apoptosis [37]. The Bcl2 homology domain 3 (BH3)-only protein Noxa is at the tip of the balance between life and death and appears to be crucial for cell death along the mitochondrial Bcl2-regulated apoptosis pathway in response to toxicant insults, presumably by sensitizing the cell toward the action of additional BH3-only protein family members [38]. BAX, the BCL-2-associated X protein, is a cardinal proapoptotic member of the BCL-2 family. BAD, a BH3-only pro-apoptotic protein, helps coordinate mitochondrial fuel metabolism and the apoptotic machinery. Both regulate the critical balance between cellular life and death [39, 40]. Death-mediating proteases, caspase-3 in particular, have been implicated in as a bifurcation point between plasticity and cell death [41]. In the present study, mice deficient in Nrf2 were highly susceptible to toxicant-induced apoptosis and necrosis, with marked upregulation of these proapoptotic components. In comparison, Nrf2 activation reduced cell death as well as the mRNA levels of these apoptosis executors.

However, graded activation of Nrf2 does not confer protection against the hepatotoxicity produced by D-galactosamine/endotoxin and the Fas ligand antibody Jo-2 (Table 2). Both hepatotoxicants produce liver injury through the modulation of the immune system. Little is known about
the effect of Nrf2 on endotoxemia and immune modulation. Keap1-KD mice and CDDO-Im-induction of Nrf2 are effective in decreasing concanavalin A-induced liver injury and the late-phase proinflammatory gene expression in the liver [42]. Our recent work shows that activation of Nrf2 suppresses IFN-γ production, while inducing the production of the Th2 cytokines IL-4, IL-5, and IL-13. Nrf2 activation also suppresses T-bet DNA binding [43], suggesting that Nrf2 activation suppresses T-bet DNA binding and promotes GATA-binding of the Th2 cytokines IL-4, IL-5, and IL-13. Nrf2 activation also represents a novel regulatory mechanism in CD4(+) T cells.

In conclusion, the present study shows that “graded Nrf2 activation” markedly decreased the hepatotoxicity of most hepatotoxins. The protective effect of Nrf2 is accompanied by induction of genes involved in antioxidant defense, and genes involved in host defense against toxicity stimuli.

4. Materials and Methods

4.1. Reagents. Carbon tetrachloride (CCL4), acetaminophen (APAP), microcystin (Microcystin-LA), phalloidin, lithocholic acid (LCA), sodium arsenite (As3+), D-galactosamine, lipopolysaccharide (LPS), furosemide, allyl alcohol, and bromobenzene were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Fas ligand antibody Jo-2 was obtained from BD Biosciences (San Jose, CA, USA). All other chemicals were reagent grade and commercially available.

4.2. Animal Husbandry and Treatment. C57BL/6 breeders were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA). Eight-week-old male mice were used for this study. Nrf2-null mice were obtained from Dr. Jefferson Chan (University of California, Irvine, CA, USA) [44]. Keap1-KD mice were supplied by Dr. Masayuki Yamamoto (Tohoku University, Sendai, Japan). In an attempt to make a hepatocyte-specific Keap1-null mouse, utilizing a loxP, Alb-Cre system, a Keap1-KD mouse was engineered, in which Keap1 was decreased throughout the body [45]. Nrf2-null mice were backcrossed into the C57BL/6 background, and >99% congenicity was confirmed by Jackson Laboratories (Bar Harbor, ME, USA). Keap1-HKO mice were generated by crossing Keap1-KD mice and AlbCre+ mice, which express Cre only in hepatocytes. All the mice were bred at the University of Kansas Medical Center, housed in a temperature-, light-, and humidity-controlled environment, and had access to Teklad Rodent Diet #8604 (Harlan Laboratories, Madison, WI, USA) and water ad libitum. The housing facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The animal treatment protocols were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

4.3. Experimental Design. Nrf2-null mice, wild-type mice, and Keap1-HKO mice were treated with acetaminophen (500 mg/kg, ip 8 h), carbon tetrachloride (25 µL/kg, 16 h), microcystin (50 µg/kg, i.p., 8 h), phalloidin (1.5 mg/kg, ip, 8 h), lithocholic acid (0.4% in diet for 7 days), sodium arsenite (13 mg/kg, i.p., 24 h), or saline (10 mL/kg, i.p.), D-galactosamine/LPS (400 mg/10 µg/kg, i.p., 8 h), furosemide (250 mg/kg, ip 24 h), bromobenzene (0.7 mL/kg), allyl alcohol (85 mg/kg, ip, 24 h), and the Fas ligand Jo-2 (5 µg/mouse). At the end of the experiments, mice were anesthetized with pentobarbital (50 mg/kg, ip). Blood and liver samples were collected. Portions of livers were fixed in 10% neutral formalin for histological analysis, and others were frozen in liquid nitrogen and stored at −80°C. The dose selection is based on our previous publications [46] and pilot experiments.

4.4. Hepatotoxicity Evaluation. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined as a biochemical indicator of hepatocellular necrosis using Pointe Scientific Liquid ALT and AST Reagent (Canton, MI, USA) according to the manufacturer’s protocol.

4.5. Histopathology. Liver samples were fixed in 10% formalin prior to routine processing and paraffin embedding. Liver sections (5 µm in thickness) were stained with hematoxylin and eosin and evaluated for hepatocellular necrosis.

4.6. Total RNA Isolation. Total RNA was isolated using RNeasy B reagent (Qiagen, USA) according to the manufacturer’s protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. The integrity of each RNA sample was evaluated by formaldehyde-agarose gel electrophoresis before analysis.

4.7. Quantification of mRNA by RT-PCR. Total RNA in mouse livers was reverse-transcribed into cDNA by High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), and the resulting cDNA was used for real-time PCR analysis using Power SYBR Green PCR Master Mix in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Oligonucleotide primers were designed with Primer3 software and listed in Supplemental Table 1 (available online at http://dx.doi.org/10.1155/2013/305861).

4.8. Statistical Analysis. Data were expressed as mean ± SEM and analyzed using a one-way ANOVA followed by Duncan’s multiple range test utilizing SPSS 13 Software (SAS, NC). The significant level was set at P ≤ 0.05.

Conflict of Interests

The authors declare that they have no conflict of interests of “Power SYBR,” “Charles River Laboratories, Inc.,” “Tel-Test, Inc.,” “Primer3 software,” “SPSS 13 Software,” financial foundation, or any other third party.

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Enhanced 4-Hydroxynonenal Resistance in KEAP1 Silenced Human Colon Cancer Cells

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Nuclear factor erythroid 2-related factor 2 (NRF2) is the transcription factor that regulates an array of antioxidant/detoxifying genes for cellular defense. The conformational changes of Kelch-like ECH-associated protein 1 (KEAP1), a cytosolic repressor protein of NRF2, by various stimuli result in NRF2 liberation and accumulation in the nucleus. In the present study, we aimed to investigate the effect of KEAP1 knockdown on NRF2 target gene expression and its toxicological implication using human colon cancer cells. The stable KEAP1-knockdown HT29 cells exhibit elevated levels of NRF2 and its target gene expressions. In particular, the mRNA levels of aldo-keto reductases (AKR1C1, 1C2, 1C3, 1B1, and 1B10) were substantially increased in KEAP1 silenced HT29 cells. These differential AKRs expressions appear to contribute to protection against oxidative stress. The KEAP1-knockdown cells were relatively more resistant to hydrogen peroxide (H₂O₂) and 4-hydroxynonenal (4HNE) compared to the control cells. Accordantly, we observed accumulation of 4HNE protein adducts in H₂O₂- or 4HNE-treated control cells, whereas KEAP1-knockdown cells did not increase adduct formation. The treatment of KEAP1-silenced cells with AKR1C inhibitor flufenamic acid increased 4HNE-induced cellular toxicity and protein adduct formation. Taken together, these results indicate that AKRs, which are NRF2-dependent highly inducible gene clusters, play a role in NRF2-mediated cytoprotection against lipid peroxide toxicity.

1. Introduction

Nuclear factor erythroid 2-related factor 2 (NRF2) is a member of cap’n’collar family of basic leucine-zipper (CNC-bZIP) transcription factors and serves as a master regulator of many cytoprotective genes. Under oxidative or electrophilic stress conditions, NRF2 translocates into the nucleus and binds to the antioxidant response element (ARE) bare in the 5’-promoter region of cytoprotective genes [1]. The products of ARE-containing murine genes can be classified into (i) direct antioxidant proteins: superoxide dismutase (Sod) and glutathione peroxidases (Gpx), (ii) thiol-containing molecules and their generating system: γ-glutamate cysteine ligase catalytic and modifier subunits (Gclc and Gclm), glutathione reductase (Gr), thioredoxin (Txn), and thioredoxin reductase (Txnrd), (iii) detoxifying enzymes: glutathione S-transferases (Gst), UDP-glucuronosyl transferases (Ugt), NAD(P)H:quinone oxidoreductase 1 (Nqo1), and aldo-keto reductases (Akr), (iv) stress-response proteins: heme oxygenase-1 (Ho-1) and ferritin heavy polypeptide (Fth1), (v) molecular chaperones and proteasomes, and (vi) drug transporters: multidrug resistance associated proteins (Mrp) [2–4]. Therefore, NRF2-mediated upregulation of these genes in murine system plays a critical role in the maintenance of cellular redox homeostasis and in the protection of cells from various endogenous/exogenous stresses.

In human cells, NRF2-target genes have been identified using several NRF2-activating chemical inducers. The genes encoding GSH-generating enzymes and detoxifying enzymes were increased with t-butylhydroquinone (t-BHQ) treatment in IMR-32 human neuroblastoma cell [5]. The expression of heme oxygenase-1 (HMOX-1) gene was induced by isothiocyanates via NRF2 signaling in HepG2 human hepatoma cells [6]. Recently, we demonstrated that the treatments of human renal epithelial cells with sulforaphane (SFN), t-BHQ, cinnamic aldehyde, and hydrogen peroxide (H₂O₂) increase multiple ARE-bearing genes, including AKRs, NQO1, and GCL [7].

Kelch-like ECH-associated protein1 (KEAPI) is a cytosolic repressor protein of NRF2 and acts as an adaptor protein...
for Cullin 3-based E3 ligase. In normal states, KEAP1 binds to NRF2 and promotes ubiquitylation and proteasome-mediated proteolysis of NRF2. Whereas various stresses induce conformational changes in the KEAP1 protein through sulfhydril modifications and result in a loss of NRF2 repressive function of KEAP1, which can consequently prevent NRF2 degradation [8–10]. The crucial role of KEAP1 in NRF2 regulation has been proved by studies with knockdown of Keap1-null mice. Keap1-null mice postnatally died from malnutrition resulting from hyperkeratosis in the esophagus and forestomach related to Nrf2-regulated changes in squamous epithelial genes. However, this lethality was rescued by breeding to nrf2-deficient mice [11]. Together with this phenotypic change, liver specific keap1-deleted mice show significantly increased mRNA levels for Nqo1, Gsts, and GSH biosynthetic enzymes and were more resistant to toxic doses of acetaminophen than wild-type mice [12]. These studies show that a disruption of keap1 expression is sufficient for the activation of Nrf2 and target gene induction. Therefore, keap1-knockout or knockdown cells can be used as a model of pure genetic activation of Nrf2. The upregulated genes by genetic Nrf2 activation were distinct from those in pharmacological Nrf2 activation: a modification of Keap1 expression primarily changes Nrf2 activity rather than chemical treatments [12, 13]. The transient KEAP1 knockdown by siRNA increased endogenous levels of NRF2 protein and elevated the expression of AKR1C1/2, GCLC, GCLM, and NQO1 in HaCaT human keratinocytes [14]. Similar NRF2-target gene expression pattern was observed in MCF10A human breast epithelial cell line which was transfected with KEAP1 siRNA [15]. In addition, human renal tubular epithelial HK-2 cells with stable KEAP1 knockdown also showed elevated expression of AKRs, GCLM, GSR, and NQO1 [7].

Lipid peroxidation, one consequence of oxidative stress, is initiitated by an attack of ROS on polyunsaturated fatty acids of cellular membrane and forms various reactive and cytotoxic aldehydes [16, 17]. Among them, 4-hydroxynonenal (4HNE) is a major product possessing many biological activities including cytotoxicity, genotoxicity, and chemotactic and antiproliferative activities [17]. Moreover, 4HNE is considered as the most toxic aldehyde due to its long half life and membrane diffusible property [18]. Within the cells, 4HNE can form adducts by nonspecific binding to various macromolecules, including proteins, lipids, and nucleic acids, which can lead to the disturbance of normal cellular physiology and the development of various pathophysiological status [19]. Indeed, elevated 4HNE adducts have been detected in human patients samples from neurodegenerative diseases and cancer [20, 21]. In particular, the levels of 4HNE were significantly increased in colorectal tumors [22]. 4HNE is one of substrates metabolized by human AKRs. AKR1C1-mediated reduction of 4HNE was reported in human hepatoma HepG2 and optic nerve head astrocytes [23, 24]. Other isoforms of AKR1C family and aldose reductase are also involved in the protection of cells against 4HNE toxicity [25, 26]. Human colon cancer LS-174 and Caco-2 cells, which were treated with isothiocyanates, showed elevated AKR1C1 expression and became resistant to toxicities by benzo[a]pyrene or H2O2 [27]. Moreover, in human colon cancer, activity of AKRIB10 contributed to the resistance to 4HNE, which was formed from treatment of anticancer mitomycin-c [28].

In the current study, we have investigated the effect of KEAP1-knockdown on NRF2 target gene expression and its toxicological implication using human colon cancer cells. HT29 and HCT116 cell lines, which are well-known human colon adenocarcinoma cells, were stably transduced by KEAP1 interfering RNA and gene expression pattern was monitored. We demonstrate that the expression of AKRs is highly elevated by this genetic activation model. Further, we explored the possible involvement of AKRs in hydrogen peroxide and 4HNE toxicities by examining the 4HNE adduct formation and cytotoxicity in KEAP1 silenced colon cancer cells.

2. Materials and Methods

2.1. Materials. All chemicals including H2O2, menadione, 4HNE, and flufenamic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The lentiviral expression plasmids for human KEAP1 short hairpin RNA (shRNA) and scRNA, Mission Lentiviral Packaging Mix, hexadimethrine bromide, and puromycin were from Sigma-Aldrich. The SYBR premix ExTaq system was obtained from Takara (Otsu, Japan). Primers for the polymerase chain reaction (PCR) were synthesized by Bioneer (Daegu, Republic of Korea). Antibodies recognizing NRF2, lamin B and β-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for AKR1C1 and AKR1C2 were from Abnova (Taipei, Taiwan) and 4HNE adduct antibody was purchased from Abcam (Cambridge, UK).

2.2. Cell Culture and Treatments. Human colon cancer cell lines HT29 (human colon adenocarcinoma grade II cell line) and HCT116 (human colorectal carcinoma cell line) were obtained from American Type Culture Collection (Manassas, VA, USA). HT29 cells were maintained in RPMI 1640 (Hyclone, Logan, Utah, USA) with 10% fetal bovine serum (FBS, Hyclone) and penicillin/streptomycin (WelGene Inc., Daegu, Republic of Korea). HCT116 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone) supplemented with 10% FBS and penicillin/streptomycin. These cells were grown at 37°C in a humidified 5% CO2 atmosphere.
were transected with 1.5 μg of pLKO.1-KEAPI shRNA (5'-CCGGGTGCGGCAATGCAACGCAATTCGAGAT-TGCTGTGATCATTGCCACATTGGTTTTGG-3'), or pLKO.1-scRNA (5'-CCGGGAAACAGATCGAAGAGCAACACT-CG-AGTTGGGTTCCTTCATCTGTGTGGTTTTTT-3') and the packaging mix by using Lipofectamine 2000 (Invitrogen). On the second day, the medium was exchanged with fresh complete medium. The medium containing lentiviral particles was harvested after 4 days.

2.4. Establishment of KEAPI-Knockdown Stable Cell Lines. HT29 and HCT116 cells seeded in 6-well plates were transduced with lentiviral particles containing pLKO.1-KEAPI shRNA or pLKO.1-scRNA in the presence of 8 μg/mL hexadimethrine bromide (Sigma-Aldrich). Transduction was continued for 48 h, followed by a 24 h recovery in complete medium. Stable transgene-expressing cells were selected by growth for 4 weeks in medium containing 1 μg/mL puromycin (Sigma-Aldrich).

2.5. Total RNA Extraction and RT-PCR Analysis. The total RNA was isolated from the cells using a TRIzol reagent (Invitrogen). For the synthesis of cDNA, reverse-transcriptase (RT) reactions were performed by incubating 200 ng of the total RNAs with a reaction mixture containing 0.5 μg/μL oligo dT12-18 and 200 U/μL moloney murine leukemia virus RT (Invitrogen). For conventional PCR analysis, PCR amplification for each gene was carried out with a thermal cycler (Bio-Rad, Hercules, CA, USA) and amplification conditions were 25–30 cycles of 40 s at 95°C, 30 s at 56°C, and 30 s at 72°C. PCR products were resolved on 1.2% agarose gels and the images were captured by using a Visi Doc-It imaging system (UVP, CA, USA). Real-time RT-PCR analysis for relative quantification of mRNA was performed using a Roche LightCycler (Mannheim, Germany) with the Takara SYBR Premix Ex Taq system (Otsu, Japan). The primer sequences for the human genes are shown in previous study [7].

2.6. Measurement of Luciferase Activity. Cells in 24-well plates were transsected with a mixture of 0.5 μg of ARE-luciferase plasmid, 0.05 μg of pRLtk control plasmid (Promega, Madison, WI, USA), and Lipofectamine 2000 reagent. After 18 h, the transfection mixture was removed, and the cells were incubated in complete medium for 24 h. The cells were then lysed, and Renilla and firefly luciferase activities were measured using the Dual Luciferase Assay System (Promega) with a luminometer (Turner Designs, Sunnyvale, CA, USA).

2.7. Nuclear Protein Extraction. Cells were lysed with homogenization buffer (2 M sucrose, 1 M Heps, 2 M MgCl₂, 2 M KCl, 30% glycerol, 0.5 M EDTA, 1 M dithiothreitol, protease inhibitor cocktail, and 10% NP-40) and followed by centrifugation at 12,000 g for 15 min to collect crude nuclear fractions. Then, nuclear proteins were extracted by incubating crude nuclear fractions with the extraction buffer containing 20 mM Heps (pH 7.9), 1.5 mM MgCl₂, 420 mM NaCl, 10% glycerol, 0.2 mM EDTA, and protease inhibitor cocktail for 30 min on ice.

2.8. Western Blot Analysis. Cells were lysed with RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% NP40) containing a protease inhibitor cocktail (Sigma-Aldrich). The protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Meridian Rd, Rockford, IL, USA). The protein samples were separated by electrophoresis on 6%–12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany) by using a Trans-Blot Semi-Dry Cell (Bio-Rad). The membrane was then blocked with 5% skim milk for 1 h and then incubated with the antibodies. The chemiluminescent images were captured using a GE Healthcare LAS-4000 mini imager (GE Healthcare, Uppsala, Sweden).

2.9. MTT Analysis. Cells were plated at a density of 5 x 10⁵ cells/well in 96-well plates. After 24 h of incubation cells were treated with varied concentration of H₂O₂, menadione, or 4HNE for 24 h. And then MTT solution (2 mg/mL) was added to each well and cells were further incubated for 4 h. Following the removal of MTT solution, 100 μL of dimethyl sulfoxide (DMSO) was added in each well and mixed for 5 min on shaking incubator. The absorbance was measured at 540 nm using a SPECTRO starNano (BMG LABTECH GmbH, Allmendgruen 8, Ortenberg/Germany).

2.10. Measurement of Cellular Total GSH Contents. For the measurement of total GSH contents, cells were grown in six-well plates for 24 h and lysed with 5% metaphosphoric acid solution. Clear cell lysate (30 μg) was incubated with 30 μL 5,5'-dithiobis(2-nitrobenzoic acid), GR, and β-NADPH, and optical densities were monitored at 405 nm for 4 min using a SPECTRO starNano.

2.11. Statistical Analysis. Statistical significance was analyzed using Student’s t-test or a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons, using Prism software (GraphPad Prism, La Jolla, CA, USA).

3. Results

3.1. KEAPI-Knockdown Stable HT29 Cell Line and NRF2 Activation. To investigate human NRF2 target genes, HT29 cells were transduced with either KEAPI shRNA or nonspecific scRNA expression lentiviral plasmid and then maintained in the presence of puromycin for more than 4 weeks for the establishment of stable cell lines (scHT29 or shKEAPI HT29). The stable expression of KEAPI shRNA reduced KEAPI mRNA level by 50% (Figure 1(a)) and, consequently, elevated ARE reporter activity by 69% (Figure 1(b)). Similar patterns were observed in NRF2 immunoblot analysis. Nuclear NRF2 levels were relatively higher in shKEAPI HT29 cells than those in the scHT29 control cells (Figure 1(c)). It should be noted that a delivery of nonspecific scRNA by lentiviral
Figure 1: Effect of KEAP1 knockdown on NRF2 activity in HT29 cells. (a) The mRNA level for KEAP1 was determined by real-time PCR analysis for relative quantification in HT29 cells expressing scRNA (sc) and KEAP1-specific shRNA (shKEAP1). At the same time, the expression levels of KEAP1 were determined in HT29, sc, and shKEAP1 cells using conventional PCR analysis. (b) ARE-driven luciferase activity was monitored in HT29, sc, and shKEAP1 HT29 cells. (c) The nuclear level for NRF2 protein was determined in HT29, sc, and shKEAP1 HT29 cells. Lamin B levels were monitored as a loading control. (d) The basal mRNA levels for NQO1 and GCLC were determined in HT29, sc, and shKEAP1 HT29 cells. (e) Cellular total GSH contents were measured in the sc and shKEAP1 HT29 cells. The values are relative levels with respect to the sc group and are the means ± SD of 3-4 experiments. *P < 0.05 compared with the sc control.
transduction did not affect nuclear NRF2 level and ARE activity in these cells (Figures 1(b) and 1(c)). In accord with elevated NRF2 levels, the basal mRNA levels of NQO1 and GCLC, which are representative target genes of NRF2 in murine cells, were increased by KEAP1 silencing compared to the control cells (Figure 1(d)). A consequence of GCLC elevation, cellular GSH level in KEAP1-knockdown cells was increased by 1.5-fold compared to the scRNA control (Figure 1(e)). These results confirm that KEAP1 silencing can effectively activate NRF2 signaling in colon cancer cell lines.

### 3.2. Effect of KEAP1-Knockdown on NRF2 Target Genes Expression in HT29.

To evaluate KEAP1 knockdown effect on NRF2-target genes expression, thirty NRF2-target genes, which play antioxidant or detoxification functions, were selected from previous reports with murine cell model [30, 31]. AKR1C1 was also selected as one of NRF2 target genes from a study of Hayes group [27]. The mRNA levels for these genes were determined by relative quantification real-time PCR analysis. For analysis, an upregulation >1.5-fold was considered to be a significant increase (Table 1). An altered gene profile reflects the effect of KEAP1 knockdown, conversely genetic NRF2 activation. Among measured thirty genes, the expression of twenty two genes was increased more than 1.5-fold by KEAP1 knockdown. These include GPX2, MT1A, GCLC, GCLM, GSR, TXN, TXNRD, GSTA3, GSTM2, UGT1A6, NQO1, EPHX1, AKRs (AKR1C1/2, 1C2, 1C3, 1B1, and 1B10), HMOX-1, FTH1, MRP2, and MRP3. In particular, the mRNA levels of AKRs were substantially increased in shKEAP1 HT29 cells: induction folds of AKR1C1/2 and AKR1C2 were 24.1- and 34.6-fold, respectively (Figure 2(a)). In addition, AKR1C3 and 1B10 were elevated more than 6-fold and AKR1B1 showed more than 2-fold increase in KEAP1 knockdown cells (Figure 2(a)). It should be noted that established KEAP1 knockdown cell line exhibits a 50% decrease in KEAPI expression and a 70% increase in ARE activity, whereas induction magnitudes of AKRs are substantial (2–35-folds) in HT29. Whereas NQO1, which is accepted as a representative Nrf2 target gene in murine cells, showed only 2.3-fold induction in KEAPI-silenced HT29 (Table 1). In consistent with elevated GSH contents, GSH-related genes, including GCLC, GCLM, GSR, GSTA2, GSTA3, and GSTM2, were upregulation (1.5–2.5-fold) by KEAPI silencing (Figure 2(b)). The expression
of drug transporters MRP2 and MRP3 was increased by 10.2-fold and 1.8-fold, respectively, in shKEAP1 HT29 cells (Figure 2(c)). These indicate that KEAP1-knockdown is an effective genetic tool to activate NRF2 signaling in colon cancer cells, and AKRs are a highly inducible gene group regulated by NRF2 in human cells.

3.3. Enhanced Resistance of KEAP1-Knockdown Human Colon Cancer Cells to H$_2$O$_2$- or Menadione-Mediated Cytotoxicity. Numerous studies have reported that increased NRF2 activity by chemical activator treatments can enhance cellular resistance to oxidative stress [32–34]. Therefore, we then explored the potential effects of KEAP1 knockdown on oxidative stress induced by H$_2$O$_2$ or menadione. The scHT29 and shKEAP1 HT29 cells were incubated with H$_2$O$_2$ (80–180 µM) or menadione (5–15 µM) for 24 h and cell viability was assessed by MTT analysis. Following 120 µM and 180 µM H$_2$O$_2$ incubation, the relative viability of the scHT29 was 45% and 25%, respectively, while the shKEAP1 HT29 showed 61% and 41% viabilities (Figure 3(a)). Similar patterns were observed in menadione-treated cells: viable cell ratios were 43% and 72% in 15 µM menadione-treated scHT29 and shKEAP1 HT29, respectively (Figure 3(b)). These results show that the activation of NRF2 signaling by KEAP1-knockdown can increase the cellular resistance to cytotoxic oxidative stress.

3.4. NRF2 Activity and Target Gene Expression Are Enhanced in KEAP1-Knockdown HCT116. In order to confirm the effect of KEAP1-knockdown on NRF2 target genes expression and oxidative stress susceptibility, another type of colon cancer cell line HCT116, which has a distinct genetic mutation profile [35–43], was used for the establishment of stable KEAP1 knockdown cell line (shKEAP1 HCT116). The stable expression of KEAP1 shRNA in HCT116 reduced the KEAP1 mRNA level by 36% and consequently elevated ARE reporter activity by 80% (Figures 4(a) and 4(b)). In the shKEAP1 HCT116 cells, the level of nuclear NRF2 protein was significantly increased compared with scRNA control (Figure 4(c)). The contents of total GSH were elevated by 56% in KEAP1-knockdown HCT116 (Figure 4(d)). The mRNA levels for representative NRF2 target genes such as GCLC, GCLM, and NQO1 were significantly increased by KEAP1 silencing (data not shown). In particular, transcript levels for AKR1C2/1, IC3, and 1B10 were also increased compared with the scRNA control although the induction magnitudes are smaller than HT29 (Figure 4(e)). As a consequence of NRF2 activation, KEAP1 knockdown HCT116 cells showed enhanced resistance to oxidative stress induced by H$_2$O$_2$ or menadione (Figures 4(f) and 4(g)). These results support that KEAP1 knockdown can upregulate AKRs expression and attenuate oxidative stress-mediated cell damages in human colon cancer cells.

3.5. Effect of KEAP1 Inhibition on H$_2$O$_2$-Mediated AKRs Expression and 4HNE Adduct Formation. Human AKRs can metabolize a wide range of substrates, including drugs, carcinogens, and endogenous substrates by reducing reactive aldehydes to corresponding alcohols. Among them, AKRs play an important role in detoxification of reactive lipid aldehydes such as 4HNE [44]. Our results show that KEAP1-knockdown colon cancer cells exhibit significantly enhanced AKRs expression and elevated cell viability in response to H$_2$O$_2$ or menadione treatments. Thus, we hypothesize that increased AKRs expression in KEAP1-knockdown cells may contribute to a rapid detoxification of 4HNE and thereby resulting in attenuated 4HNE adduct formation. To evaluate the association of KEAP1-knockdown-induced AKRs expression with H$_2$O$_2$ response, the mRNA levels for AKRs were assessed following H$_2$O$_2$ incubation (40 and 80 µM, 24 h). The control sc HT29 cells showed notable increases in AKR1C1, 1C2, 1C3, 1B1, and 1B10 transcripts following H$_2$O$_2$ incubation in a concentration-dependent manner (Figures 5(a)–5(e)), whereas the basal and inducible levels of AKRs in KEAP1-knockdown cells were significantly higher than those of control cells. Similarly, protein levels of AKR1C1 were greater in KEAP1-knockdown HT29 (Figure 5(f)). These indicate that AKRs are highly inducible genes upon oxidative stress condition and imply the involvement of AKRs in H$_2$O$_2$ cytotoxicity. As one of cytotoxic mechanisms of H$_2$O$_2$, ROS from H$_2$O$_2$ can attack lipid compositions and generate lipid peroxide 4HNE. Therefore, in order to ask the involvement of 4HNE and AKRs in H$_2$O$_2$ cytotoxicity, levels of 4HNE protein adducts were monitored using western blot analysis. When 200 µM H$_2$O$_2$ was incubated in cells for 4–12 h, levels of 4HNE adducts were increased at 8 and 12 h incubation in the control sc HT29 cells (Figure 6(a)), indicating the generation of 4HNE in H$_2$O$_2$–treated cells, whereas KEAP1 knockdown cells did not show an increase in 4HNE adduct level. Elevated antioxidant and detoxification capacities in KEAP1-knockdown cells may be responsible for this reduction. Thus, we next tested the association of AKRs with H$_2$O$_2$ resistance using a pharmacological inhibitor of AKR1C1-IC3 [45–47]. The sc HT29 and shKEAP1 HT29 cells were coincubated with flufenamic acid (20 µM) and H$_2$O$_2$ (80 µM), and cell viability was assessed. The treatment of cells with flufenamic acid further enhanced cytotoxic effect of H$_2$O$_2$ in both sc and shKEAP1 cell lines: cell viability was reduced from 50% to 19% in the sc control and 70% to 42% in the KEAP1-knockdown cells by flufenamic acid (Figure 6(b)). This indicates that AKRIC isozymes are associated with the cytoprotection from H$_2$O$_2$ in HT29 cells. Of note, differential cell viabilities shown in flufenamic acid coincubated sc and shKEAP1 cells may imply the involvement of other antioxidant components in 4HNE cytoprotection.

3.6. Effect of KEAP1 Inhibition on 4HNE-Mediated Cytotoxicity. Next we investigated the direct linkage between KEAP1-knockdown-mediated AKRs induction and 4HNE cytotoxicity by determining cell viability and protein adduct formation. When the sc HT29 control and shKEAP1 HT29 cells were incubated with 4HNE (0–160 µM) for 24 h, the KEAP1 knockdown cells showed enhanced cell viabilities compared with the control cells (Figure 7(a)). Similarly, the incubation of shKEAP1 cells with 4HNE for 48 h exhibits 60% viability, while less than 10% of the sc control cells
Figure 2: Effect of KEAP1 knockdown on the expression of NRF2 target genes. (a) The basal mRNA levels for AKRs (AKR1C1/2, 1C2, 1C3, 1B1, and 1B10) were determined by real-time PCR analysis for relative quantification in the sc and shKEAP1 HT29 cells. (b) The basal mRNA levels for GCLC, GCLM, GSR, GSTA2, GSTA3, and GSTM2 were determined in the sc and shKEAP1 HT29 cells. (c) The basal mRNA levels for MRP2 and MRP3 were monitored in the sc and shKEAP1 HT29 cells. The values are relative levels with respect to the sc control group and are the means ± SD of 3-4 experiments. *P < 0.05 compared with the sc control.

Figure 3: Effect of KEAP1 knockdown on cell viability upon oxidative stress. (a) Cell viabilities were measured using MTT analysis following the incubation of the sc control and shKEAP1 cells with H$_2$O$_2$ (80–180 µM) for 24 h. (b) Cell viabilities were assessed following the incubation of cells with menadione (5–15 µM) for 24 h. The values are means ± SD from 8 wells. *P < 0.05 compared with the sc group.
Figure 4: Effect of KEAP1 knockdown on NRF2 activity in HCT116 cells. (a) The mRNA level for KEAP1 was determined in HCT116 cells expressing scRNA (sc) and KEAP1-specific shRNA (shKEAP1). (b) ARE-driven luciferase activity was monitored in HCT116, sc, and shKEAP1 HCT116 cells. (c) The nuclear level for NRF2 protein was determined in HCT116, sc, and shKEAP1 HCT116 cells. Lamin B levels were used as a loading control. (d) Cellular total GSH contents were measured in HCT116, sc, and shKEAP1 HCT116 cells. (e) The basal mRNA levels for AKRs (AKR1C1/2, 1C2, 1C3, 1B1, and 1B10) were determined in HCT116, sc, and shKEAP1 HCT116 cells. Values are relative levels with respect to sc group and are the means ± SD of 3–4 experiments. (f) Cell viabilities were measured using MTT analysis following the incubation with \( \text{H}_2\text{O}_2 \) (350–650 \( \mu \text{M} \)) for 24 h. (g) Cell viabilities were measured following the incubation of cells with menadione (5–15 \( \mu \text{M} \)) for 24 h. The values are means ± SD from 8 wells. *P < 0.05 compared with the sc control.
Figure 5: Induction of AKRs by H$_2$O$_2$ treatment. The sc and shKEAP1 HT29 cells were incubated with H$_2$O$_2$ (40 or 80 μM) for 24 h. The mRNA levels for AKR1C1 (a), 1C2 (b), 1C3 (c), 1B1 (d), and 1B10 (e) in the sc and shKEAP1 HT29 cells were assessed by using real-time PCR analysis. At the same incubation conditions, the protein levels for AKR1C1 and AKR1C2 were estimated following H$_2$O$_2$ (40 or 80 μM) incubation in the sc or shKEAP1 HT29 cells (f). The values are relative levels with respect to sc vehicle group and are the means ± SD of 3-4 experiments. *P < 0.05 compared with the sc group.
survived (Figure 7(b)). In accord with the resistance to 4HNE cytotoxicity, the increase in 4HNE protein adducts was substantially reduced in KEAP1-knockdown HT29 compared to that in the control cells (Figure 7(c)). The involvement of AKR1C enzymes in 4HNE cytotoxicity could be confirmed by pharmacological inhibitor flufenamic acid treatment. The coincubation of flufenamic acid with 4HNE slightly increased 4HNE adduct formation and exacerbated 4HNE-mediated cell death in the shKEAP1 HT29 (Figures 7(d) and 7(e)). However, in the presence of flufenamic acid, KEAP1-knockdown cells still remained to be relatively more resistant to 4HNE toxicity, which implies the involvement of additional NRF2-target genes in 4HNE detoxification. Overall, these results indicate that the KEAP1-knockdown HT29 cells can be protected from 4HNE adduct formation and cytotoxicity, and elevated AKRs may be participating in facilitated 4HNE detoxification.

4. Discussion

Comparative gene analysis using nrf2 knockout mice and chemical activator treatments revealed the key role of Nrf2 in the regulation of multiple antioxidants and detoxifying enzymes. The gene expression of GSH-related enzymes such as Gcl and detoxifying enzymes such as Nqo1 was upregulation by the treatment with Nrf2 activators (dithiolethione and SFN) in wild-type mice, but not in nrf2 knockout mice

Figure 6: Effects of AKR1C on H₂O₂-mediated cytotoxicity. (a) The levels of 4HNE adducts were measured in the sc and shKEAP1 HT29 cells following the incubation with H₂O₂ (200 μM) for 0–12 h. The bar graph represents relative intensities of 4HNE adducts/β-tubulin. Average intensities of two marked bands were measured and normalized with each β-tubulin intensity. (b) The sc and shKEAP1 HT29 cells were coincubated with flufenamic acid (F, 20 μM) and H₂O₂ (80 μM) for 24 h and cell viabilities were assessed using MTT analysis. The values are relative levels with respect to each vehicle group and are the means ± SD of 8 wells. aP < 0.05 compared with the sc control cell line. bP < 0.05 compared H₂O₂ alone treated scHT29 cells.
Figure 7: Continued.
Figure 7: Effect of AKR1C on 4HNE protein adducts formation and cytotoxicity by 4HNE. (a) Cell viabilities were determined in the sc and shKEAP1 HT29 cells following the incubation with 4HNE (10–160 μM) for 24 h. (b) Cell viabilities were monitored following the incubation with 40 μM 4HNE for 48 h. The values are relative levels with respect to each vehicle group and are the means ± SD of 8 wells. *P < 0.05 compared with the sc control cell line. (c) The sc and shKEAP1 HT29 cells were incubated with 4HNE (160 μM) for 0.5 and 1 h and the levels of 4HNE adducts were measured using western blot analysis. The bar graph represents quantified intensities of 4HNE adducts/levels of 4HNE protein adducts were measured in cell lysates from the sc and shKEAP1 HT29 cells. The bar graph represents quantified intensities of 4HNE adducts/levels of 4HNE protein adducts were measured and normalized with each vehicle group and are the means ± SD of 8 wells. *P < 0.05 compared with the sc control cell line. (d) The sc and shKEAP1 HT29 cells were coincubated with flufenamic acid (Flu, 20 μM) and 4HNE (40 μM) for 3 h. The levels of 4HNE protein adducts were measured in cell lysates from the sc and shKEAP1 HT29 cells. The bar graph represents quantified intensities of 4HNE adducts/β-tubulin. Average total intensities of 4HNE adducts were monitored using western blot analysis. The bar graph represents quantified intensities of 4HNE adducts/β-tubulin. *P < 0.05 compared with the sc control cell line. (e) The cell viabilities were assessed following the coincubation with flufenamic acid (F) and 4HNE. The values are relative levels with respect to each vehicle group and are the means ± SD of 8 wells. *P < 0.05 compared with the sc control cell line. 

Moreover, hepatocyte-specific keap1-disruption in mice confirmed elevated levels of Gsts and Nqo1 in their livers [30, 31]. In rodent system, Gsts and Nqo1 are highly inducible genes by Nrf2 activation. The primary mechanism for Nrf2 activation is the dissociation of Nrf2 from Keap1. Since Keap1 is a cysteine-rich protein, modifications of sulfhydryl residues of the Keap1 protein result in an alteration of protein conformation, consequently easy to be dissociated from Nrf2 [49]. The oxidation of cysteine residues can be caused by various oxidative stress and exogenous chemicals. Reactive cysteine residues of Keap1 were identified by several studies following modification of Keap1 protein with dexamethasone (Cys 257, Cys273, Cys288, and Cys297) [50], dithiolethiones, and SFN (Cys 273 and Cys288) [51, 52], and t-BHQ (Cys151) [53, 54].

In the present study, we investigated human genes whose expression is highly dependent on NRF2 in colon cancer cells and elucidated its physiological relevance to oxidative stress-mediated toxicity. For this, we established stable colon cancer cell lines with KEAP1 knockdown as a model of pure genetic activation of NRF2 and monitored expression levels of thirty NRF2 target genes, which were known from studies with the murine system. These genes were mainly related with antioxidant and detoxification functions, and the basal and inducible expression of them is supressed in nrf2-knockout mouse tissues [30, 31]. In KEAP1 knockdown HT29 cells, among thirty genes monitored, the expression of AKRIC1/2, 1C3, and 1B10 is substantially elevated compared to other known target genes (AKRIC1/2, 24.1-folds; AKRIC2, 34.6-fold). NRF2-dependent expression of AKRs was also confirmed in other type of colon cancer cell line HCT116. However, in HCT116, the induction folds of NRF2-target genes were smaller than in HT29: AKRs expressions were only elevated by 2–4-folds by KEAP1 knockdown. Based on this, we could expect that shKEAP1 HT29 cells can be more resistant to H2O2 or menadione treatment than shKEAP1 HCT116 cells. However, in our results, the resistance to H2O2-induced oxidative stress appears to be similar in both cell lines. This phenomenon could be explained by distinct genetic profiles between these cell lines. HT29 cells harbor mutations in adenomatous polyposis coli (APC) and p53 but have wild-type genotypes in β-catenin and RAS oncogene [35–38]. On the contrary, HCT116 bears mutations in β-catenin and RAS and has normal genotypes in APC and p53 [39–43]. These distinct genetic backgrounds may be associated with differential induction folds of AKRs as well as the resistance to oxidative stress damage. AKRs have been shown as NRF2-dependent and highly inducible genes in several types of human cells. In a siRNA-mediated transient KEAP1 inhibition approach, AKR1C1, IC2, IC3, and other NRF2 target genes were increased in both human keratinocytes and breast cancer cell line. These studies showed that AKRIC induction was much greater than other NRF2 target genes [14, 15]. Similarly, in human renal tubular epithelial HK-2 cell, AKRIC1 was the most inducible gene following chemical activator treatment, and its induction was completely abolished.
in NRF2 knockdown HK-2. Furthermore, in KEAP1 silenced HK-2, the expression of AKRs and NQO1 was increased with great magnitude [7].

AKRs are soluble NAD(P)H oxidoreductases that reduce aldehydes and ketones to their corresponding primary and secondary alcohols in cytoplasm [55]. The human AKRs are classified to AKR1, AKR6, and AKR7 and have their own physiologic roles. AKR1B1 and 1B10 are aldehyde reductases that reduce sugar aldehyde and lipid-derived aldehydes. AKR1C1-IC4 genes share high sequence homologies, but they catalyze different substrates. AKR1C1 and 1C2 metabolize progesterone and 5α-dehydrotestosterone, and AKR1C3 is involved in the formation of testosterone and prostaglandin F [56]. In addition to endogenous substrates, AKR1C1 and 1C2 have been implicated in metabolism of various exogenous substrates, including drugs (e.g., cancer chemotherapeutics), carcinogens (e.g., polycyclic aromatic hydrocarbon, aflatoxin dialdehyde), and reactive aldehydes such as 4HNE. Human AKR expression is regulated by multiple transcription factors, including AP-1, aryl hydrocarbon receptor, and NRF2 [57, 58]. Human AKR1C1, 1C2, and 1C3 genes are known to have core AREs in their promoters. Functional AREs of AKR1C1 and AKR1C2 genes are located in the –6.3-kb and –5.5-kb upstream promoter regions, respectively [59]. In AKR1C3, essential AREs were identified at –1.4 and –6.8 kb upstream regions [60]. Our results imply that human AKRs, including 1C1/2, 1C3, and 1B10, can be upregulated through NRF2 and were highly inducible by KEAP1 inhibition.

Under oxidative stress conditions, elevated ROS (superoxide anion radicals, hydroxyl radicals, and H2O2) attack the polyunsaturated fatty acids of cellular membrane and produce reactive lipid aldehydes. 4HNE, the cytotoxic lipid aldehyde, reacts with various intracellular biomolecules and forms covalent adducts with proteins, DNA, and lipids. These adducts interfere with normal cell physiology and play a role as an underlying mechanism of various pathogenesis by oxidizing conditions [19]. It has been shown that NRF2 activity controls 4HNE metabolism. The inhibition of NRF2 expression resulted in a reduction of GSTA4 expression and GSH-4HNE formation and increased sensitivity to 4HNE-mediated antiproliferation and apoptosis in prostate cancer cells [61]. Activated NRF2 by SFN and carnosic acid significantly attenuated 4HNE-induced mitochondrial dysfunction [62]. In our study, high levels of AKRs expression in KEAP1 knockdown colon cancer cells appear to be associated with the resistance to 4HNE toxicity and diminished protein adducts formation. Moreover, pharmacological inhibition of AKRIC in KEAP1 knockdown cells using flufenamic acid reduced resistance against 4HNE toxicity. Coincubation of 4HNE with flufenamic acid increased levels of 4HNE protein adducts and exacerbated 4HNE cytotoxicity. Similarly, it was observed that cytotoxic effects of H2O2 were reduced in KEAP1-silenced HT29 and HCT116 with a concomitant reduction in 4HNE adducts levels. These results suggest that KEAP1 knockdown-mediated AKRs induction can contribute to 4HNE detoxification and cytoprotection from oxidative stress. It has been known that 4HNE can be metabolized to 1, 4-dihydroxy-2-nonene by AKR1C1, 1C2, and 1C3 [23, 25], and 1, 2-dihydroxynonenone by AKR1B1 [25]. Other than AKRs, GSH conjugation, aldose reductase, and HO-1 have been shown to be involved in 4HNE detoxification process [26, 62, 63]. In our study, a pharmacological inhibition of AKRIC in KEAP1 knockdown cells did not show a complete reversion in 4HNE toxicity. This can be explained by the involvement of increased AKRIB expression, elevated cellular GSH levels, and enhanced HO-1 activity in these cells.

Collectively, our results show that AKRs are the most inducible human genes regulated by NRF2 in colon derived epithelial cells, and this induction is associated with cytotoxic lipid peroxy 4HNE detoxification. Particularly, from the observation that 4HNE has a strong relevance to colon carcinogenesis in humans [22, 64], our results support the anticancer activity of the NRF2 pathway in colon tissues.

**Abbreviations**

AKRs: Aldo-keto reductases
shKEAP1: KEAP1 knockdown cell line
sc: Nonspecific scrambled RNA control cell line
ARE: Antioxidant response element
ROS: Reactive oxygen species
GSH: Glutathione
NQO1: NAD(P)H:quinone oxidoreductase-1
GST: Glutathione S-transferase
GCLC: Catalytic subunit of γ-glutamate cysteine ligase
GCLM: Modulatory subunit of γ-glutamate cysteine ligase
SOD: Superoxide dismutase
GSR: Glutathione reductase
TXNRD: Thioredoxin reductase
UGT: UDP glucuronosyl transferase
EPHX: Epoxide hydrolase
FTH: Ferritin heavy polypeptide
MRP: Multidrug resistance-associated protein
HMOX-1: Heme oxygenase-1
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMO: Dimethyl sulfoxide
H2O2: Hydrogen peroxide
4HNE: 4-Hydroxynonenal.

**Conflict of Interests**

The authors certify that there is no conflict of interests with any financial organization regarding the material discussed in the paper.

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References


Research Article

The Amelioration of N-Acetyl-p-Benzoquinone Imine Toxicity by Ginsenoside Rg3: The Role of Nrf2-Mediated Detoxification and Mrp1/Mrp3 Transports

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1. Introduction

Ginseng, one of the most commonly used herbal medicines, has been reported to be adaptogenic in the endocrine, immune, cardiovascular, and central nervous systems [1,2]. Previously, we reported that ginseng has the potential to protect against benzo[a]pyrene and acetaminophen (APAP) [3,4]. The ginseng saponins, referred to as ginsenosides, play a key role for most of the physiological and pharmacological activities of ginseng [5]. In angiogenesis, the ginsenosides Rb1 and Rg1 showed opposing activities [6]. Ginsenoside Rg3 is responsible for various pharmacological actions of red ginseng, including antitumor activity, antihyperglycemic activity, and cardio protection [7–9]. We demonstrated that Rg3, but not Rb1, Rc, or Rgl, accounted for the significant induction of glutathione S-transferase A2, which may facilitate catalysis of glutathione conjugation with APAP metabolites [4].

Hepatotoxicity induced by excess APAP, a widely used analgesic and antipyretic drug, is the most common cause of death by acute liver failure [10]. N-acetyl-p-benzoquinone imine (NAPQI), a toxic metabolic intermediate, is a major cause of hepatic necrosis as a result of high doses of APAP [10]. The reactive electrophile is subsequently converted to an inactive product through additional metabolic processes involving phase II detoxifying enzymes, which eliminate toxic metabolites [11]. However, overdose of APAP rapidly depletes hepatic glutathione (GSH), and the remaining NAPQI results in hepatocellular necrosis. N-acetylcysteine, a precursor of GSH, is the primary therapeutic treatment for APAP overdose [12]. However, a low therapeutic window and critical timing of N-acetylcysteine therapy is a limiting factor for treatment of APAP poisoning because the GSH level alone is not enough to protect against it [12,13].

Antioxidant defense systems are composed of GSH and its synthesis, phase II detoxifying enzyme, and reactive
oxygen species inactivating enzymes, which play key roles in protecting cells upon oxidative damage including that caused by NAPQI [14]. Glutamate cysteine ligase (GCL) is the rate-limiting enzyme for de novo GSH synthesis and comprises heterodimeric proteins formed of a catalytic and a modulatory subunit (GCLC and GCLM, resp.) [15]. Phase II drug-metabolizing enzymes, such as glutathione S-transferase and NAD(P)H: quinone oxidoreductase 1 (NQO1), serve to decrease the damage caused by reactive intermediates. The transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) that binds to the antioxidant response element (ARE) exerts an essential effect on the transcriptional activation of this gene induction [16].

In addition to the GSH pool and hepatic detoxification, Nrf2 stimulates hepatic multidrug resistance-associated protein (Mrp) transport, which exerts efflux of xenobiotics and metabolites instead of their accumulation [17]. Mrp2 transports conjugates of glucuronate, sulfate, and GSH into the bile (from hepatocyte) or urine (from renal proximal tubular cells), whereas Mrp3 and Mrp4 in the basolateral membrane transport these into the bloodstream towards renal excretion [17, 18]. High levels of APAP glucuronide in the plasma resulting from hepatic Mrp3 induction are excreted in the kidney, which suppresses APAP toxicity [14, 19, 20]. The dysregulation of ARE in the protective antioxidant defense system and transporters may be attributed to high sensitivity to APAP hepatotoxicity in the Nrf2-knockout mice [11, 14]. The coordination of detoxification and transport pathways by Nrf2 may enhance action in the mitigation of cellular injury.

In the present study, we demonstrated for the first time that Rg3 attenuated NAPQI-induced toxicity, which is attributed to GSH repletion, as well as induction of cellular defense genes including GCLC, GCLM, and basolateral Mrp transports via Nrf2 activation. We conclude that the beneficial effects of the ginsenoside Rg3 may contribute to detoxification and excretion of NAPQI metabolites suggesting that Rg3 should be considered as a potential hepatoprotective agent against NAPQI-induced damage.

2. Materials and Methods

2.1. Reagents. Anti-GCLC, Anti-GCLM, Anti-LaminA, and Anti-Nrf2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-goat IgGs were purchased from Zymed Laboratories (San Francisco, CA, USA). NAPQI, 20(S)-ginsenoside Rg3 (Figure 1), and other reagents in the molecular studies were acquired from Sigma Chemical (St. Louis, MO, USA). Nrf2 small interfering RNA (siRNA) and nontargeting scrambled RNA (control siRNA) were obtained from Bioneer Co. (Daejeon, Republic of Korea). Murine embryonic fibroblasts (MEFs) from wild-type and Nrf2-disrupted mice were kindly received by M. K. Kwak, Catholic University, Gyeonggi, Republic of Korea [21].

2.2. Cell Culture. H4IIE cells (ATCC CRL-1548), a rat hepatocyte-derived cell line, were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 50 units/mL penicillin, and 50 µg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO2. MEFs from wild-type and Nrf2-disrupted mice were maintained in Iscove’s modified Dulbecco’s medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum, 4 mM L-glutamine, Hepes and 50 units/mL penicillin, and 50 µg/mL streptomycin at 37°C.

2.3. Determination of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) Levels. The activities of ALT and AST, as markers of liver injury, were measured in the media using cobas 6000 chemistry analyzer (Roche, Mannheim, Germany).

2.4. Glutathione Assay. The GSH levels of cell homogenates were determined by using assay kit GSH BIOXYTECH GSH-400 (Oxis International Inc., Portland, OR, USA) and operated in accordance with the instruction.

2.5. Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from cells with TRiZol (Invitrogen, Carlsbad, CA, USA). RT-PCR amplification was conducted as previously described [22]. The number of amplification cycles was empirically determined for each primer pair to identify the logarithmic phase. The selective primer sets for GCLC, GCLM, Mrp1, Mrp2, Mrp3, Mrp4, and β-actin were designed to have a Tm of approximately 55°C and a GC content of ~50%; BLAST searches were used to confirm the specificity of the selected nucleotide sequences. Band intensities of the amplified DNAs were compared after visualization on a UV transilluminator.

2.6. Immunoblot Analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analyses were performed in lysates and nuclear protein from cells according to previously published procedures [22, 23]. The resulting image was developed using the ECL chemiluminescence detection kit (Amersham Biosciences, Amersham, UK). Equal loading of proteins was verified by β-actin or lamin A immunoblotting. Changes in the protein levels were
2.7. siRNA Knockdown. H4IIE cells were transiently transfectected with siRNA directed against rat Nrf2 or control siRNA (20 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction. The Nrf2 knockdown was confirmed in the protein samples.

2.8. Statistical Analysis. One-way analysis of variance (ANOVA) procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparisons of multiple group means. All statistical tests were two sided.

3. Results

3.1. The Protective Effect of Rg3 against NAPQI. A high dose of NAPQI causes hepatic toxicity [4, 10]. We found increases in ALT and AST levels as a result of treatment with NAPQI at a dose greater than 200 μM for 6 h in H4IIE, hepatocyte-derived cells (data not shown). To examine whether Rg3
is protective against NAPQI-induced toxicity, cells were pretreated with Rg3 for 24 h prior to treatment with NAPQI (400 μM) for 6 h. The ALT or AST leakage in the supernatant of cells treated with NAPQI was suppressed in a dose-dependent manner by Rg3 treatment (Figures 2(a) and 2(b)). Excess NAPQI rapidly depletes GSH, and the remaining NAPQI results in hepatic necrosis [10]. Additionally, we assessed the GSH level after the administration of a high dose of NAPQI for 2 h with or without Rg3. The suppression of GSH levels by NAPQI was 60% of the control level (Figure 2(c)). In contrast, the cells pretreated with 1–10 μg/mL Rg3 reversed the NAPQI-mediated GSH suppression. These results suggest that increasing GSH content by Rg3 treatment may be a beneficial mechanism for protection against NAPQI.

3.2. The Effects of Rg3 on the GSH Synthesis Gene Expression and GSH Content. GCL, which is the rate limiting step in GSH
3.4. The Role of Nrf2 in the GCLC and GCLM Induction by Rg3. To assess whether the activation of Nrf2 by Rg3 is critical for gene induction, we performed Nrf2-gene knockdown with Nrf2 targeting siRNA in H4IIE cells. Treatment with Rg3 (3 μg/mL) at 12 h increased GCLC and GCLM mRNA levels in the control group but not in Nrf2-specific siRNA transfected cells (Figure 5(a), left). To determine whether the changes in the mRNA levels were accompanied by changes in the protein expressions, we performed a western blot analysis of cell lysates treated with Rg3 (3 μg/mL) for 24 h in both cell groups. Rg3 significantly elevated the GCLC and GCLM protein expression by 4.5-fold and 3-fold, respectively, which was consistent with mRNA levels (Figure 5(a), right). In contrast, the induction of GCLC and GCLM expression by Rg3 was attenuated in Nrf2-gene knockdown cells (Figure 5(a), right). The baseline control expression of GCLC and GCLM was very low in Nrf2-siRNA transfected cells. The gene knockdown by Nrf2 siRNA at 48 h was confirmed using western blottting (Figure 5(a), right up). Next, we analyzed gene expressions that were induced by Rg3 in the Nrf2−/− cells to confirm the role of Nrf2. Rg3 increased GCLC and GCLM mRNA levels by ∼3-fold in the control group, whereas this induction was attenuated in the Nrf2-knockout group (Figure 5(a), left). Rg3 increased GCLC and GCLM protein expression by 4-fold and 3.5-fold, respectively, in the wild-type MEF but not in the Nrf2−/− MEF. This difference in gene expression in response to Rg3 between control and Nrf2 deficiency supports the idea that Nrf2 activation is critical for gene inductions by Rg3, which leads to mitigated NAPQI toxicity.

3.5. Attenuation of GSH Reversal by Rg3 against NAPQI in the Nrf2-Gene Knockdown. Reduced GSH repletion is necessary for cell survival under conditions of GSH depletion and cytotoxicity [24]. To prove the role of Nrf2 in the reversal of GSH by Rg3 in response to NAPQI, we determined GSH levels after NAPQI exposure with or without Rg3 (3 μg/mL) in gene knockdown cells using Nrf2-targeting siRNA. Treatment with NAPQI (200 μM) at 2 h readily suppressed GSH levels, and this effect was significantly reversed by Rg3 pretreatment in the control group (Figure 6). However, Rg3 failed to reverse NAPQI-mediated GSH inhibition in the Nrf2-knockdown group. These findings prove that Nrf2 is required for the Rg3-mediated GSH regulation in hepatocytes in response to NAPQI toxicity.

3.6. The Differential Gene Expressions of Mrp Family Members Induced by Rg3. GCL and Mrp coexpression in many systems suggests that these two genes are coordinately regulated [18]. Mrp family transport expression determines the efflux of APAP metabolites, resulting in alteration of susceptibility to APAP hepatotoxicity [14, 18]. Hence, we measured the Mrp family mRNA levels induced by Rg3 at the indicated doses using quantitative RT-PCR. Interestingly, Rg3 differentially regulated Mrp mRNA levels (Figure 7). Mrp1 mRNA levels were significantly increased in a dose-dependent manner after Rg3 treatment, showing a maximal induction of 3-fold at 12 h, although the basal expression of Mrp1 in the liver is very low compared to the other isozymes. Unexpectedly, Rg3 caused dose-dependent suppression of Mrp2 mRNA synthesis, controls the biosynthesis of reduced GSH form [24]. We examined GCLC, and GCLM gene expression to address the role of GSH synthesis in GSH production. Treatment of Rg3 at a dose of 3–10 μg/mL significantly increased GCLC and GCLM mRNA levels, with maximal inductions of 4.5-fold and 3-fold at 12 h, respectively (Figure 3(a)). We further evaluated whether these gene inductions lead to an increase in GSH levels. As shown in Figure 3(b), the GSH level was increased in a dose-dependent manner by Rg3, showing a saturated pattern at a dose of 1 μg/mL. These results show that a significant increase in GSH content is accompanied by gene inductions of GCLC and GCLM by Rg3.

3.3. Rg3-Mediated Nuclear Translocation of Nrf2. The nuclear Nrf2 plays a key role in the transactivation of GCLC and GCLM [11]. To determine whether Rg3 induces Nrf2 nuclear translocation, we evaluated Nrf2 expression in the nucleus of cells treated with 1–10 μg/mL Rg3. The nuclear translocation of Nrf2 after Rg3 treatment was significantly increased in a dose-dependent manner at 8 h (Figure 4). This data suggests that Nrf2 activation by Rg3 might be associated with the regulations of GCLC and GCLM.
levels. Rg3 at the dose of 10 μg/mL significantly increased the levels of Mrp3 mRNA by 4.5-fold, whereas the level of Mrp4 mRNA was not significantly altered. When we considered these effects on Mrp gene expression, it is possible that Rg3 will cause NAPQI metabolite efflux into the basolateral membrane and inhibited transport into bile.

3.7. Nrf2 Knockdown Blocks the Alteration of Mrp Family Transporter Gene Expressions by Rg3. Mrp family genes, which include Mrp1, Mrp2, Mrp3, and Mrp4, were reported to be Nrf2 target genes [18, 25]. We compared the Mrp family gene regulations by Rg3 in the Nrf2 knockdown or knockout with that in their respective controls. Rg3 increased Mrp1 mRNA levels by 2.5-fold in each control group in Figures 8(a) and 8(b), whereas the induction by Rg3 was not observed in either the Nrf2-siRNA transected cells or the Nrf2-knockout MEF (Figure 8). Slight Mrp2 suppression by Rg3 was observed in control siRNA-transfected H4IIE cells, whereas Rg3 did not inhibit Mrp2 mRNA level in Nrf2 siRNA group, suggesting that Nrf2 may be a negative regulator of Rg3-mediated Mrp2 mRNA regulation (Figure 8(a)). Unexpectedly, Mrp2 mRNA by Rg3 was unchanged in Nrf2+/+ cells, and Mrp2 mRNA was enhanced by Rg3 in Nrf2−/− MEF (Figure 8(b)). This discrepancy of Mrp2 gene expression by Rg3 between H4IIE cells and MEF remains to be further assessed. Rg3 significantly increased Mrp3 mRNA in both of the control groups but not in either of the Nrf2-loss groups. Abolishment of the increased mRNA levels of Mrp1 and Mrp3 by Rg3 was evident in Nrf2-deficiency models, implying that Nrf2 is required for the induction of these genes by Rg3. On the other hand, there was no difference in the Mrp4 mRNA levels induced by Rg3 between the genotypes. These findings demonstrate that the Mrp mRNA levels that were differentially regulated by Rg3 were in part mediated by Nrf2.
4. Discussion

Previously, we found the beneficial effect of ginseng in response to hepatotoxicants via metabolic regulation involving suppression of CYP and induction of GSTA2 [3, 4]. Several studies have shown that ginsenosides have diverse pharmacological actions [5]. Among these constituents of ginseng, Rg3, which is produced from raw ginseng by the steaming process, is one of the red ginseng-specific components. Rg3 has been reported to exert various biological activities, including anti-inflammatory, antiallergy, antitumor, and vascular relaxation [6–8]. Recently, work with Rg3 has focused on the major stereoisomer 20(S)-Rg3, that is, converted from protopanaxadiol-type ginsenosides Rb1, Rb2, Rc, and Rd [26]. We demonstrated for the first time that 20(S)-Rg3, not Rg1 and Re, is an active ingredient responsible for the GSTA2 induction for hepatoprotection by Korean red ginseng [4].

The liver, the primary organ for the metabolism and detoxification of APAP, is the main target for its protection [4]. APAP is metabolized in the liver first by the CYP450 system and subsequently conjugated with glucuronide, sulfate, and GSH. CYP2E1-knockout mice were resistant to toxic doses of APAP, which suggested that CYP2E1 is involved in the formation of NAPQI [27]. We found that Korean red ginseng suppressed CYP2E1 expression, leading to retention of intact APAP [4]. NAPQI generated from APAP binds reduced GSH to form a stable conjugate and readily depletes intrahepatic GSH. The remaining NAPQI reacts with cellular proteins and causes formation of an adduct [10]. Here, the approach with NAPQI rather than APAP itself has allowed us to focus directly on the toxicity of the active metabolite. NAPQI at concentrations greater than 200 μM resulted in significant increases in ALT and AST levels. This toxic range of NAPQI is consistent with a previous report in primary hepatocytes [10]. As shown in Figure 2, treatment of Rg3 followed by NAPQI attenuated the toxicity. Treatment with 1–50 μg/mL Rg3 did not affect the viability of H4IIE cells (data not shown). These beneficial effects of Rg3, a natural component, give rise to therapeutic potential against hepatotoxicity of APAP.

NAPQI detoxification occurs primarily by GSH conjugation. In the clinic, N-acetylcysteine was reported to produce only a ~25% reduction in mortality from APAP insult [28]. Therefore, development has focused on potential alternative agents as therapeutic targets against APAP toxicity by restoring hepatic GSH content. A genome-wide association study suggested that the GSH pathway is partially associated with variations in NAPQI toxicity [29]. The current study showed that GSH was depleted, with subsequent increases in ALT and AST activities, when cells were exposed to NAPQI at doses of 200 μM or higher. Rg3 significantly reversed the GSH depletion that was caused by a toxic dose of NAPQI. Interestingly, a low concentration of NAPQI (100 μM) caused a mild increase in GSH level without producing toxicity (data not shown), which may have resulted from cellular defensive responses including upregulations of detoxifying genes to moderate oxidative stress [17]. However, a lethal dose of APAP fails to produce enough defense pathways to block the toxicity [4]. Collectively, this suggests that Rg3-mediated GSH repletion against NAPQI toxicity may be necessary for the hepatoprotection.

One possible explanation for the reversal of GSH depletion during NAPQI challenge is GSH biosynthesis [II]. As mentioned previously, GCL, also known as gamma-glutamylcysteine synthetase, is the rate-limiting enzyme of its synthesis. One study suggested that a cysteine prodrug is the recommended antidote for APAP, because it acts as a supplement to cysteine for GSH synthesis [30]. Although N-acetyl cysteine is still used today as the only approved drug to treat APAP toxicity, more direct mechanisms besides a GSH supply are required to protect cells from APAP insult [31]. Thus, the induction of both catalytic and regulatory GCL mRNA by Rg3 may facilitate GSH biosynthesis. The observed gene induction by Rg3 was accompanied by a dose-dependent increase in GSH levels. These results demonstrate that GCL-dependent GSH biosynthesis plays an important role in Rg3-mediated protection of NAPQI toxicity.

The ARE region(s) in the 5'-flanking regions of many Nrf2 target genes including GSTA and NQO1 play a key role in gene regulation [32, 33]. We reported that Rg3 caused a great increase in luciferase activity in the ARE of the ~1.65 kb GSTA2 promoter but not in the Nrf2-deleted promoter construct [4]. The significant enhancement of GSTA2 by Rg3 was attributable to convergence of 2 transcription factors, Nrf2 and C/EBP. As shown in Figure 4,
Rg3 significantly increased the nuclear translocation of Nrf2 in a dose-dependent manner. The Nrf2 activation by a low concentration of Rg3 suggested that Nrf2 is likely to be the primary target of Rg3. Other studies such as the antiangiogenesis study used greater doses of Rg3 than what we used in this study [7]. We surmised that GCL gene induction by Rg3 is Nrf2 dependent as Nrf2 functions at the GSTA2 promoter [4]. The functional role of Nrf2 in the transactivation of these genes was confirmed in the Nrf2-deficient systems. Rg3 did not reverse the GSH depletion by NAPQI in the Nrf2 deficiency, supporting the previous studies findings that Nrf2 deficiency is highly susceptible to APAP-mediated toxicity in vivo [14]. In addition to its effects on the GSH biosynthesis genes, Rg3 also significantly induced antioxidant defense genes including NQO1, GSTA, thioredoxin reductases, and superoxide dismutase 1, which are Nrf2-controlled target genes, implying that Rg3 leads to coordinated gene inductions that protects cells in response to oxidative insults (data not shown). These observations provide evidence that the activation of Nrf2 by Rg3 produces effects on multiple targets involved in protection against NAPQI toxicity.

Recently, Mrp family genes were identified as Nrf2 target genes [18, 34]. We expected that Rg3 would upregulate Mrp family genes because Rg3 increased nuclear translocation of Nrf2 with transactivation of ARE. Indeed, Rg3 significantly increased mRNA levels of Mrp1 and Mrp3. Mrp1 contributes to Nrf2-dependent GCLC upregulation [35]. Interestingly, Rg3 suppressed Mrp2 mRNA levels. We also found that Korean red ginseng inhibited Mrp2 mRNA levels and protein expression (data not shown). Some research has demonstrated that other regulatory mechanisms (other nuclear receptors including PXR, FXR and CAR) might control Mrp2 gene expression, but only localized to the canalicular membrane [36]. Rg3 was reported to have mild glucocorticoid- and estrogen-like activities [5, 8]. Mrp2 suppression by Rg3 seems to be involved in other nuclear receptors for regulation, which are currently unknown. The MEF from wild-type mice showed that Rg3 induced mRNA levels of Mrp1 and Mrp3, which are consistent with effects in H4IIE cells. Unexpectedly, the decline of Mrp2 by Rg3 was not observed in the MEF cells. We assumed that this discrepancy of Mrp2 mRNA between H4IIE cells and embryonic fibroblasts might be due to their different machinery. The changes in mRNA levels of Mrp family genes by Rg3 were accompanied by changes in the protein expressions (data not shown).

The Nrf2 knockdown abrogated Rg3-induced Mrp1/Mrp3 gene induction and reversed Rg3-mediated Mrp2 suppression. Mrp2 mRNA by Rg3 was noticeably enhanced in Nrf2-knockout MEF. The role of Nrf2 in Mrp2 is still controversial. Two ARE sequences in the promoter region of the Mrp2 exist, and Nrf2 activators generated coinduction of Mrp and GCLC gene [18]. Conversely, the distal ARE sequence in Mrp2 most likely has a disposition for Maf homodimerization, which potentially negatively regulates Mrp2 [37]. A previous report demonstrated that Nrf2 plays a pivotal role in the constitutive levels of Mrp1 and Mrp3 but not those of Mrp2, suggesting that Mrp2 regulation is distinct and that other mechanisms may be involved [17]. Additional study of the distinct Mrp2 gene expression of Rg3 and its underlying mechanism is required.

Resistance to APAP toxicity was attributed to Mrp3 upregulation by Nrf2, which accelerated NAPQI excretion by efflux of APAP conjugates into the blood and suppression of
Figure 8: The effect of Nrf2 loss on the Mrp family gene expressions by Rg3. (a) H4IIE cells were transiently transfected with Nrf2-specific siRNA or control siRNA and subsequently treated with Rg3 (3 μg/mL) or vehicle for 12 h. The representative blots of mRNA after Rg3 treatment were assessed by RT-PCR. β-actin was used as the loading control. (b) MEFs from wild-type (Nrf2+/+) or Nrf2-deficient mice (Nrf2−/−) were treated with Rg3 (3 μg/mL) or vehicle for 12 h. The mRNA levels for the genes were analyzed as described above (a). The multiple analyses of 3 independent experiments were assessed by scanning densitometry. Each value represents the mean ± S.E. (** P < 0.01, * P < 0.05, significantly different from each control; ## P < 0.01, # P < 0.05, significantly different from Rg3 in the control group).
its enterohepatic recirculation [20]. Conversely, low expression of Mrp3 in Nrf2 knockout decreased efflux of APAP conjugates [14, 18]. Intriguingly, Mrp2 in the canalicular portion of Mrp3 in Nrf2 knockout decreased efflux of APAP (C0037058). The authors thank Mi-Kyoung Kwak for generously providing the Nrf2-knockout MEFs.

**Abbreviations**

ALT: Alanine aminotransferase  
APAP: Acetaminophen  
ARE: Antioxidant response element  
AST: Aspartate aminotransferase  
GCLC: Glutamate cysteine ligase catalytic subunit  
GCLM: Glutamate cysteine ligase modulatory subunit  
GSH: Glutathione  
Mrp: Multidrug resistance-associated protein  
NAPQI: N-acetyl-p-benzoquinone imine  
Nrf2: NF-E2-related factor 2  
RT-PCR: Reverse transcription-polymerase chain reaction.

**Conflict of Interests**

There is no conflict of interests to disclose for any of the authors.

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Role of the Nrf2-ARE Pathway in Liver Diseases

1. Introduction

The liver is a multifunctional organ that is responsible for detoxification and metabolic homeostasis. It has two blood supply sources: the hepatic artery delivers oxygenated blood from the general circulation and the portal vein supplies deoxygenated but nutrient-rich blood from the intestinal region [1]. Many cell types compose the liver. The parenchymal cells, which are the most abundant in the liver, are hepatocytes (80% by volume) [2]. The nonparenchymal cells such as endothelial cells, Kupffer cells, smooth muscle cells, hepatic stellate cells, and oval cells are other important cell components in the liver [2]. All of these cells can modulate the progression of liver diseases and activate multiple signaling pathways.

The liver is the first organ exposed to orally administered xenobiotics after absorption from the intestine, and it is a major site of biotransformation and metabolism. Since the liver is a metabolically active organ, it is particularly susceptible to reactive oxygen species (ROS). ROS are produced in liver cells as byproducts of normal metabolism and detoxification. Therefore, a wide range of antioxidant systems have developed in the liver, so that when produced, ROS are rapidly destroyed [3]. However, sustained and excessive ROS cause cellular damage and have been linked to a variety of liver diseases. Viral hepatitis and alcoholic or nonalcoholic steatohepatitis are the 3 major causes of chronic liver diseases, which are highly associated with oxidative stress, lead to liver fibrosis, cirrhosis, and end-stage hepatocellular carcinoma (HCC). Therefore, it is generally accepted that oxidative stress plays a key role in promoting the progression of these liver diseases [4].

Elevated ROS and electrophiles induce a series of antioxidant genes through the activation of antioxidant response element (ARE) to protect cells against oxidative stress [5]. ARE-containing gene expression is primarily regulated by NF-E2-related factor-2 (Nrf2), a member of the cap’n’ collar family of bZIP transcription factors [6]. Nrf2 is activated in response to oxidative stress and electrophiles in a variety of tissues and cells and plays a role as a multigorgan protector through target gene induction [7]. Keap1 is a negative regulator of Nrf2 and acts as an adapter protein for functional E3 ubiquitin ligase complex with Cul3 and Rbx1 [8, 9]. In agreement with that, Nrf2 is constitutively accumulated in nuclei in Keap1-knockout mice [10].

Nrf2 activation is observed in nonparenchymal cells including hepatic stellate cells and Kupffer cells as well as in parenchymal hepatocytes [11, 12]. Moreover, many kinds of...
Nrf2 target genes are also expressed in the liver. Nrf2 plays complex and multicellular roles in hepatic inflammation, fibrosis, hepatocarcinogenesis, and regeneration via its target gene induction (Figure 1). Therefore, the protective roles of Nrf2 activation in the pathogenesis of liver diseases have been extensively investigated. Nrf2-knockout mice and Keap1-knockout mice have been made available and used extensively to investigate the functions of Nrf2 in many hepatic injury models. In this review, we highlight recent advances in Nrf2 signaling in liver pathophysiology and discuss the potential application of Nrf2 as a therapeutic target to prevent and to treat liver diseases.

2. Role of Nrf2 in Viral Hepatitis

Viral hepatitis is a common infectious disease worldwide. There are at least six main hepatitis viruses, referred to as types A, B, C, D, E, and G, but types B and C viruses are more prevalent and might lead to liver cirrhosis and eventually cancer. An estimated 350 million people worldwide and 1.4 million people in the United States have chronic hepatitis B virus (HBV) infection [13]. An estimated 250 million people worldwide and 2.7 million people in the United States have chronic hepatitis C virus (HCV) infection [13]. Chronic viral hepatitis may lead to cirrhosis in about 20% of infected patients. Although the incidence of chronic HBV and HCV varies depending on several factors, it is well known that both infectious diseases are associated with oxidative stress [14]. However, only limited studies to date have examined the impact of Nrf2 on viral hepatitis, because hepatitis virus only infects humans and chimpanzees, having virtually no effect in other species, meaning conventional rat or mouse animal models are ineffective. Further studies are needed to identify the role of Nrf2 in viral hepatitis patient samples or an adequate animal model.

2.1. Nrf2 Signaling in HCV. The HCV genome is a single-stranded positive-sense RNA molecule of ~9,600 bases in length and encodes a large polyprotein, that is, cleaved into 11 structural (core, E1 and E2) and six nonstructural (NS) proteins (NS2-NS5B) [15]. HCV is associated with oxidative stress in human liver cells as indicated by oxidative stress markers, such as malondialdehyde, nitric oxide, and myeloperoxidase activity, which are much higher in chronic HCV patients than in healthy control subjects [16]. HCV gene expression is also reported to induce oxidative stress through Ca²⁺ signaling in the endoplasmic reticulum [17–19]. HCV infection in Huh-7 cells, a human hepatocarcinoma cell line, increases nuclear translocation of Nrf2 in a time-dependent manner and Nrf2-dependent gene induction, which contribute to cell survival against HCV infection [20]. Mitogen-activated kinases, casein kinase 2, phosphoinositide-3 kinase, and protein kinase C are involved in the phosphorylation and subsequent nuclear translocation of Nrf2 in HCV-infected cells [20, 21] (Figure 2). Consistent with this, HCV-induced Nrf2 activation is abrogated in the presence of antioxidants or Ca²⁺ chelators [20]. However, Carvajal-Yepes et al. recently reported that HCV impaired the induction of Nrf2-ARE-regulated genes by increasing the amount of small Maf proteins [22], which negatively regulate the expression of Nrf2-ARE-mediated genes [23]. Colocalization and direct interactions between small Maf and the nonstructural protein NS3, but not structural core proteins, are observed in HCV-replicating cells [22]. The controversial reports on the effect of Nrf2 activation in the antiviral defense might be due to the difference in the experimental design. Burdette et al. mainly focused on the basal level of Nrf2 translocation and target gene expression after HCV infection, whereas Carvajal-Yepes et al. evaluated the effect of HCV infection on Nrf2 overexpression- or tert-butylhydroquinone-induced Nrf2 activation.

2.2. Nrf2 Signaling in HBV. HBV is a circular DNA virus of the Hepadnaviridae family, and the HBV genome encodes two regulatory proteins: PreS2 activator large surface protein (LHBs) and the Hepatitis B virus X protein (HBx). Both proteins regulate a series of different intracellular signaling cascades [24]. Schaedler et al. recently showed that in a human HBV genome introduced-stable cell line, HepAD38 or HepG2.2.15, the expression of diverse cytoprotective genes that are regulated by Nrf2-ARE pathway in vitro and in vivo was increased compared with that in HBV-negative HepG2 cells. The HBV-mediated gene induction is primarily initiated by the two regulatory proteins of HBV, HBx and LHBs, and is triggered by the kinases, c-Raf and MEK. The Nrf2 activation results in better protection of HBV-infected cells against oxidative damage as compared with that in control cells. Moreover, HBV infection in the cells increased the Nrf2-regulated proteasomal subunit PSMB5, and HBV-positive cells have higher constitutive proteasome activity and decreased immunoproteasome activity compared with control cells. However, Nrf2 activation does not affect HBV replication [25].

3. Role of Nrf2 in Drug- or Chemical-Induced Hepatitis

The liver plays a major role in metabolizing xenobiotics such as alcohol, drugs, chemicals, and toxins. It breaks them down and eliminates them in separate steps, called phase I and phase II metabolism. The metabolism of xenobiotics could generate highly reactive intermediates which reduce molecular oxygen directly to produce ROS. Hundreds of drugs, chemicals, and toxins can cause reaction in the liver and lead to damage, that is, similar to that in acute viral hepatitis.

3.1. Nrf2 Signaling in Acetaminophen- (APAP-) Induced Hepatotoxicity. Drug-induced liver injury is a significant public health problem, accounting for over half of all cases of acute liver failure [26]. Worldwide, APAP is one of the most widely used nonprescription drugs for its analgesic and antipyretic activities. Even though it is a safe drug at a normal therapeutic dose, the possibility of hepatotoxicity remains. Indeed the major cause of drug-induced liver failure and death in the United States is APAP overdose poisonings as evidenced by over 100,000 cases each year [27, 28]. When used at
therapeutic doses, APAP is primarily metabolized in the liver by glucuronidation (52–57% total urinary metabolites) catalyzed by UDP-glucuronosyltransferase (UGT) and sulfation (30–44%) by sulfotransferases and oxidation (<5%) [29, 30]. When used at higher doses, APAP is metabolized by several cytochrome P450 enzymes into the highly reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI) [31, 32], which is normally detoxified through conjugation with glutathione (GSH) both nonenzymatically and enzymatically in a reaction catalyzed by glutathione S-transferases (GSTs) [33]. In situations when sulfation and glucuronidation become saturated and cellular GSH production and conjugation systems are defective, excess NAPQI covalently binds to cellular macromolecules, resulting in oxidative stress and cytotoxicity [34]. Because Nrf2 can transcriptionally regulate genes that are responsible for the biotransformation and excretion of APAP, namely, UGT, glutamate cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), GST, and NAD(P)H quinone oxidoreductase 1 (NQO-1) [6], Nrf2 activation is considered a prominent therapeutic target for APAP-induced hepatotoxicity (Figure 3). Two independent studies with Nrf2-knockout mice have shown that acetaminophen hepatotoxicity is exacerbated by Nrf2 deficiency. Large doses of APAP cause liver injury through oxidative stress, and Nrf2-knockout mice died sooner and at lower doses of APAP through the GSH synthesis pathway [35, 36]. Moreover, Reisman et al. reported that the elimination of APAP metabolites was decreased as a result of Mrp reduction in Nrf2-knockout mice. Furthermore, increased Mrp expression in Keap1-knockdown mice enhanced the efflux of APAP metabolites [37]. These results were confirmed by liver-specific Keap1-knockout mice which were significantly more resistant to toxic doses of acetaminophen than control animals which accompanied by the Nrf2 accumulation [10]. Goldring et al. reported that administration of APAP increased Nrf2 nuclear translocation in mouse liver as early as 60 minutes after treatment, with concomitantly increased expression of several downstream Nrf2 target genes [38]. Similar studies have confirmed the effect of APAP on hepatic induction of the Nrf2 target genes hemeoxygenase-1 (HO-1) and NQO-1 [39–41]. Elevation of NQO-1 is also observed in human liver tissues obtained during APAP overdose [42]. Moreover, NAPQI selectively modifies cysteine residues located within the intervening region of Keap1 [43]. It was recently reported that pharmacological stimulation of autophagy with rapamycin protects against APAP-induced hepatotoxicity [44]. Moreover, hepatic specific knockout of Atg5 mice, which is required specifically for autophagy, showed that persistent activation of Nrf2 and increased basal hepatic GSH levels and a faster...
recovery of GSH after APAP intoxication, which result in increased hepatocyte proliferation and protect against APAP-induced liver injury [45]. Furthermore, natural compounds with antioxidant activity such as isoliquiritigenin, sauchinone, oleanolic acid, and CDDO-Im protect against APAP-induced hepatotoxicity by activating Nrf2 [46–49].

3.2. Nrf2 Signaling in Chemical Hepatotoxin-Induced Hepatotoxicity. Randle et al. reported the ability of chemical hepatotoxins such as bromobenzene, carbon tetrachloride (CCl4), and furosemide to induce hepatic Nrf2 nuclear translocation and Nrf2-regulated gene expression [50]. Repair of the liver injury after a single treatment with CCl4 was severely delayed in Nrf2-deficient mice [51]. 1-Bromopropane (1-BP), which is an alternative to ozone-depleting solvent, exhibited hepatotoxicity [52]. The Nrf2-knockout mice showed greater susceptibility to liver injury with reduced antioxidant response from 1-BP exposure compared to wild-type mice [53]. In contrast, Nrf2 activator such as curcumin attenuated dimethylnitrosamine-induced liver injury in rats [54]. For this reason, Nrf2 activation may be a therapeutic target for conditions that involve drug- or chemical hepatotoxin-induced hepatitis.

4. Role of Nrf2 in Alcoholic Steatohepatitis

Alcoholic liver disease represents a broad spectrum of hepatic disorders, ranging from simple fatty liver (steatosis) to more severe forms of liver injury, including alcoholic steatohepatitis, cirrhosis, and HCC. Alcohol is a major contributor to liver disease-mediated deaths worldwide [55]. Alcohol is metabolized through several steps or pathways in the liver. First, alcohol dehydrogenase, cytochrome P450 2E1 (CYP2E1), and catalase metabolize alcohol to acetaldehyde, a highly toxic intermediate and well-known carcinogen. Then, acetaldehyde is further metabolized by aldehyde dehydrogenase to a less active byproduct called acetate. Increases in Nrf2 protein and mRNA levels were observed in liver tissues or hepatocytes from chronic alcohol-fed mice. HepG2 cells overexpressing CYP2E1 (E47 cells) showed increased Nrf2 mRNA and protein expression compared with control HepG2 (C34 cells). Nrf2 is activated in E47 cells as shown by an increase in nuclear translocation of Nrf2 and Nrf2-ARE binding activity and upregulation of Nrf2-regulated genes such as GCLC and HO-1 [56]. Lamlé et al. reported a critical role for Nrf2 in the protection against ethanol-induced liver injury. Nrf2-knockout mice given chronic ethanol administration showed significantly increased mortality associated with liver failure compared to wild-type mice. Reduced ability to detoxify acetaldehyde was detected in Nrf2-knockout mice, leading to accumulation of the toxic metabolite. Loss of Nrf2 caused a marked steatosis and inflammatory response mediated by Kupffer cells in ethanol-fed mice. Furthermore, chronic ethanol consumption led to a progressive depletion of total and mitochondrial reduced GSH, which was associated with more pronounced structural and functional changes to mitochondria of Nrf2-knockout mice [57]. Consistent with this, Nrf2 activation through Keap1 knockdown and hepatocyte-specific knockout blunted the increase in serum triglyceride and hepatic-free fatty acid in livers of ethanol-treated mice [58].
5. Role of Nrf2 in Nonalcoholic Steatohepatitis (NASH)

Obesity and insulin resistance are highly associated with nonalcoholic fatty liver disease, which includes nonalcoholic fatty liver and NASH. Yates et al. carried out a global analysis of mouse hepatic gene expression and revealed that both genetic and pharmacologic activation of Nrf2 induce a larger cluster of genes associated with lipid metabolism [59]. Thus, Nrf2 activation seems to play an important role in energy metabolism, especially during the development and progression of fatty liver diseases. When mice are fed high-fat diets (HFDs), the mRNA levels of Nrf2 and its target genes are reduced in wild-type mice. However, another report showed that long-term feeding of HFD increased the mRNA level of Nrf2 [60]. Although Nrf2 expression after HFD feeding remains controversial, Nrf2 appears to be linked to metabolic liver diseases by diverse pathways. Severe liver injuries were observed in Nrf2-null mice compared to wild-type mice fed HFD [61]. Consistent with this, Nrf2-knockout mice exhibited a considerable increase in micro- and macrovesicular steatosis, and a massive increase in the number of neutrophil recruitments compared to those of wild-type mice when they are fed a methionine- and choline-deficient (MCD) diet. Livers of Nrf2-knockout mice fed MCD diet suffered more oxidative stress, iron accumulation, fibrosis and inflammation than wild-type mice [62–64]. Liver X receptor-α (LXRα), a member of the orphan nuclear receptor superfamily of ligand-activated transcription factors, regulates de novo fatty acid synthesis that stimulates hepatic steatosis [65, 66]. A recent report claimed that Nrf2 activation inhibits LXRα activity and LXRα-dependent liver steatosis. Increased hepatic steatosis parameters by treatment with LXRα synthetic ligand T0901317 were further enhanced by Nrf2 deficiency. Moreover, Nrf2 activator sulforaphane (SFN) inhibited T0901317-induced SREBP-1c and lipogenic genes in hepatocytes [67]. Kay et al. also showed that Nrf2 activation promoted deacetylation of farnesoid X receptor (FXR), inducing the FXR-target gene small heterodimer partner (SHP), which was responsible for LXRα repression. In agreement with that, the transcripts of LXRα and SREBP-1c were inversely correlated with those of Nrf2, FXR, and SHP in human samples of steatosis [67].

6. Role of Nrf2 in Cholestatic Liver Injury

Impaired hepatic bile flow can lead to excessive accumulation of toxic bile acids in liver cells, causing hepatic cholestasis and liver injury. The bile acid pumps, such as bile salt export pump and Mrp, are members of the ATP-binding cassette superfamily of transporters, and Nrf2 is a key regulator of induction of certain hepatobiliary transporters, as well as of hepatic detoxification and antioxidant mechanisms [68, 69]. Reduced rates of biliary bile acid, GSH excretion, and higher levels of intrahepatic bile acids were observed in Nrf2-knockout mice compared with wild-type mice after bile duct ligation (BDL). Moreover, the hepatic bile acid transporter gene expression was altered in Nrf2 deficiency. mRNA expression of efflux basolateral transporters such as Mrp3 and Mrp4 and bile acid synthetic enzymes CYP7a1 and CYP8b1 were reduced in Nrf2-knockout mice [70] (Figure 4). BDL is a useful animal model that leads to accumulation of bile acids in the liver and results in liver injury. However, Nrf2-knockout mice are not more susceptible to hepatic injury after BDL as shown by alanine aminotransferase (ALT) and histology data due to the compensatory response of bile acid transporters and nuclear receptors pregnane X receptor [70]. In contrast, BDL-induced liver injury is significantly attenuated in Keap1-knockdown mice compared with wild-type mice, through an enhancement of antioxidative stress systems, accompanied by Mrp efflux transport [71]. Lithocholic acid (LCA) is the most toxic secondary bile acid produced in the intestine, and elevated circulating levels of LCA induce cholestatic liver injury in rodents. Nrf2-knockout mice treated with LCA had significantly more severe multifocal liver necrosis compared with wild-type mice. This was accompanied by inflammation of bile ducts and necrosis of the ductal epithelium. Serum ALT and alkaline phosphatase levels were higher in Nrf2-knockout mice administered LCA than in wild-type mice [72]. Ursodeoxycholic acid (UDCA) improves clinical and biochemical indexes in a variety of cholestatic liver diseases [73]. UDCA significantly increased nuclear Nrf2 expression in livers of wild-type mice, and the treatment produced maximal hepatic induction of Mrp2, Mrp3, and Mrp4 in an Nrf2-dependent manner [74]. Moreover, UDCA treatment enhanced hepatic Nrf2 expression and phosphorylation and upregulated hepatic thioredoxin and thioredoxin reductase 1 protein expression in primary biliary cirrhosis patients [75]. These results indicate that Nrf2 activation is very useful for prevention or treatment of cholestatic liver injury.

7. Role of Nrf2 in Liver Fibrosis and Cirrhosis

A recent report showed that treatment with SFN inhibited the development and progression of early stage hepatic...
fibrosis induced by BDL in mice, accompanied by reduced expression of profibrogenic genes or hepatic stellate cell activation marker such as type I collagen or α-smooth muscle actin, respectively. In addition, SFN treatment suppressed transforming growth factor-β-induced Smad signaling and plasminogen activator inhibitor-1 expression [76]. After long-term CCl₄ treatment, liver damage was also strongly aggravated in the Nrf2-knockout mice. Nrf2 deficiency enhanced and prolonged inflammatory and profibrogenic responses [51]. Further study is needed to define the role of Nrf2 and its molecular mechanisms in liver fibrosis and cirrhosis.

8. Role of Nrf2 in HCC

HCC, one of the most frequent tumor types worldwide, results in over 1 million deaths per year. It is the fifth most common cancer and the third leading cause of cancer death [77]. Interestingly, many well-documented chemopreventive drugs from natural products have beneficial effects on suppression of carcinogenesis and many other chronic diseases through the activation of Nrf2. Recent studies with Nrf2-deficient mice demonstrated the role of Nrf2 in protecting liver from xenobiotic-mediated hepatocarcinogenesis. During long-term treatment of 2-amino-3-methylimidazo[4,5-f]quinoline, a carcinogenic and mutagenic heterocyclic amine derivative, the multiplicity and incidence of liver tumors in male and female were significantly higher in Nrf2-knockout mice than in wild-type mice [78]. Pomegranate, an ancient fruit with antioxidant properties, reduced hepatocarcinogenesis in the diethylnitrosamine, a dietary carcinogen, exposed rat model via Nrf2 upregulation and its target gene (e.g., hepatic antioxidant genes and carcinogen detoxifying enzymes) induction [79]. Nrf2 is thus regarded as a potential molecular target for cancer chemoprevention.

9. Role of Nrf2 in Liver Regeneration

Liver regeneration is a very complicated process orchestrated through a series of signaling cascades induced by cytokines, growth factors, and hormones [80]. In contrast to Nrf1-knockout mice, Nrf2-knockout mice were fertile and did not have developmental deficits [9], suggesting that Nrf2 is not required for development. But, it was recently reported that liver regeneration is significantly impaired in Nrf2-knockout mice after partial heptectomy. Oxidative stress and hepatocyte apoptosis were enhanced after partial heptectomy in Nrf2-knockout mice compared with those in wild-type mice in accordance with Nrf2-target gene repression. Nrf2 deficiency resulted in oxidative stress-mediated insulin/insulin-like growth factor resistance through impaired mitogen-activated protein kinases and Akt, which increased hepatocyte death and delayed proliferation [81]. The Notch signaling is essential for embryogenesis of mice and affects differentiation, proliferation, and apoptosis. Notch1 is activated and plays a crucial role in cell proliferation during liver regeneration after partial heptectomy. Mouse embryonic fibroblast (MEF) cells isolated from wild-type and Nrf2-disrupted mice showed that Notch1 and its downstream target gene expression were repressed in Nrf2-knockout MEF cells. Furthermore, a functional ARE site was found in the promoter of Notch1. Nrf2-knockout mice showed lower levels of Hes1 transcripts, which reflect Notch1 signaling, following partial heptectomy in Nrf2-knockout mice. However, constitutive expression of the gene encoding Notch1 and Notch1 signaling in the hepatocytes of Nrf2-knockout mice following partial heptectomy enhanced liver regeneration to a level comparable to that in wild-type mice [82]. These results indicate that Nrf2 activation is essential for liver regeneration via alleviation of oxidative stress and regulation of hepatocyte proliferation.

10. Conclusion

Oxidative stress is implicated in the pathogenesis of liver disease. During periods of oxidative stress, Nrf2 is activated to protect the liver through target gene expression. Consistent with this, Nrf2-knockout mice are more susceptible to stresses such as chemical hepatotoxins. Moreover, most Nrf2 inducers are used for chemoprevention to detoxify carcinogens. These results show the pivotal role of the Nrf2-ARE pathway in liver pathophysiology. Moreover, they suggest the potential of Nrf2 as a therapeutic target to prevent and treat liver diseases.

Authors’ Contribution

S. M. Shin and J. H. Yang contributed equally to this work.

Conflict of Interests

The authors have declared no conflict of interest.

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Research Article

Exacerbated Airway Toxicity of Environmental Oxidant Ozone in Mice Deficient in Nrf2

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Ozone (O₃) is a strong oxidant in air pollution that has harmful effects on airways and exacerbates respiratory disorders. The transcription factor Nrf2 protects airways from oxidative stress through antioxidant response element-bearing defense gene induction. The present study was designed to determine the role of Nrf2 in airway toxicity caused by inhaled O₃ in mice. For this purpose, Nrf2-deficient (Nrf2⁻/⁻) and wild-type (Nrf2⁺/⁺) mice received acute and subacute exposures to O₃. Lung injury was determined by bronchoalveolar lavage and histopathologic analyses. Oxidation markers and mucus hypersecretion were determined by ELISA, and Nrf2 and its downstream effectors were determined by RT-PCR and/or Western blotting. Acute and subacute O₃ exposures heightened pulmonary inflammation, edema, and cell death more severely in Nrf2⁻/⁻ mice than in Nrf2⁺/⁺ mice. O₃ caused bronchiolar and terminal bronchiolar proliferation in both genotypes of mice, while the intensity of compensatory epithelial proliferation, bronchial mucous cell hyperplasia, and mucus hypersecretion was greater in Nrf2⁻/⁻ mice than in Nrf2⁺/⁺ mice. Relative to Nrf2⁺/⁺, O₃ augmented lung protein and lipid oxidation more highly in Nrf2⁻/⁻ mice. Results suggest that Nrf2 deficiency exacerbates oxidative stress and airway injury caused by the environmental pollutant O₃.

1. Introduction

Ozone (O₃) is a highly reactive gaseous oxidant air pollutant. Elevated levels of ambient O₃ have been associated with increased hospital visits and respiratory symptoms including chest discomfort, breathing difficulties, coughing, and lung function decrement [1, 2]. Moreover, subjects with preexisting asthma and rhinitis are known to be particularly vulnerable to O₃ and are at risk of exacerbations [3]. Controlled O₃ exposure studies in healthy volunteers found oxidant generation and temporal antioxidant depletion in fluid lining compartments of the airways or sputum [4]. Inhaled O₃ in experimental animal models causes airway inflammation and hyperresponsiveness, reactive oxygen species (ROS) production, mucus overproduction, and epithelial damage and compensatory proliferation predominantly in ciliated cells of the upper respiratory tract and Clara cells in terminal bronchioles. Long-term exposure of O₃ may cause lung tumors in certain strains of mice [5].

Many studies have investigated the roles of inflammatory mediators in the pathogenic airway response to O₃. Infiltration of neutrophils into the interstitium and airways contributes to O₃-induced nasal mucous cell metaplasia and airway hyperreactivity [6, 7], although some studies demonstrated uncoupling of airway inflammation and hyperreactivity [8, 9]. Tumor-necrosis-factor-α (TNF-α), a susceptibility gene for O₃ toxicity in mice [10], has a significant role in O₃-induced inflammation and airway hyperreactivity in rodent lungs mediated through nuclear factor-κB and activator protein-1 [10–13]. Toll-like receptor 4 and inflammasome proteins (e.g., Nlrp3) also contribute to O₃-induced airway hyperpermeability and hyperreactivity, respectively, in mice [14–16]. O₃ is thought to initiate toxicity by oxidation of biomolecules including proteins and lipids in epithelial lining
2.1. Mice. Breeding colonies of Nrf2+/+ and Nrf2−/− mice [25] were backcrossed to ICR (Taconic, Hudson, NY, USA) as previously published [26] and maintained in the National Institute of Environmental Health Sciences (NIEHS) animal facility. Mice were provided with modified AIN-76A diet and water ad libitum.

2.2. Inhalation Exposure. After acclimation, mice were placed in individual stainless-steel wire cages within a whole-body inhalation chamber (Hazelton 1000; Lab Products, Maywood, NJ, USA) equipped with a charcoal and high-efficiency particulate air-filtered air supply. Mice had free access to water and food. For the sub-acute model, mice were exposed continuously to 0.3 ppm O3 for 6, 24, 48, or 72 hr. For the acute model, mice were exposed continuously to 2 ppm O3 for 3 hr and recovered in room air for 3, 6, or 24 hr. O3 was generated from ultrahigh purity air (<1 ppm total hydrocarbons; National Welders Inc., Raleigh, NC, USA) using a silent arc discharge O3 generator (Model L-11, Pacific Ozone Technology, Benicia, CA, USA). Constant chamber air temperature (72 ± 3°F) and relative humidity (50 ± 15%) were maintained. O3 concentration was monitored continually (Dasibi model 1008-PC, Dasibi Environmental Corp.). Parallel exposure to filtered air was done in a separate chamber for the same duration. Immediately following each exposure, mice were euthanized by sodium pentobarbital overdose (104 mg/kg). All animal use was approved by the NIEHS Animal Care and Use Committee.

2.3. Measurement of Airways Reactivity. At the end of designated exposure duration, mice were anesthetized with urethane (1.5 g/kg in 0.125 μg/mL PBS, i.p.), placed on a temperature controlled heating pad, and connected to an EKG monitor. A tracheal cannula was surgically inserted and attached to a small animal ventilator equipped with a nebulizer. After loss of responses to pain stimulus (foot pinch), mice were paralyzed with pancuronium bromide injection (0.8 mg/kg as 0.08 mg/mL PBS) and subjected to a deep lung inflation. Lung function was measured using a computer controlled flow-type body plethysmograph system (FlexiVent; SciReq Inc., Montreal, QC, Canada). Mice were ventilated at a respiratory rate of 150 breaths/min and tidal volume of 10 mL/kg against a positive end expiratory pressure of 3 cm H2O. Following baseline resistance measurements, mice were challenged with increasing doses of acetylcholine aerosol (6.25, 12.5, or 25 mg/mL). Lung function parameters were acquired by fitting pressure and volume data to the single compartment model and the constant-phase model measuring parameters including resistance of the whole respiratory system as described by the manufacturer. From the plot of resistance against acetylcholine concentration, area under the curve (AUC) of resistance was calculated.

2.4. Bronchoalveolar Lavage (BAL) Analyses. Right lungs from each mouse were lavaged in situ with HBSS, and BAL returns were analyzed for total protein content and cell differentials as described previously [11].

2.5. Lung Histopathology. Left lung tissues from each mouse were inflated gently with 10% neutrally buffered formalin, fixed under constant pressure for 30 min, and proximal (around generation 5) and distal (approximately generation 11) levels of the main axial airway were sectioned for paraffin embedding. Tissue sections (5 μm thick) were stained with H&E and AB/PAS.

2.6. Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) of Mucin. Secreted mucin 5, subtypes A and C (Muc5AC) protein was determined with adaptation of a published method [27, 28]. Briefly, an aliquot of BAL fluid (20 μL) was loaded in each well of an ELISA plate containing a polyclonal anti-Muc5AC capture antibody (1: 40 dilution; sc-19603, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in pH 9.5 bicarbonate-carbonate coating buffer (BD OptEIA Reagent; BD Biosciences Pharmingen, San Diego, CA, USA). The plate was incubated at 48°C until the reaction was dry (>5 hr). The wells were washed and blocked overnight with an assay diluent containing 10% fetal bovine serum (BD Opt EIA) at 4°C. The samples were then incubated with a 1:100 diluted biotinylated monoclonal anti-Muc5AC detection
antibody (Clone 45M1; Thermo Scientific/Lab Vision Co., Fremont, CA, USA) for 1.5 hr at 37°C. Following incubation with a peroxidase-conjugated secondary antibody (1:2500, goat anti-mouse-IgG-HRP), color change was developed by adding the TMB substrate solution. Optical density was measured at 450 nm after the stop buffer was added.

2.7. Redox Measurement. The amount of oxidized protein was quantified in lung protien aliquots by colorimetric detection of protein carbonyls [29]. Briefly, total lung protein samples (1 µg) were adsorbed onto a 96-well plate (OxiSelect Protein Carboxyl ELISA; Cell Biolabs Inc., San Diego, CA, USA) overnight at 4°C. After derivatization of the protein carboxyl moieties by adding 2,4-dinitrophenylhydrazine (DNP), the protein samples were incubated with an anti-DNP antibody and a secondary antibody in turn following the manufacturer’s instructions. The protein carboxyl contents were quantified by absorbance at 450 nm using a standard curve from predetermined reduced and oxidized BSA standards. Lung lipid oxidation was determined by measuring the amount of malondialdehyde (MDA) which forms 1:2 adduct with thiobarbituric acid (TBA). Briefly, an aliquot of lung homogenates (equivalent to 50 µg proteins) was incubated with TBA reactive substances (OxiSelect TBARS Assay; Cell Biolabs Inc.) at 95°C for 1 hr. Color change indicating MDA-TBA adducts were measured spectrophotometrically at 532 nm, and MDA was quantified using a standard curve. Total glutathione levels in airway ELF were quantified by a kinetic method in an aliquot of BAL fluid (20 µL) following the manufacturer’s instruction (OxiSelect Total Glutathione kit, Cell Biolabs Inc.). Briefly, oxidized glutathione (GSSG) in the sample was reduced to GSH by adding glutathione reductase in the presence of NADPH and subsequently adding chromogen for reaction with the thiol group of GSH, which produced a colored compound that was detectable at 405 nm. Total GSH concentration proportional to the rate of chromophore production was determined by comparison with the predetermined GSH standard curve.

2.8. RT-PCR. cDNA was prepared from total lung RNA of each mouse (n = 3–4/group), and quantitative PCR was performed following a published procedure [30] using 240 nM of primer sets specific for glutathione peroxidase 2 (GPx2) 381 forward 5'-tgc aac cag ttc gca cat c-3', 531 reverse 5'-agg cca aga cag gat gct c-3', HO-1 (901 forward 5'-aga tca gca cta gct cat ccc-3', 1074 reverse 5'-gcc agg cca gag cat tct ccc tta-3'), or NAPDH:quinone oxidoreductase 1 (NQO1) 1141 forward 5'-agg gag ctc gaa aat act ct-3', 1303 reverse 5'-gcc gat tgt tta ctt tga gc -3') in a 7700 prism sequence detection system (Applied Biosystems, Carlsbad, CA, USA). Semiquantitative PCR was done for Nrf2 message [29].

2.9. Western Blot Analysis. Lung total proteins (50 µg) isolated from RIPA homogenates were separated on appropriate percentage Tris-HCl SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed by routine Western blotting using specific antibodies against Nrf2 (Santa Cruz Biotechnology Inc.) and pan-actin (Santa Cruz Biotechnology Inc.). Representative protein blot images from duplicates were scanned using the Bio-Rad Gel Doc system.

2.10. Statistics. SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA) was used to compare means. One-way ANOVA followed by Student-Newman-Keuls test for a posteriori comparisons was used for Nrf2 mRNA data sets. Two-way ANOVA followed by Student-Newman-Keuls test was used for other data sets. Data were expressed as group mean ± SEM. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Lung Injury Parameters in BAL. Overall, compared to acute O₃ exposure, sub-acute O₃ exposure caused greater pulmonary protein edema determined by total protein concentration and airway cell lysis determined by lactate dehydrogenase level by 72 hr exposure. In contrast, acute O₃ exposure caused more pronounced inflammatory cell influx to the airways than sub-acute exposures. The degree of airway epithelial cell exfoliation was similar in both models.

3.1.1. Sub-Acute O₃. With the exception of epithelial cells, no significant differences in the mean number of cellular phenotypes were found between Nrf2⁻/⁻ and Nrf2²⁺/⁺ mice after air exposure. However, 0.3 ppm O₃ caused significant lung edema, cellular injury, and inflammatory cell influx in both genotypes of mice, which were maximal after 72 hr exposure (Figure 1). Relative to Nrf2²⁺/⁺ mice, significantly heightened lung cell cytotoxicity indicated by BAL lactate dehydrogenase level, edema indicated by total BAL protein concentration, and epithelial exfoliation were found in Nrf2⁻/⁻ mice (Figure 1). However, no significant difference was observed in mean numbers of BAL neutrophils between the genotypes after O₃ (Figure 1).

3.1.2. Acute O₃. No significant differences in mean BAL phenotypes were found between Nrf2⁻/⁻ and Nrf2²⁺/⁺ mice after air exposure. Relative to sub-acute O₃ exposure that caused mild-to-moderate BAL phenotype changes, 2 ppm O₃ caused acute phase inflammatory responses characterized by neutrophilic influx (Figure 2). Significantly greater mean numbers of BAL neutrophils, epithelial cells, and total protein concentration were found as early as 3 hr postexposure (PE) in Nrf2⁻/⁻ mice compared to Nrf2²⁺/⁺ mice (Figure 2). BAL cell lysis was also significantly greater in Nrf2⁻/⁻ mice than in Nrf2²⁺/⁺ mice at 24 hr PE (Figure 2).

3.2. Airway Reactivity. Total airway response to acetylcholine indicated by AUC was measured at 24 hr PE after 2 ppm O₃ exposure. Mice exposed to either air or O₃ did not respond differently to aerosolized acetylcholine compared to vehicle (see Supplementary Figure 1 available online at http://dx.doi.org/10.1155/2013/254069). Although dose response pattern to acetylcholine was observed in AUC
Figure 1: Lung injury after sub-acute O₃ exposure. Lactate dehydrogenase levels (a), total cells (b), neutrophils (c), lymphocytes (d), epithelial cells (e), and total protein concentrations (f) in fluid recovered by bronchoalveolar lavage (BAL) from Nrf2⁺/⁺ and Nrf2⁻/⁻ mice after 6, 24, 48, or 72 hr exposure to 0.3 ppm O₃. Control mice were exposed to filtered air. All data are presented as mean ± SEM. *Significantly different from genotype-matched air controls (P < 0.05). †Significantly different from exposure-matched Nrf2⁺/⁺ mice (P < 0.05). n = 5 (air) or 12 (O₃) per group.
Figure 2: Lung injury after acute O₃ exposure. Lactate dehydrogenase levels (a), total cells (b), neutrophils (c), lymphocytes (d), epithelial cells (e), and total protein concentrations (f) in fluid recovered by bronchoalveolar lavage (BAL) from Nrf2⁺/⁺ and Nrf2⁻/⁻ mice 3, 6, or 24 hr after 3 hr exposure to 2 ppm O₃. Control mice were exposed to filtered air. All data are presented as mean ± SEM. * Significantly different from genotype-matched air controls (P < 0.05). † Significantly different from exposure-matched Nrf2⁺/⁺ mice (P < 0.05). n = 5–8 per group.
regardless of the genotype and exposure, genetic deletion of Nrf2 did not significantly alter airway responsiveness basally or after O₃ (Supplementary Figure 1).

3.3. Pulmonary Histopathology. Compared to air exposure, 0.3 ppm O₃ caused mild histologic changes in Nrf2⁺/⁺ lungs characterized by thickening of epithelium lining bronchioles and terminal bronchioles indicating epithelial cell proliferation and by neutrophil influx in air spaces after 72 hr (Figure 3(a)). More severe proliferation was found in Nrf2−/- mice exposed to 0.3 ppm O₃, which extended to alveolar epithelium in addition to terminal bronchial epithelium and coincided with inflammatory cell accumulation (Figure 3(a)). Consistent with the BAL phenotypes, 2 ppm O₃ caused histologically evident inflammatory cell influx to the air spaces particularly in Nrf2−/- mice from 6 hr PE (Figure 3(a)). The abundance of AB/PAS-positive mucus-bearing goblet cells in main stem airway epithelium was increased in both genotypes after 0.3 ppm O₃, while this mucous cell hyperplasia was more manifest in Nrf2−/- mice than in Nrf2⁺/⁺ mice (Figure 3(b)). Acute O₃ also caused bronchial mucous cell hyperplasia and airway mucus hypersecretion more noticeably in Nrf2−/- mice than in Nrf2⁺/⁺ mice (Figure 3(b)). As assessed by Muc5AC protein amounts in BAL fluids, mucus hypersecretion was found earlier and/or in greater amounts in Nrf2−/- mice compared to Nrf2⁺/⁺ mice after sub-acute and acute exposures (Figure 3(c)).

3.4. Pulmonary Redox Status. Significant pulmonary lipid peroxidation was found after 48 hr exposure to 0.3 ppm O₃ and 24 hr PE to 2 ppm O₃ in Nrf2⁺/⁺ mice (Figure 4(a)). Compared to Nrf2⁺/⁺ mice, we found significantly greater and earlier lung lipid peroxidation in Nrf2−/- mice during 0.3-ppm O₃ (6 hr) while O₃-induced lipid oxidation was similar between two genotypes at other time points (Figure 4(a)). Acute O₃ exposure caused significantly greater lung lipid peroxidation at 24 hr PE in Nrf2−/- mice than in Nrf2⁺/⁺ mice (Figure 4(a)). The kinetics of lung lipid peroxidation and protein oxidation were not the same in the two O₃ exposure models (Figures 4(a) and 4(b)). Mean protein carbonyl groups were greater in Nrf2−/- mice than in Nrf2⁺/⁺ mice after air exposure (Figure 4(b)). The amount of protein carbonyl group was significantly increased over the air control after 3 d exposure to 0.3 ppm O₃, and the O₃-induced protein oxidation was significantly greater in Nrf2−/- mice than in Nrf2⁺/⁺ mice after 2-3 d exposure. The effects of 2 ppm O₃ on protein oxidation were found at 3 h PE, and no significant effect of genotype was found (Figure 4(b)). Different from lung tissue levels [29], no Nrf2-dependent glutathione depletion was found in ELF of air-exposed control mice (Figure 4(c)). Total glutathiones (oxidized GSSG and reduced GSH) in BAL fluids were significantly enhanced after 6 hr of 0.3-ppm O₃ in both genotypes. Glutathione level in Nrf2⁺/⁺ mice remained elevated up to 72 hr of 0.3 ppm O₃, while it significantly declined from 48 hr O₃ in Nrf2−/- mice (Figure 4(c)); this decline occurred simultaneously with increases in protein and lipid oxidations in these mice (Figures 4(a) and 4(b)). Acute exposure to O₃ also significantly increased total BAL glutathione in Nrf2⁺/⁺ mice but not in Nrf2−/- mice (Figure 4(c)).

3.5. Pulmonary Nrf2 and Antioxidant Activation. Compared to air-exposed controls, mRNA expression of lung Nrf2 in Nrf2⁺/⁺ mice was significantly enhanced after 6 and 24 hr exposure to 0.3 ppm O₃ and declined thereafter (Figure 5(a)). Lung protein level of Nrf2 remained elevated after 72 hr O₃ (Figure 5(a)). Following acute exposure to 2 ppm O₃, Nrf2 message level appeared to increase relative to air-exposed mice, but these increases were not statistically significant (Figure 5(a)). Relative to air control mice, lung Nrf2 proteins also increased 3 hr after exposure to 2 ppm O₃ (Figure 5(a)). We also characterized expression profiles of pulmonary ARE-responsive genes GPx2, HO-1, and NQO1 after O₃ exposure. The kinetics of message levels for the genes were largely similar to those of Nrf2 (Figure 5(b)), with increases after 6 and 24 hr exposure to 0.3 ppm O₃ and increases at 3 and 6 hr PE to 2.0 ppm O₃. Nrf2-dependent differences in mean gene expression levels were found after air exposure in HO-1, after exposure to 0.3 ppm O₃ in GPx2 (48 and 72 hr), HO-1 (6, 24, and 48 hr), and NQO1 (24 hr), and after exposure to 2.0 ppm O₃ in GPx2 (3 and 6 hr PE), HO-1 (6 hr PE), and NQO1 (6 hr PE) (Figure 5(b)).

4. Discussion

Among components of ambient pollutions, O₃ is one of the most intensively studied oxidants. However, despite the extensive research on health effects of exposure to O₃, mechanisms of differential susceptibility among exposed humans and animals remain unclear. In the present study we found that, relative to wild-type mice, mice with targeted deletion of the transcription factor Nrf2 had greater numbers of inflammatory cells and markers of oxidative stress and diminished antioxidant capacity following exposure to 0.3 or 2.0 ppm O₃. These studies support the hypothesis that Nrf2 has an important role in protecting the lung against the inflammation and injury induced by exposure to O₃ and may lead to means for preventing injury induced by inhaled oxidants.

High concentrations of O₃ (≥2 ppm) are not encountered in the outdoor environment. However, short exposures to high concentrations have been used to predict a possible human exposure during vigorous exercise at a high O₃ concentration of approximately 0.4 ppm [31]. Acute exposures also provide a reproducible tool to examine molecular and cellular events underlying acute lung injury caused by oxidant overload. Sub-acute exposure (up to 72 hr) to 0.3 ppm O₃ represents a more environmentally relevant dosing regimen and also elicits airways inflammation though airways hyperreactivity is not a strong feature of this model. Based on National Ambient Air Quality Standards for ambient O₃ (8 hr average 0.075 ppm; details in http://www.epa.gov/air/criteria.html) and results from dosimetry studies in which rodents require 4-5-fold higher doses of O₃ than humans to create an
**Figure 3:** Lung histopathology and mucus hypersecretion. (a) Epithelial proliferation lining terminal bronchioles and alveoli accompanying air space infiltration of inflammatory cells in Nrf2 \(^{+/+}\) (top panels) and Nrf2 \(^{−/−}\) (bottom panels) mice after air (left panels), 72 hr exposure to 0.3 ppm O\(_3\) (middle panels), and 6 hr postexposure to 2 ppm O\(_3\) (right panels). Representative light photomicrographs of H&E-stained lung tissue sections are presented. Arrows indicate proliferation of epithelial cells. Arrow heads indicate infiltrated inflammatory cells. AV: alveoli; BR: bronchi or bronchiole; TB: terminal bronchiole; BV: blood vessel. Bars = 100 \(\mu m\). (b) AB/PAS-positive mucous goblet cells in Nrf2 \(^{+/+}\) (top panels) and Nrf2 \(^{−/−}\) (bottom panels) mice after air (left panels), 72 hr exposure to 0.3 ppm O\(_3\) (middle panels), and 6 hr postexposure to 2 ppm O\(_3\) (right panels). Inlets are higher magnification of mucus stored in bronchial epithelial goblet cells. Representative light photomicrographs of AB/PAS-stained lung tissue sections are presented. Arrows indicate intraepithelial mucosubstances. Arrow heads indicate secreted mucus in air space. Bars = 100 \(\mu m\). (c) Amount of Muc5AC proteins in secreted mucus determined by ELISA in BAL returns from Nrf2 \(^{+/+}\) and Nrf2 \(^{−/−}\) mice after air or O\(_3\) exposure. All data are presented as mean ± SEM (n = 3-4/group). * Significantly different from genotype-matched air controls (P < 0.05). † Significantly different from exposure-matched Nrf2 \(^{+/+}\) mice (P < 0.05).
Figure 4: Lung redox status. (a) Malondialdehyde (MDA) levels conjugated with the substrate TBARS in lung homogenates from Nrf2<sup>+/+</sup> and Nrf2<sup>−/−</sup> mice after 6 hr or 24, 48, and 72 hr exposure to 0.3 ppm O<sub>3</sub> (left) and 3, 6, and 24 hr after 3 hr exposure to 2 ppm O<sub>3</sub> (right). n = 3/group. (b) Oxidized protein levels in lung homogenates from Nrf2<sup>+/+</sup> and Nrf2<sup>−/−</sup> mice after exposure to air, 0.3 ppm O<sub>3</sub> (left), or 2 ppm O<sub>3</sub> (right). n = 3/group. (c) Total glutathione (GSH) in bronchoalveolar lavage returns (100 μL) from Nrf2<sup>+/+</sup> and Nrf2<sup>−/−</sup> mice after exposure to air, 0.3 ppm O<sub>3</sub> (left), or 2 ppm O<sub>3</sub> (right). n = 3/group. All data are presented as mean ± SEM. *Significantly different from genotype-matched air control mice (P < 0.05). †Significantly lower than exposure-matched Nrf2<sup>+/+</sup> mice (P < 0.05).
Figure 5: Continued.
Figure 5: Lung Nrf2 and antioxidant expression. (a) 

O$\text{3}$-induced changes in expression of Nrf2 mRNA (left panels) and protein (right panels) in lung homogenates from Nrf2 $^{+/+}$ mice after exposure to air, 0.3 ppm O$\text{3}$ (top), or 2 ppm O$\text{3}$ (bottom). Data presented as mean ± SEM ($n=3-4$ / group) after normalization to air controls. * Significantly different from air control mice ($P < 0.05$). For Western blots, pan-actin was measured as a loading control. Representative band images from replicates are shown. (b) mRNA expression of antioxidants glutathione peroxidase 2 (GPx2), heme oxygenase-1 (HO-1), and NAD(P)H:quione oxidoreductase 1 (NQO1) in lung homogenates from Nrf2 $^{+/+}$ mice after exposure to air, 0.3 ppm O$\text{3}$ (top), or 2 ppm O$\text{3}$ (bottom). Data present fold differences of each gene expression relative to Nrf2 $^{+/+}$ air after normalization to corresponding 18 s rRNA expression. Group mean ± SEM presented ($n=3$-4/group). * Significantly different from genotype-matched air control ($P < 0.05$). * Significantly different from exposure-matched Nrf2 $^{+/+}$ mice ($P < 0.05$).

equal deposition and pulmonary inflammatory response [31], either level of O$\text{3}$ used in the current study is a reasonable exposure level which is comparable with humans exposures. Interestingly, some of the protective effects of Nrf2 were specific to the two exposure regimens. For example, significantly greater number of total cells and neutrophils were found in Nrf2 $^{-/-}$ mice relative to Nrf2 $^{+/+}$ mice after acute exposure to 2 ppm O$\text{3}$, while no genotype effects were found after exposure to 0.3 ppm O$\text{3}$. One reason for this difference may be attributed to a difference in the magnitude of the injury induced by two concentrations of O$\text{3}$ in the current models. The acute exposure model elicited a larger cellular inflammatory response (e.g., $20 \times 10^3$ versus $2 \times 10^3$ neutrophils), and it is possible that the protective effect of Nrf2 may not be manifested until greater injury and subsequent sequelae initiate Nrf2 activation. Conversely, loss of Nrf2 caused increased BAL protein, epithelial cell loss, histopathological changes, and Muc5AC production in both models. The different protective effects of Nrf2 in the two models illustrate the complexity of the pulmonary response to oxidant stimuli and suggest that Nrf2 may have different protective capacities against environmental stressors that are dose-dependent.

A role for Nrf2 in response to other air pollutants has also been demonstrated. Particulate matter (PM) is known to be proinflammatory and generates ROS in airway cells and tissues, and studies have suggested a role for the Nrf2-ARE pathway in pulmonary defense against ambient PM exposures. For example, diesel exhaust particles (DEP) increased Nrf2 levels and ARE responses in airway epithelial cells [32]. Nrf2-deficient mice were significantly more susceptible to lung DNA adduct formation and allergic airway inflammation induced by DEP, compared to similarly exposed wild-type mice [33, 34]. Chronic exposure to nanosized PM also enhanced Nrf2 and ARE-responsive detoxifying enzymes in the lung [35]. Williams et al. [36] demonstrated that dendritic cells from Nrf2 $^{-/-}$ mice heightened Th2-type allergic responses including expression of surface antigens and production of interleukins 10 and 12 against ambient PM, compared to dendritic cells derived from wild-type mice. Supporting a role for Nrf2 in inflammatory allergic responses against airborne particles, polymorphisms in Nrf2 and ARE-responsive antioxidant genes (GSTPI, SOD2) were associated with a trend toward increased risk of hospitalization during periods of high outdoor PM in an asthma/COPD cohort [37]. In extra pulmonary tissues, potential protective roles of Nrf2-ARE in particulate toxicity have been addressed using mouse models of atherosclerosis [38], insulin resistance, and risk of type 2 diabetes [39].

Both O$\text{3}$ exposure regimens diminished total glutathione and increased markers of oxidant stress (oxidized proteins and lung lipid peroxidation) in the BAL fluid from Nrf2 $^{+/+}$ and Nrf2 $^{-/-}$ mice. In general, these effects were greater in Nrf2 $^{-/-}$ mice than in Nrf2 $^{+/+}$ mice. These results are consistent with the hypothesis that absence of Nrf2 suppresses antioxidant capacity and leads to greater O$\text{3}$-induced production of oxidized molecules which contributes to enhanced inflammatory response in Nrf2 $^{+/+}$ mice compared to Nrf2 $^{-/-}$ mice. Although health effects of environmental O$\text{3}$ have been broadly examined (e.g., http://www.epa.gov/
variable responses to O₃ on the role of Nrf2. In addition, mice genetically deficient to protect the epithelial barrier against O₃[5, 42]. Antioxidants in cells and the lining fluid are thought oxidative DNA fragmentation and adduct (8-oxo-dG) formation [41], which could involve the weak carcinogenic response in mouse lung after chronic exposure [5, 42]. Antioxidants in cells and the lining fluid are thought to protect the epithelial barrier against O₃ or its reaction products. Therefore potentially important mechanisms contributing to respiratory pathogenesis of O₃ include the imbalance between ROS and antioxidant capacity, and Nrf2 may have an important role in maintaining the balance.

Results of our investigation lead to the possibility that dietary supplementation with antioxidants may prevent or suppress the toxic effects of exposure to O₃. However, the effectiveness of antioxidant supplements (e.g., vitamins A, C, and E, N-acetylcysteine) remains inconclusive in human studies of O₃ exposure [43]. In laboratory rodents, supplementation with gamma-tocopherol significantly attenuated allergic responses and mucus production in upper airways [44]. Servais et al. [45] found that immature (3 wk old) rats were more sensitive to O₃ (0.5 ppm, 12 hr/d, and 7 d) in body weight loss and DNA adduct formation than adult (6 wk old) rats, and they attributed this difference to relatively lower SOD, GPx, and catalase in the immature rats compared to the adults. Moreover, mice overexpressing Cu/Zn SOD (SOD1) were also resistant to acute O₃ (0.8ppm, 3 hr)-induced edema, inflammation, and lipid peroxidation in the lung [46]. Recent studies demonstrated that ambient level of O₃ increases Nrf2 and ARE responses in airway cells or in the lung [22–24], though little attention has focused on the role of Nrf2. In addition, mice genetically deficient in phase 2 detoxifying enzymes, direct Nrf2 effectors, have variable responses to O₃. Enhanced inflammation, vascular permeability, and DNA adduct formation were found in the lung of metallothionein (Mt1/Mt2) null mice after sub-acute O₃ (0.3 ppm, 65 hr) exposure [47]. In contrast, with 70% depletion of glutathione, reduced lung injury was found in mice deficient in modifier subunit of glutamate cystein ligase (Gclm) relative to their wild-type controls [48]. The authors suggested that compensatory magnification of antioxidant defenses such as metallothioneins, alpha-tocopherol transporter protein, and solute carrier family 23 member 2 (sodium-dependent vitamin C transporter) in Gclm−/− mice may confer increased resistance to O₃-induced lung injury [48]. Similarly, mice genetically deficient in peroxiredoxin (Prdx1) were more protected against acute O₃ (2 ppm, 6 hr)-induced lung inflammation compared to wild-type mice, and Prxl as a potent pro-inflammatory factor activating toll-like receptor 4/NF-κB signaling was thought to recruit the inflammatory regulators in the model [22]. Overall, deletion of single defense enzyme may not be sufficient to affect airway pathogenesis by acute or sub-acute O₃. The protective effect of Nrf2 in O₃-exposed lung was noticeable in anti-inflammation and redox balance as well as protection of airway cell death and exfoliation and mucus overproduction in either or both exposure periods. Inasmuch as emerging evidence indicates that Nrf2 not only modulates antioxidant enzymes but also affects various pathways including cell cycle and immunity directly through ARE target genes or indirectly through interaction with other signaling networks [26, 49, 50], Nrf2 may exert its defensive effect against O₃ not only through antioxidant defense but also through mechanisms such as activation of macrophage scavenger receptor [51] or inhibition of the inflammasome pathway [52].

Acute exposure to 2 ppm O₃ did not alter airways reactivity in wild-type mice, and any effect of Nrf2 deficiency on airway hyperreactivity in response to O₃ could not be evaluated in the current study. It has been noted that changes in airways reactivity and inflammation/injury in response to O₃ are not always codependent in rodents [53] or in human subjects [54, 55]. Furthermore, airways reactivity to acetylcholine is strain dependent [53]. The background strain (ICR) of the current study may have contributed to the low acetylcholine reactivity basally and after O₃ exposure, considering that ICR mice are more like Th1-responders as they lack pulmonary eosinophilia and serum IgE induction after airway viral infection [29], compared to Th2-responder strains such as BALB/c. Alternatively, as severe mucus overproduction and hyper-secretion are the key phenotypes in the O₃-susceptible Nrf2−/− mice, it is also possible that airway plugging by excess mucus may hinder the access of aerosolized acetylcholine to the muscarinic receptors and interrupt the measurement of airway functions in these mice. Further investigations with targeted deletion of Nrf2 on different strain backgrounds should provide insight to the role of Nrf2 on airway reactivity.

5. Conclusion

Genetic loss of Nrf2 augmented pulmonary cellular toxicity including inflammatory cell influx, epithelial injury, and mucus cell hyperplasia leading to mucus hyper-secretion against ambient levels of O₃. Heightened pulmonary oxidative stress indicated by lipid peroxidation after acute O₃ exposure and protein oxidation after sub-acute O₃ exposure parallel with suppressed antioxidant defense in Nrf2−/− mice relative to their wild-type controls explain the protective role of Nrf2. Results suggest that therapeutic intervention of Nrf2 inducers for respiratory disorders may protect individuals at risk to environmental oxidants.

Abbreviations

AB/PAS: Alcan blue/periodic acid Schiff
ANOVA: Analysis of variance
ARE: Antioxidant response element
Conflict of Interests

The authors declare that they have no conflict of interests.

Authors’ Contribution

H.-Y. Cho and S. R. Kleeberger designed the research; H.-Y. Cho and W. Gladwell conducted the research, and M. Yamamoto provided the animals; H.-Y. Cho analyzed data and wrote the paper; S. R. Kleeberger edited the paper. All authors have read and approved the final paper.

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Research Article

Activation of the Nrf2 Pathway by Inorganic Arsenic in Human Hepatocytes and the Role of Transcriptional Repressor Bach1

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Previous studies have proved that the environmental toxicant, inorganic arsenic, activates nuclear factor erythroid 2-related factor 2 (Nrf2) pathway in many different cell types. This study tried to explore the hepatic Nrf2 pathway upon arsenic treatment comprehensively, since liver is one of the major target organs of arsenical toxicity. Our results showed that inorganic arsenic significantly induced Nrf2 protein and mRNA expression in Chang human hepatocytes. We also observed a dose-dependent increase of antioxidant response element- (ARE-) luciferase activity. Both the mRNA and protein levels of NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) were all upregulated dramatically. On the other hand, entry and accumulating of Nrf2 protein in the nucleus, while exporting the transcriptional repressor BTB and CNC homology 1 (Bach1) from nucleus to cytoplasm, were also confirmed by western blot and immunofluorescence assay. Our results therefore confirmed the arsenic-induced Nrf2 pathway activation in hepatocytes and also suggested that the translocation of Bach1 was associated with the regulation of Nrf2 pathway by arsenic. Hepatic Nrf2 pathway plays indispensable roles for cellular defenses against arsenic hepatotoxicity, and the interplay of Bach1 and Nrf2 may be helpful to understand the self-defensive responses and the diverse biological effects of arsenicals.

1. Introduction

Inorganic arsenic is a ubiquitous environmental contaminant and has been identified as a human carcinogen by International Agency for Research on Cancer (IARC, 2004). Arsenic exposure could result in both chronic and acute toxicity in humans. The main cause of the widespread chronic arsenicosis is the consumption of underground drinking water naturally contaminated with arsenic. Chronic exposure to drinking water containing high levels of inorganic arsenic is associated with various skin diseases, diabetes, cardiovascular diseases, and cancers of several organs [1]. Acute arsenic poisoning is relatively less common but has been documented after accidental ingestion of insecticides or pesticides and attempted suicides or murders with arsenicals [2]. Acute exposure to inorganic arsenic in humans usually results in cardiac failure, neuropathy, anemia, leucopenia, and death [3–7]. On the other hand, interestingly, arsenic-containing compounds have been proven to be effective as therapeutic agents in treating cancer such as leukemia [8], chronic inflammatory disease [9], and parasitic infection [10]. It is well known that oxidative stress is an important mechanism for arsenic pathogenesis [11]. However, the exact molecular targets and signaling pathways that account for most of the biological effects of arsenic remain to be determined.

Nuclear factor erythroid 2-related factor 2 (Nrf2), a cap "n" collar (CNC) basic leucine zipper protein, is a redox-sensitive transcription factor, and the Nrf2 pathway is commonly recognized to augment the cellular defenses against elevated oxidative damages [12]. When cells are exposed to oxidative stress, Nrf2 escapes from the Kelch-like ECH-associated protein 1- (Keap1-) mediated repression in the cytoplasm then translocates to the nucleus, forms heterodimers with the small Maf proteins, and binds to the antioxidant response element (ARE) sequence to activate transcription of antioxidant enzymes and phase II drug-metabolizing enzymes (e.g., NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), glutathione transferases (GSTs),
and glutamate-cysteine ligase (both subunits GCLC/GCLM)) [13, 14]. The induction of these enzymes is now regarded as a strategy for cellular protection against the adverse effects of excess reactive oxygen species (ROS) production.

Cell cultures and animal experiments have shown that arsenic is an inductor of the Nrf2 pathway [15]. It has been reported that arsenic increased the protein level of Nrf2, as well as induced increase in NQO1 gene expression and enzyme activity in mouse hepatoma hepG2c7 cells [16]. Furthermore, arsenic is proved to induce the association of Nrf2 with Mafs by chromatin immunoprecipitation (ChIP) assay [16]. Our previous studies have also shown a quick and significant activation of Nrf2 protein and upregulation of HO-1 mRNA and protein by sodium arsenite treatment [17]. However, the more detailed aspects of Nrf2 pathway, including the ARE-luciferase activity (representing the transcriptional activity of Nrf2), and the expressions of other Nrf2-regulated downstream genes and proteins by arsenic exposure still need to be explored in hepatocytes, since the liver is one of the target organs of arsenical toxicity [18]. Besides, liver is the most important site of arsenic biotransformation and methylation, supported by studies showing a marked improvement of arsenic methylation in patients with end-stage liver disease following liver transplantation [19].

On the other hand, studies on Nrf2 pathway recent years lead to the discovery of Bach1 (BTB and CNC homology 1), a kind of nuclear transcriptional repressor of Nrf2 activation. Similar to Nrf2, Bach1 also belongs to the CNC family and could form heterodimers with the small Maf proteins that bind to ARE just like the Nrf2/small Maf heterodimers [20]. However, it has been suggested that only when Bach1 dissociates from ARE and exports from the nucleus upon oxidative stress, imported nuclear Nrf2 could become accessible to ARE and initiate the transcription of Nrf2 target downstream genes. It is reported that the existing of Bach1 in the heterodimers with small Maf could compete with Nrf2 for ARE-binding and therefore inhibits the gene expressions of HO-1 [21], NQO1 [22], and GCLC and GCLM [23], the rate-limiting enzyme of GSH biosynthesis. However, as to inorganic arsenic exposure, whether Nrf2 activation is associated with Bach1 export from nucleus remains to be investigated. Understanding the role of Bach1 in Nrf2 pathway and the relations between Bach1 and Nrf2 are important for exploring the diverse biological effects of arsenic.

In this paper, we first observed the arsenic-induced activation of Nrf2 mRNA and protein and then investigated the increase of Nrf2 transcriptional activity, as well as the upregulation of Nrf2 downstream genes in Chang human hepatocytes. We also demonstrated that arsenic-induced nuclear import of Nrf2 was accompanied with nuclear export of Bach1 in these cells.

2. Materials and Methods

2.1. Reagents. Sodium arsenite (NaAsO₂, ≥99.0%), dimethylsulfoxide (DMSO), tert-butylhydroquinone (tBHQ, ≥97.0%), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), puromycin, hexadimethrine bromide, and paraformaldehyde were purchased from Sigma Chemical (St. Louis, MO, USA). Oligonucleotides used for primers were purchased from Takara (Dalian, China) and Sangon Biological Engineering Technology (Shanghai, China). Real-time polymerase chain reaction (real-time PCR) Kit was from Takara Co. (Japan). Primary antibodies of Nrf2 (H-300: sc-13032), NQO1 (A180: sc-32793), HO-1 (H-105: sc-10789), Bach1 (C-20: sc-14700), β-actin (I-19: sc-1616), Lamin B (C-20: sc-6216), and secondary antibodies conjugated with horseradish peroxidase IgG were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents and chemicals used were of the highest grade available.

2.2. Cell Culture. Chang human hepatocyte line (number Z11003, Cell Bank of Chinese Academy of Sciences, Shanghai, China) was maintained in RPMI medium 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Tianjin Bio, China) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Sigma, USA). All cell cultures were maintained in a humidified and 37°C incubator with 5% CO₂ and 95% air. Cells were subcultured with 0.25% trypsin (Gibco, USA) at a ratio of 1: 3 and used at 75~80% confluence for the following experiments.

2.3. Preparation of Protein Extracts and Western Blot Assay. Cells were treated with NaAsO₂ (10, 25, and 50 μmol/L) for different time intervals as detailed in respective figure legends. After washing three times with ice-cold phosphate-buffered saline (PBS), whole-cell extracts were obtained with cell lysis buffer (50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 1% Nonidet P-40 (NP-40), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 0.5% sodium deoxycholate) [24]. Nuclear and cytosolic extracts were prepared using a Nuclear Extraction Kit from KeyGEN (KeyGEN Biotechnology, Nanjing, China) according to the manufacturer’s protocol. Protein concentrations were measured using a Protein Assay Kit (Bio-Rad, CA, USA) according to the manufacturer’s recommendation. An equal amount (30 μg) of protein for each sample was resolved on 10% or 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, USA). Blots were probed with the primary antibodies of Nrf2 (1:1000), NQO1 (1:1000), HO-1 (1:1000), and Bach1 (1:1000) 4°C overnight, followed by incubation with secondary antibodies conjugated to horseradish peroxidase, respectively (Santa Cruz, CA, USA). Blots were then incubated with chemiluminescence reagents (PicoWest Super Signal, Pierce Biotechnology, IL, USA) and visualized using Electrophoresis Gel Imaging Analysis System (MF-ChemiBIS 3.2, DNR Bio-Imaging Systems, Israel). β-actin (1:2000) and Lamin B (1:1000) were used as the internal control. Incubation, isolation, and western blot analysis were performed 3 times for each condition.

2.4. Total RNA Isolation and Real-Time PCR Analysis. Chang human hepatocytes were seeded at a density of
1 × 10^5 cells/mL in a six-well plate for 24 h and then treated with NaAsO_2 (10, 25, and 50 μmol/L) for 6 h. Total RNA was extracted using the TRIZOL (Invitrogen, Grand Island, NY, USA). 500 ng of total RNA was reverse transcribed to cDNA using PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time, Takara, Japan), and PCR amplification was performed by SYBR Premix Ex Taq II Kit (Perfect Real Time, Takara, Japan). Real-time PCR was performed by using 7500 Real-Time PCR system (ABI, USA) according to the manufacturer’s instructions. PCR amplification conditions were 1 cycle of initial denaturation (95°C for 30 s), 40 cycles of amplification (95°C for 5 s and 60°C for 34 s). Primers for human genes were designed and synthesized by Takara (Dalian, China) as follows: hNrf2 (Accession number NM-006164.4), forward (AGCCGACACTCCAGTCAG) and reverse (TGGATGAGATGGATGGGAG) and reverse (AGGGGAGACTGGAATATCAC), 223 bp (280–502); hHO-1 (accession number NM-002133.2), forward (TTGGAAGGTTCTGCAACTCC), 150 bp (622–771); hGAPDH (accession number NM-000903.2) (from Sangon Biotechnological Engineering Technology, Shanghai, China), forward (TGCATGCACTCATCAAAGTGTTAAG), 99 bp (819–917); hNQO1 (accession number NM-000903.2) (from Sangon Biotechnological Engineering Technology, Shanghai, China), forward (AGCCCAGCACATCCAGTCAG) and reverse (TCAGCAGCTCTGCAACTCC), 150 bp (622–771); hGAPDH (accession number NM-000903.2), forward (GCACCGTCAAGGCTTGGCCACCAAGTTC) and reverse (TGGTGAAGACGCCAGTTGGA), 138 bp (275–412). All primer sets were tested prior to use in this work to ensure that only a single product of the correct size was amplified. Triplicate reactions were performed for each sample. Cycle threshold (Ct) values were obtained graphically for both different target genes and GAPDH. The Ct values of different target genes were first normalized to GAPDH in the same sample and expressed as ΔCt values. Then ΔΔCt values were obtained by subtracting the ΔCt values of the control samples from that of the treated samples, and 2^−ΔΔCt values were calculated to represent the amounts of different target genes. The final values presented were expressed as ratio to control cells.

2.5. Antioxidant Response Element (ARE) Reporter Assay. Cignal Lenti ARE reporter was obtained from SABiosciences (Frederick, MD, USA), which was a ready-to-transduce ARE-responsive lentiviral firefly luciferase reporter for monitoring the transcriptional activity of Nrf2. Lentiviral transfection of Chang human hepatocyte line was performed as described previously [25]. Briefly, Chang human hepatocytes were plated in 6-well plates at ~40–50% confluence in RPMI medium 1640. The following day, hexadimethrine bromide (Sigma), a transfection enhancer, was added to each well at a concentration of 8 μg/mL, and viral particles were added to each well at a concentration of 2 × 10^5 transducing units (TU)/mL. Following overnight incubation, medium containing viral particles was removed and replaced with fresh medium containing 1.2 μg/mL of puromycin. Transfected Chang human hepatocytes were grown to ~90% confluency, seeded in 96-well plates, and then exposed to tert-butylhydroquinone (tBHQ, Sigma) (10 and 50 μmol/L) or NaAsO_2 (10, 25, and 50 μmol/L) for 6 h, respectively. Luciferase activity was finally measured using the Luciferase Reporter Gene Assay Kit (Beyotime Institute of Biotechnology, China) and normalized to cell viability, which was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously [24]. Luciferase activity was finally expressed as ratio to control cells.

2.6. Immunofluorescence Assay. The subcellular localization of Bach1 in Chang human hepatocytes was detected by indirect immunofluorescence assay as described previously [26]. In brief, cells were inoculated in 8-well Lab-Tek Chamber slides at a density of 1 × 10^5 cells/mL in RPMI 1640 medium overnight, followed by treated with 25 μmol/L NaAsO_2 for 4 h. Cells were gently washed twice with PBS, fixed in 4% paraformaldehyde for 10 min, and then permeabilized with 0.2% Triton X-100 for 15 min. After washing 3 times with PBS, the cells were incubated with primary antibody of Bach1 (1:100) overnight at 4°C and then incubated with secondary tetramethyl rhodamine isothiocyanate- (TRITC-) conjugated IgG (Eartbox LLC, CA, USA) for 1 h. After washing 3 times with PBS, the slide was mounted with 4',6-diamidino-2-phenylindole (DAPI, Sigma) counterstaining to visualize the nuclei (blue). The cells were examined immediately by Fluorescence Microscope with Digital CCD Imaging System (BX61/DP71, OLYMPUS, Japan).

2.7. Statistical Analysis. All the experiments were repeated three times and carried out at least in triplicate. Data were presented as mean ± standard deviation (SD). Statistical significances were determined by one-way analysis of variance (ANOVA) followed by Student-Newman-Keul’s posthoc comparison (SPSS 10.0, SPSS, Chicago, IL). Difference with P < 0.05 was considered statistically significant.

3. Results

3.1. NaAsO_2 Increases Total Cellular Nrf2 Protein Levels in Chang Human Hepatocytes. Previous studies have shown that inorganic arsenic could induce the activation of Nrf2 pathway in UROtsa (human bladder cell line) [27], HaCaT (human keratinocyte line) [15], endothelial cells [28], and some other cell types [29]. As an indicator of Nrf2 activation, we monitored the Nrf2 protein levels in whole-cell lysates, since these values could reflect relative Nrf2 levels in the nuclear fractions of arsenic-treated cells [30]. In this report, we first observed the total cellular Nrf2 protein levels after 10 μmol/L NaAsO_2 treatment for different time intervals. The results of western blot analysis showed that the total cellular Nrf2 protein levels increased after 2 h, peaked at 4 to 12 h, and subsequently decreased to control levels after 24 h (Figure 1(a)). However, low level of Nrf2 protein was found in 36 h group, which may be due to increased cell death with the prolonged NaAsO_2 exposure. Accordingly, in all the next experiments, we chose the 24 h as the longest exposure time. 25 and 50 μmol/L NaAsO_2 also caused a rapid increase in Nrf2 protein levels, peaked at 4 h to 12 h, and decreased thereafter (Figures 1(b) and 1(c)), consistently with the results of 10 μmol/L NaAsO_2 treatment.
In addition, we also observed the dose-effect response of intracellular Nrf2 proteins induced by different levels of arsenic. We found that the Nrf2 protein levels increased with the higher doses as well, after 6 h exposed to 10, 25, and 50 μmol/L of inorganic arsenic in Chang hepatocytes (Figure 1(d)).

3.2. NaAsO₂ Induces Nrf2 mRNA Expression in Chang Human Hepatocytes. Nrf2 has been shown to be regulated by arsenic at both transcriptional [15] and posttranscriptional [31] levels. In this study, we examined the changes of Nrf2 mRNA by sodium arsenic treatment. The results of real-time PCR showed that different doses of NaAsO₂ treatment could...
increase the expression of Nrf2 mRNA in hepatocytes to some extent (Figure 2). Together with results in Figure 1, we suggested that the elevated levels of Nrf2 protein in arsenic-treated Chang human hepatocytes were, at least in part, due to an increase in Nrf2 gene transcription.

3.3. NaAsO₂ Increases ARE-Luciferase Activity with a Dose-Effect Response in Chang Human Hepatocytes. The Cignal Lenti ARE reporter is ready-to-transduce lentiviral particles for monitoring the transcriptional activity of Nrf2, which could easily and rapidly monitor the activation of ARE-regulated signaling pathway in cells. In this report, Chang human hepatocytes stably transduced with the Cignal Lentil ARE reporter showed a dose-dependent increase of luciferase activity after TBIQ (a confirmed Nrf2 activator) treatment, indicating that the transfected cells were responsive to Nrf2 activation (Figure 3(a)). We subsequently treated these ARE reporter cells with different dose of NaAsO₂ (10, 25, and 50 μM) for 6 h and observed a dose-dependent increase in ARE-luciferase activity, suggesting the activation of ARE-regulated downstream pathway by arsenic exposure (Figure 3(b)).

3.4. NaAsO₂ Induces Nrf2-Regulated NQO1 and HO-1 Expressions in Chang Human Hepatocytes. To examine whether Nrf2 activation by arsenic could upregulate the expression of NQO1 and HO-1 proteins in hepatocytes by western blot analysis. As shown in Figure 4(a), NaAsO₂ treatment for 6 h moderately upregulated the NQO1 protein expression, while strongly upregulated HO-1. What is more, the dose-dependent induction of NQO1 and HO-1 proteins was also evident (Figure 4(a)).

We next determined the NQO1 and HO-1 gene expressions by real-time PCR. Both NQO1 and HO-1 gene expressions were increased obviously by arsenic treatment (Figures 4(d) and 4(e)), consistent with the increase of ARE-luciferase activity shown in Figure 3(b). Meanwhile, the induction of HO-1 gene was more dramatic than NQO1, which was also in accordance with our results of NQO1 and HO-1 protein expressions (Figure 4(a)).

3.5. NaAsO₂-Induced Nuclear Import of Nrf2 Is Accompanied with the Nuclear Export of Bach1. The relationship between Nrf2 and Bach1, a kind of nuclear transcriptional repressor, was also explored in this experimental study. We treated the hepatocytes with 25 μmol/L of NaAsO₂ at different time points, and the subcellular protein localization of Nrf2 and Bach1 was examined (Figures 5(a), 5(b), 5(c), and 5(d)). We found that very low level of Nrf2 nuclear fractions was present in control cells, whereas nuclear Nrf2 accumulation was elevated rapidly and dramatically following NaAsO₂ treatment at 0.5, 1, 2, and 4 h (Figures 5(a) and 5(c)). In contrast, however, Bach1 levels gradually decreased in the nucleus, while increasing correspondingly in the cytoplasm (Figures 5(a), 5(b), and 5(d)). These transformations suggested that the nuclear export of Bach1 may be related to the nuclear import and activation of Nrf2. We also found there was little or no Nrf2 accumulation in the cytoplasmic fractions after NaAsO₂ exposure (Figures 5(b) and 5(c)).

To further confirm the nuclear export of Bach1 by NaAsO₂ in Chang human hepatocytes, an immunofluorescence assay was used to assure the intracellular localization of Bach1. In control cells, Bach1 fluorescence was mostly concentrated in the nuclei. However, after 25 μmol/L of NaAsO₂ treatment for 4 h, nuclear fluorescence levels of Bach1 decreased, at the same time Bach1 fluorescence mostly appeared in the cytoplasm (Figure 5(e)). Together with the subcellular protein localization of Nrf2 and Bach1 in Figures 5(a) and 5(b), our results strongly suggested that Bach1 could translocate from the nucleus to the cytoplasm by arsenic, which may be related to arsenic-induced Nrf2 nuclear accumulation and Nrf2 pathway activation.

4. Discussion

To counteract the detrimental effects of environmental insults, mammalian cells have evolved a hierarchy of sophisticated sensing and signaling mechanisms to turn on the endogenous defensive responses accordingly. One of the major cellular protective responses is the induction of antioxidative and detoxification enzymes through the Nrf2/ARE target gene system [12]. Previous literatures have proved that one of the environmental toxicant, inorganic arsenic, could activate the NrF2 pathway in human bladder cells [27], keratinocytes [15], endothelial cells [28], and some other cell types [29]. In this study, we tried to explore the hepatic Nrf2 pathway upon arsenic treatment comprehensively, since liver is one of the major target organs of arsenical toxicity. Our results showed that inorganic arsenic could quickly and significantly induce the nuclear transcription factor Nrf2.
protein expression in Chang human hepatocytes. In addition, our results also found a dose-dependent increase of Nrf2 transcriptional activity (indicated by the enhancement of ARE-luciferase activity), as well as both the mRNA and protein levels of NQO1 and HO-1, two downstream targets of Nrf2, were upregulated dramatically after arsenic invasion. Induction of hepatic Nrf2 pathway in our results, unanimously with many in vitro studies using other cell types altogether [32, 33], indicates that the Nrf2 pathway activation by arsenic is a kind of cellular ubiquitous phenomenon, and the hepatic Nrf2 pathway might play indispensable roles for the cellular defense against arsenic hepatotoxicity.

Studies have clarified that Nrf2 is sequestered in the cytoplasm by Keap1-mediated ubiquitination and the proteasomal degradation system, and that oxidative stress activates Nrf2 by permitting its translocation into the nucleus, suggesting that the regulation of Nrf2 transcriptional activity is mainly mediated by posttranscriptional mechanisms [31]. In our results, we also observed the remarkable nuclear accumulation of Nrf2 protein and the enhancement of Nrf2 transcriptional activity with sodium arsenite treatment. However, as demonstrated by some other studies that Nrf2 mRNA levels could be affected by arsenic [15], our results also found a moderate improvement of Nrf2 mRNA levels by arsenic exposure. It is therefore suggested that multiple mechanisms might be involved in Nrf2 activation, including both the transcriptional and the posttranscriptional events as far as inorganic arsenic is concerned.

On the other hand, a kind of transcriptional repressor, Bach1, has gained close attentions in recent years. In general, Bach1 serves as a repressor of the oxidative stress responses. It forms a heterodimer with the small Maf proteins (MafF, MafG, and MafK) to bind ARE in the nucleus [22], thus competes with the ARE-binding sites and represses the binding activity of Nrf2. Some researches indicate that activation of Nrf2 requires the inactivation of the transcriptional repressor Bach1 [34], and it is therefore argued that even when Nrf2 enters and accumulates in the nucleus, Nrf2 could not bind to the ARE site to initiate the Nrf2-mediated antioxidant responses unless Bach1 inactivates and probably exits out of the nucleus. What is more, it is also found that some of the Nrf2-regulated gene transcription is related to Bach1. Sun et al. [21] have shown that HO-1 was constitutively expressed at higher levels in many tissues of Bach1-deficient mice. Similarly, it has been demonstrated that knockdown of Bach1 in human keratinocytes specifically upregulated the gene expression of HO-1 [35]. In addition, Sakamoto et al. [36] demonstrated the overexpression of varying concentrations of Bach1 in HepG2 cells could result in decrease of NQO1 protein and repression of NQO1 activity. It seems that nuclear factor Bach1 might act as a negative regulator of Nrf2 pathway, which gives the Nrf2/ARE system a high range of plasticity to adapt to adverse cellular conditions.

As to inorganic arsenic exposure, Reichard et al. [37] have reported that sodium arsenite decreased Bach1 protein levels in the nucleus, promoted dissociation of Bach1 from the HO-1 enhancers, and increased Nrf2 expression. They also proved that the inactivation of Bach1 was necessary and sufficient for Nrf2 activation and the subsequent transcriptional induction of HO-1 in human keratinocytes. Consistent with their studies, our results here also found that sodium arsenite could regulate the intracellular localization of Bach1. Bach1 protein levels gradually decreased in the nucleus, while increased correspondingly in the cytoplasm after arsenic treatment. In addition, Bach1 fluorescence was transferred from the nucleus to the cytoplasm. Our results therefore confirmed that the nuclear import and accumulation of Nrf2 by arsenic were associated with Bach1 export from nucleus in hepatocytes. As a result, Nrf2 could be able to bind to the ARE-binding sites to initiate the downstream gene transcription. About the mechanism of Bach1 inactivation and translocation, Kaspar and Jaiswal demonstrated that antioxidant-induced phosphorylation of tyrosine 486 was essential for the nuclear export of Bach1 [38]. Another study
reported that arsenite regulated the Bach1 cysteine residues C557 and C574 to regulate the Bach1 function in human microvascular endothelial cells [28]. The relations between Bach1 and Nrf2 and the details of Bach1 translocation by arsenic still need to be confirmed and investigated.

In our results, we also found that both the mRNA and the protein levels of NQO1 and HO-1 were all increased when exposed to different concentrations of sodium arsenite. As the downstream target genes of Nrf2 pathway, NQO1 and HO-1 are all believed to have imperative cytoprotective functions. NQO1 is one of the phase II enzymes, capable of converting reactive electrophiles to less toxic and more readily excretable products, thus protecting cells against various chemical stresses and carcinogenesis [39]. Heme oxygenase-1 (HO-1) is the inducible form of the first and rate-limiting enzyme of heme degradation, which degrades heme into carbon monoxide, Fe$^{2+}$, and biliverdin. HO-1 possesses cytoprotective properties such as antioxidative, immunomodulatory, anti-inflammatory, and antiapoptotic functions [40, 41]. What is more, a recent study that used a high-throughput chromatin immunoprecipitation with parallel sequencing methodology identified more than 600 Nrf2
Figure 5: NaAsO$_2$-induced nuclear import of Nrf2 is accompanied with the nuclear export of Bach1. (a) Chang human hepatocytes were treated with 25 𝜇mol/L of NaAsO$_2$ for different time interval (0.5, 1, 2, and 4 h). Nuclear and cytosolic proteins were extracted separately to perform the western blot assay. Representative immunostained bands of nuclear (left) and cytosolic (right) proteins illustrated the changes of subcellular localization of Nrf2 and Bach1. (b) and (c) were quantitative analysis of nuclear and cytosolic Nrf2 and Bach1 proteins as shown in (a). β-actin and lamin B were used as internal control, accordingly. (e) The subcellular location of Bach1 was detected by immunofluorescence. Chang human hepatocytes were treated with 25 𝜇mol/L of NaAsO$_2$ for 4 h, immunostained with anti-Bach1 and TRITC-conjugated second antibodies (red, far left) and counterstained with DAPI to show the nucleus (blue, middle). The far right panels represented the overlay of Bach1 and DAPI fluorescence images. The results shown here were representative of three separate experiments.
target genes, further confirming the essential role of Nrf2 as the central regulator of cell protective and survival responses against numerous oxidative and electrophilic chemicals [42]. Induction of other Nrf2 downstream molecules by arsenicals and clarifying their potential roles in maintaining the cellular redox homeostasis and limiting arsenic-caused oxidative damage are still under investigation in our laboratory.

In summary, our results showed that arsenic accelerated the Nrf2 mRNA and protein expression in hepatocytes, promoted Nrf2 protein entry, accumulated in the nucleus, and enhanced the Nrf2 transcriptional activity. On the other hand, we found in this study that transcriptional repressor Bach1 exported from the nucleus to the cytoplasm. In addition, the mRNA and protein levels of NQO1 and HO-1, two Nrf2 downstream genes, increased correspondingly, which may exert their antioxidant and detoxification roles to against damages of arsenic treatment. The results of our study confirmed the arsenic-induced Nrf2 pathway activation in hepatocytes and attempted to uncover tentatively the interplay of Bach1 and Nrf2, which may be helpful to further understand the cellular self-defensive responses as well as the diverse biological effects of arsenicals.

**Abbreviations**

Nrf2: Nuclear factor erythroid 2-related factor 2  
Keap1: Kelch-like ECH-associated protein 1  
ARE: Antioxidant response element  
Bach1: BTB and CNC homology 1  
HO-1: Heme oxygenase-1  
NQO1: NADP(H):quinone oxidoreductase 1  
ROS: Reactive oxygen species  
tBHQ: Tert-butylhydroquinone  
DCFH-DA: 2′,7′-dichlorofluorescein diacetate

**Conflict of Interests**

The authors declare that they have no conflict of interests.

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**References**


Review Article

Nrf2 Is a Protective Factor against Oxidative Stresses Induced by Diesel Exhaust Particle in Allergic Asthma

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Epidemiological studies have shown that air pollutants, such as diesel exhaust particle (DEP), are implicated in the increased incidence of allergic airway disorders. In vitro studies of molecular mechanisms have focused on the role of reactive oxygen species generated directly and indirectly by the exposure to DEP. Antioxidants effectively reduce the allergic inflammatory effects induced by DEP both in vitro and in vivo. On the other hand, Nrf2 is a transcription factor essential for the inducible and/or constitutive expression of phase II and antioxidant enzymes. Disruption of Nrf2 enhances susceptibility to airway inflammatory responses and exacerbation of allergic inflammation induced by DEP in mice. Host responses to DEP are regulated by a balance between antioxidants and proinflammatory responses. Nrf2 may be an important protective factor against oxidative stresses induced by DEP in airway inflammation and allergic asthma and is expected to contribute to chemoprevention against DEP health effects in susceptible individuals.

1. Introduction

Air pollution is a complex mixture of compounds in gaseous and particle phases and the strongest evidence from many epidemiological studies linking air pollution with human health effects [1–4] centers around the particulate components. Particles are classified according to their aerodynamic diameter into size fractions such as particulate matter (PM) 10 (thoracic particles, ≤10μm), PM2.5 (fine particulate matter of diameter, ≤2.5μm), and ultrafine particles (UFP, fine particulate matter of diameter ≤0.1μm) [5]. Many epidemiologic studies suggest that PM2.5 is associated with increased respiratory morbidity and mortality [6–8]. Particulate matter includes primary particles that are emitted directly from sources such as fossil-fuel combustion, for example, diesel exhaust particle (DEP), and secondary particles that are generated from gases through chemical reactions [5].

Air pollution from motor vehicles has been implicated as an important factor responsible for the increased prevalence of allergic diseases [9]. There is epidemiologic evidence of the impact of diesel exhaust or “near roadway” effects on asthma in humans, particularly children [4]. DEPs are the major components of ambient PM2.5 [10], especially in urban areas, and many in vivo and in vitro studies have been performed to clarify the association between DEP and pulmonary disorders such as asthma [11–15]. In vitro studies have shown that most of the effects of DEP were due to reactive oxygen species (ROS) generated by exposure to DEPs and the subsequent generation of the oxidative stress response within exposed cells [16–21].

A nuclear factor, erythroid-derived 2-like 2 (Nfe2l2) or NF-E2-related factor 2 (Nrf2), is a redox-sensitive basic leucine zipper transcription factor that is involved in the transcriptional regulation of many antioxidant genes [22].
Extensive studies have suggested that Nrf2 contributes to protection against various pathologies, including asthma [23–25], chronic obstructive pulmonary disease (COPD) [26], lung fibrosis [27], carcinogenesis [28], atherosclerosis [29], inflammatory disorders [30], and environmental oxidants, including hyperoxia [31, 32] and cigarette smoke [33]. Furthermore, it is implicated that Nrf2 subsides host defense through modulation of complex pathways, including well-characterized antioxidant activation in airway inflammation and allergic asthma induced by DEP [21]. A study of a small number of patients with allergic rhinitis showed that susceptibility to the adverse risks induced by DEP is partially dependent on glutathione-S-transferases (GST) genotypes in humans [34]. Oxidant stress seems to be involved in diesel-associated increases in airway inflammation [35] and allergic asthma [36], as evidenced by experiments in Nrf2 knockout mice. The present review describes recent research demonstrating the effect of Nrf2 in allergic asthma implicated in DEP exposure.

2. Characteristics of Diesel Exhaust

DEP produced by diesel engines is a major component of particulate atmospheric pollution, especially in urban areas. DEP has a complex structure characterized by a carbonaceous core with adsorbed organic compounds such as polyaromatic hydrocarbons (PAHs) and quinones. Their small size (0.1–0.3 μm) allows them to penetrate deeply into the respiratory tract and reach the pulmonary alveoli [37]. The PAHs and their oxygenated derivatives (e.g., quinones) have attracted special attention because they are able to participate in the redox cycle and generate ROS in target cells [38]. The main effect observed in healthy human volunteers exposed to DEP is inflammation characterized by an increase of inflammatory cells and chemokines and immunoglobulin E levels in nose lavages [13], which could account for the epidemiological association between chronic exposure to particulate matter and the increase of allergic diseases such as asthma and rhinitis [37].

3. Nrf2 as a Key Regulator of Phase II Detoxifying Enzyme Genes and Antioxidant-Responsive Genes

Nrf2 was discovered as a ubiquitous transcriptional regulator of antioxidant and detoxification genes. Nrf2 is a transcription factor essential for the inducible and/or constitutive expression of phase II and antioxidant enzymes. For instance, several GST isoforms and NAD(P)H:quinone oxidoreductase 1 (NQO1) were found to be uninducible by xenobiotics in the Nrf2 germ line mutant mouse [22]. These findings showed that Nrf2 has a major role in transcriptional activation through antioxidant responsive elements.

The expression of phase II detoxifying enzyme genes is clearly induced in wild-type and heterozygous Nrf2 knockout mice, but the inducible expression of these genes is markedly reduced in homozygous Nrf2-knockout mice [39]. Insufficient induction of cytoprotective enzyme genes causes an increased susceptibility to various xenobiotics [40, 41] and components such as DEP [35, 42].

4. DEP Induces ROS In Vitro and In Vivo, and Subsequent Nrf2 Activation Leads to Antioxidant Gene Expression

Studies of molecular mechanisms have focused on the role of ROS generated directly and indirectly by exposure to DEP. ROS play an important role in proinflammatory reactions in airways. Enhanced inflammation involving the activation of alveolar macrophages following DEP exposure leads to the generation of ROS indirectly [43]; however, it is reported that intratracheal exposure to DEP caused the formation of 8-hydroxydeoxyguanosine in the murine lung [44]. DEP chemicals [45] and metals [46] could directly produce ROS such as superoxide and hydroxyl radical. These observations suggest that DEP can generate ROS, leading to oxidative stress-dependent pulmonary damage. DEP induce inflammatory cytokines such as interleukin- (IL-) 8 expression mediated by nuclear factor- (NF-)κ B in vitro, and these effects are blocked by antioxidant agents such as N-acetyl cysteine (NAC) [16, 18]. These observations also suggest that DEP-induced activation of signal pathways and transcription factors is due to ROS derived primarily from DEP.

In vitro research suggests that cytoprotective pathways are induced by the Nrf2 transcription signal pathway at the lowest levels of oxidative stress from DEP and can induce the transcription of antioxidant genes in the earliest level of defense. This may constitute the first tier of a hierarchical oxidative stress response. If these enzymes fail to neutralize the effects of ROS, proinflammatory effects constitute a second tier or superimposed level of oxidative stress. The final tier or superimposed level of oxidative stress is cytotoxicity, including the initiation of programmed cell death [19]. Nrf2 regulates antioxidant defense that is constituted as a main defense action against the proinflammatory and oxidizing effects of DEP [21].

In vivo studies with low-level and repeated DEP exposure (100 μg/m³) showed that DEP exposure induced airway inflammation in mice [47–50]. Host responses to DEP are regulated by a balance between antioxidant defenses and proinflammatory responses [38]. Studies of two different strains in mice demonstrated that there was a susceptibility difference to DEP exposure, and that certain antioxidant enzymes could be candidates as susceptibility genes [49, 50]. DNA adduct formation has been shown to be accelerated in the lungs of Nrf2 knockout mice exposed to DEP [42]. Nrf2 knockout mice exposed to low-dose DEP for 8 weeks showed significantly increased airway hyperresponsiveness and counts of lymphocytes and eosinophils, together with increased concentrations of IL-12 and IL-13, and thymus and activation-regulated chemokine (TARC) in bronchoalveolar lavage (BAL) fluid compared with wild-type mice. In contrast, the expression of antioxidant enzyme genes was significantly higher in wild-type mice than in Nrf2 knockout mice [35]. This study strongly suggested that DEP-induced
oxidative stress and host antioxidant responses were regulated by Nrf2.

It is known that DEP induces and exaggerates allergic airway inflammation in vivo [13, 51, 52]. Studies of a murine model of asthma, where mice received repeated low-level exposure to DEP, showed that DEP induced and exaggerated allergic airway inflammation, and NAC treatment reduced these allergic inflammatory responses caused by DEP [53]. Nrf2 knockout mice exposed to low-dose DEP showed significantly increased airway hyperresponsiveness and counts of lymphocytes, neutrophils, and eosinophils, together with increased concentrations of TARC in BAL fluid compared to wild-type mice in an asthma model [36]. TARC is a pivotal chemokine for the development of Th2-dominated experimental allergen-induced asthma with eosinophilia and airway hyperresponsiveness [54]. Increased inflammatory cells and PAS staining-positive mucus cell hyperplasia were evident in Nrf2 knockout mice. In contrast, the expression of GSH/GSSG (reduced glutathione/oxidized glutathione) was higher in wild-type mice than in Nrf2 knockout mice [36]. These results highlighted the role of DEP-induced oxidative stress and host antioxidant responses in the exaggeration of allergic airway inflammation in mice. It has been reported that the responsiveness of the Nrf2-directed antioxidant pathway acts as a major determinant of susceptibility to allergen-mediated asthma [23–25]. These reports suggest that the synergistic effects of oxidative stress caused by DEP and allergens contribute to the major pathway of the exaggeration of allergic asthma.

Disruption of Nrf2 enhances susceptibility to allergic airway inflammatory responses induced by low-dose DEP (100 μg/m³) in mice, but the data did not show any adjuvant activity of DEP for IgE production [36]. The effect of oxidative stresses caused by DEP may be crucial for the induction and exaggeration of allergic airway inflammatory responses due to DEP exposure in vivo.

5. Future Direction: Nrf2 Is a Key Factor As a Potential Target of Chemoprevention

The production of ROS was closely implicated in airway inflammation, allergy, and asthma; therefore, antioxidants may become a prophylactic strategy against adverse health effects of DEP. Chemoprevention by antioxidants has been reported to reduce the allergic inflammatory effects of DEP in mice [55]. NAC is widely known as an antioxidant drug. DEP-induced oxidants stress and the resultant inflammatory changes were blocked by NAC in asthma model [53]. Sulforaphane, a compound found in broccoli sprouts and broccoli, is also known to be a potent Nrf2 activator and is capable of preventing the toxicity of organic chemicals [56, 57]. Sulforaphane-stimulated phase II enzyme induction inhibits cytokine production by airway epithelial cells stimulated with DEP [58].

Observations from these studies highlight the importance of the Nrf2-antioxidant pathway and may provide new therapeutic strategies for acute respiratory distress syndrome implicated in oxidative stress from DEP exposure.

6. Conclusion

Epidemiological, human, and animal experimental studies together suggest that DEP is involved in the recent increased prevalence of allergic diseases. Studies of molecular mechanisms have focused on the role of ROS generated directly and indirectly by exposure to diesel exhaust. Chemoprevention against DEP health effects in susceptible individuals may become a choice for a future environmental protection policy, and Nrf2 is a key potential target of chemoprevention.

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The NRF2 Activation and Antioxidative Response Are Not Impaired Overall during Hyperoxia-Induced Lung Epithelial Cell Death

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Lung epithelial and endothelial cell death caused by pro-oxidant insults is a cardinal feature of acute lung injury/acute respiratory distress syndrome (ALI/ARDS) patients. The NF-E2-related factor 2 (NRF2) activation in response to oxidant exposure is crucial to the induction of several antioxidative and cytoprotective enzymes that mitigate cellular stress. Since prolonged exposure to hyperoxia causes cell death, we hypothesized that chronic hyperoxia impairs NRF2 activation, resulting in cell death. To test this hypothesis, we exposed nonmalignant small airway epithelial cells (AECs) to acute (1–12 h) and chronic (36–48 h) hyperoxia and evaluated cell death, NRF2 nuclear accumulation and target gene expression, and NRF2 recruitment to the endogenous HMOX1 and NQO1 promoters. As expected, hyperoxia gradually induced death in AECs, noticeably and significantly by 36 h; ∼60% of cells were dead by 48 h. However, we unexpectedly found increased expression levels of NRF2-regulated antioxidative genes and nuclear NRF2 in AECs exposed to chronic hyperoxia as compared to acute hyperoxia. Chromatin Immunoprecipitation (ChIP) assays revealed an increased recruitment of NRF2 to the endogenous HMOX1 and NQO1 promoters in AECs exposed to acute or chronic hyperoxia. Thus, our findings demonstrate that NRF2 activation and antioxidant gene expression are functional during hyperoxia-induced lung epithelial cell death and that chronic hyperoxia does not impair NRF2 signaling overall.

1. Introduction
The induction of antioxidant gene expression in lung-resident and infiltrated inflammatory cells in response to oxidative stress plays a significant role in pulmonary defense mechanisms [1, 2]. However, disequilibrium between prooxidant load and antioxidant defenses, leading to redox imbalance, could potentially enhance tissue susceptibility to oxidative stress, thereby contributing to the lung pathogenesis of many acute and chronic airway diseases. These diseases include idiopathic pulmonary fibrosis, emphysema, bronchopulmonary dysplasia, acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), and lung cancer [3–6]. Supplemental oxygen (hyperoxia) is used as therapy to treat ALI/ARDS patients. In mice, chronic exposure to hyperoxia results in endothelial and alveolar epithelial cell death accompanied by pulmonary edema and respiratory impairment; these pathologic features are similar to those observed in ALI/ARDS patients [7]. Thus, understanding the mechanisms by which hyperoxia contributes to lung
pathogenesis is crucial to limiting the potentially harmful effects of oxygen toxicity in the clinical setting.

We have previously shown that NF-E2-related factor 2 (Nfe2l2, also known as Nrf2), a bZIP transcription factor, is crucial for the induction of several antioxidant and cytoprotective genes in response to various pro-oxidant stimuli, including hyperoxia [8, 9]. Nrf2-deficient mice are more susceptible than wild-type mice to inflammatory and hyperpermeability responses to hyperoxic insult; this response has been generally attributed to a diminished or low expression level of several antioxidant enzymes (AOEs), including gene encoding NQO1, HMOX1, GCLC, GCLM, and GPXs [8, 9], which detoxify reactive oxygen species (ROS) and/or nitrogen (RNS) species. We have also shown that a loss of Nrf2 impairs the resolution of hyperoxia-induced acute lung injury and inflammation and also exacerbates bacterial infection in adult mice following hyperoxic insult [10].

One-day old Nrf2-deficient pups, when exposed to hyperoxia for 72 h, develop greater levels of alveolar simplification (septal growth arrest) at the 4th day [11] and 14th day [12] than do Nrf2-sufficient pups. Nrf2 deficiency enhances cellular stress and susceptibility to oxidant-induced lung epithelial cell death [13], and its overexpression confers cellular protection against hyperoxia in lung epithelial cells [14] as well against proapoptotic stimuli in nonlung epithelial cells [15, 16]. These observations suggest an important role for the Nrf2-driven transcriptional response in mitigating cellular stress induced by prooxidants.

Since chronic exposure to hyperoxia causes the death of lung epithelial cells, despite the presence of NRF2, we hypothesized that dysfunctional NRF2 signaling may contribute to this cell death. To test this hypothesis, we have now analyzed the nature of NRF2 activation (nuclear accumulation) and recruitment to the antioxidant gene (HMOXI and NQO1) promoters in human nonmalignant lung small airway epithelial cells during acute and chronic hyperoxia exposure. Here, we report that chronic hyperoxia does not impair NRF2 nuclear accumulation or antioxidant gene expression during the hyperoxia-induced death of lung epithelial cells.

2. Materials and Methods

2.1. Human Lung Epithelial Cell Culture and Hyperoxia Exposure. The human normal small airway epithelial cell line (hereafter referred to as AECs) was established by the ectopic expression of human telomerase reverse transcriptase (hTERT). Cells were grown in Dulbecco’s Modified Eagle Medium with Ham’s F12 nutrient mixture (DMEM/F12) in the presence of 10% FBS and antibiotics [17]. Cells were plated in equal number, grown to 70%–80% confluence and then exposed to hyperoxia in complete medium. For generating hyperoxic condition, cells were kept in modular incubator chamber (Billups-Rothenberg, Del Mar, CA) and filled with gas mixture containing 95% O₂ and 5% CO₂ and chambers placed in 37°C incubator. The chambers were refilled with the gas mixture every 12 h. As room air control group, cells were placed in the regular cell culture incubator with room air and 5% CO₂ at 37°C. Culture medium was changed every 24 h during the exposure period.

2.2. Gene Expression Analysis. Cells were exposed to either room air (RA) or hyperoxia (95% O₂ and 5% CO₂) for the indicated time periods. Total RNA was isolated using Trizol reagent (Gibco-BRL/Life Technologies, Grand Island, NY) and reverse transcribed using the qScript cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD). Target gene expression was assessed by quantitative RT-PCR (qRT-PCR) using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). For Immunoblot analyses, total protein was extracted in lysis buffer consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 5 mM β-glycerophosphate, and 1 μg/mL leupeptin. Comparable amount of total protein (~40 μg) from each sample was separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the membranes were probed with antibodies specific for NRF2 (Santa Cruz Biotech, Santa Cruz, CA), HMOX1 (Santa Cruz Biotech, Santa Cruz, CA), NQO1 (Abcam, Cambridge, UK), GCLC (kindly provided by Dr. Terrance Kavanagh, University of Washington, Seattle, WA). β-actin (Sigma, St. Louis, MO) antibody was used as the loading control. The blots were developed using an ECL kit (HyGlo, Denville Scientific Inc., Metuchen, NJ).

2.3. Cell Viability Assays. Cells in equal number were plated and exposed to hyperoxia as described above. Cell viability was quantified by CellTiter-Glo kit (Promega, Madison, WI) and MTT assay. LDH release was measured by CytoTox 96 NonRadioactive Cytotoxicity assay kit (Promega, Madison, WI). Viability and LDH release was calculated as a percentage of increase or decrease over their respective room air controls.

2.4. Chromatin Immunoprecipitation (ChIP) Assays. ChIP assays were performed using the EZ-ChIP assay kit (Millipore, Billerica, MA). Briefly, AECs were exposed to either room air or hyperoxia for indicated time points, cross-linked with formaldehyde, and chromatin fragmentation was carried out as detailed in the kit procedure. Diluted soluble chromatin solution was incubated with rabbit anti-NRF2 (Santa Cruz Biotechnology, Santa Cruz, CA) for 18 h at 4°C with rotation. Nonimmune rabbit IgG was used as a negative control to determine the binding specificity. Following incubation with protein A/G agarose beads, the bound products were washed, and DNA was eluted. DNA was subjected to PCR with primers encompassing the functional antioxidant response elements (AREs) located upstream of transcriptional start site of HMOXI (F: 5’-CCCTGCTGAGTAACTCCTTTGCAGA-3’ and R: 5’-ATGTCCCAGCTCCAGATCTCCA-3’) and NQO1 (F: 5’-GTTGAAGTCTCCGAAGAGA-A-3’ and R: 5’-TGTCCTCCCAGAGACTCTTCAG-3’) to determine the binding of NRF2 in ChIP assays.
2.5. Transfections and Reporter Gene Analyses. Cells were transfected with the NQO1 (NADPH: quinone oxidase reductase-1) promoter reporter (luciferase) construct [18] (kindly provided by Jeffrey Johnson, University of Wisconsin). To normalize transfection efficiency between wells, the cells were cotransfected with 5 ng of the Renilla luciferase plasmid pRL-TK (Promega Corp., Madison, WI). At 24 h after transfection, cells were exposed to either room air or hyperoxia, and extracts were assayed for firefly and the Renilla luciferase activities using a dual luciferase kit (Promega Corp., Madison, WI). Firefly luciferase activity was normalized to that of Renilla.

2.6. Statistical Analyses. Data were expressed as the mean ± SD (n = 3–9) as indicated in the legends. The significance between the exposures was calculated by one-way or two-way (for cell viability and LDH release) analysis of variance followed by the Bonferroni post hoc tests by GraphPad PRISM 4 Software. A P value of ≤0.05 is considered statistically significant. We also performed Student’s t-test to confirm one-way analysis.

3. Results

Malignant human lung epithelial cells express higher levels of NRF2 than do nonmalignant lung epithelial cells mainly because of mutations in the inhibitor of NRF2, KEAP1, which is known to facilitate NRF2 degradation under basal conditions [19–21]. To overcome this problem and to determine whether chronic hyperoxia promotes lung epithelial cell death by suppressing the NRF2-mediated transcriptional response, we have utilized a nononcogenic human lung (small airway) epithelial cell line immortalized by telomerase [17]. To assess the effect of hyperoxia on airway epithelial cells (AECs), we subjected the cells to hyperoxia for 24 to 48 h and measured cell viability by using CellTiter-Glo and MTT reagents; LDH release was evaluated by Cytotox. No significant difference in cell viability was observed between room air and hyperoxia during the first 24 h of exposure (Figure 1(a)). However, after 36 h, hyperoxia had produced a significant decline (~42%) in cell viability (as determined by CellTiter-Glo), which fell to ~55% at 48 h. To verify this result, we evaluated cell viability using MTT assay (Figure 1(b)). Hyperoxia caused a significant decline (~37% loss) in cell viability after 36 h, and the loss of viability increased ~49% at 48 h; in contrast, hyperoxia exposure for 24 h had no significant effect on cell viability when compared to room air controls.

We also analyzed hyperoxia-induced cellular toxicity, as measured by LDH release into the culture medium. A significant increase in LDH release was detected in cells exposed to hyperoxia for 24 h (Figure 1(c)), when compared to their room air-exposed counterparts. However, the LDH released by the cells exposed to hyperoxia for 36 h to 48 h was markedly higher than the amount in the corresponding room air controls (Figure 1(c)). The discordance in results between the cell viability (Figures 1(a) and 1(b)) and LDH measurement (Figure 1(c)) at the 24 h time point may be related to the differences in the sensitivity of the assays.

To determine the impact of chronic hyperoxia on the regulation of the NRF2-dependent antioxidant transcriptional response, we subjected cells to either acute (1–12 h) or chronic (36–48 h) hyperoxia and determined the expression levels of HMOX1, GCLC, and NQO1 by qRT-PCR and immunoblot analysis. We selected these genes because they are upregulated by cellular stress and are putative transcriptional targets of NRF2 and because they are known to play key roles in cellular detoxification processes (reviewed in [22]). As anticipated, acute hyperoxia markedly stimulated GCLC (2.3-fold) and NQO1 (2.7-fold) mRNA expression by as early as
6 h, and the levels remained high up to 12 h (3.6-fold and 5.8-fold increases for GCLC and NQO1, resp.) (Figure 2(a)). In contrast, a significant increase in HMOX1 mRNA expression was noticed during the acute phase as early as 3 h (3.3-fold), and its expression remained high (12.1-fold) up to 12 h. To verify that the changes in mRNA expression also occurred at the protein level, we performed western blot analyses using anti-HMOX1, -GCLC, and -NQO1 antibodies on total protein extracts prepared from cells exposed to hyperoxia or normoxia. In agreement with the RT-PCR data, western blot analyses showed an increased expression of these genes (Figure 2(b)). HMOX1 expression was increased by 1.8-, 1.8-, and 1.9-fold at 3 h, 6 h, and 12 h, respectively, when compared to the room air control group. Likewise, NQO1
expression was increased by 1.7-fold and 2.3-fold at 3 h and 6 h, respectively. No increase was found at the 12-h time point. In the case of GCLC, the protein expression was increased by 1.4-fold at the 12-h time point only.

We next analyzed the expression levels of these genes in cells exposed to chronic hyperoxia in order to determine whether their lack of induction by NRF2 could be attributed to cell death. Intriguingly, we found increased levels of \( HMOX1 \) (57.5-fold), \( GCLC \) (7.5-fold), and \( NQO1 \) (6.3-fold) mRNA expression in cells exposed to chronic hyperoxia (36 h) when compared to those exposed to room air (Figure 3(a)). The induction of \( HMOX1 \) (246-fold), \( GCLC \) (15.5-fold), and \( NQO1 \) (11.3-fold) mRNA expression remained high up to 48 h. The results of the western blot analyses were correlated with a significantly increased expression of \( HMOX1 \) (2.39-fold at 36 h and 8.34-fold at 48 h), \( GCLC \) (1.37-fold at 36 h and 1.92-fold at 48 h) and \( NQO1 \) (1.57-fold at 36 h and 2.57-fold at 48 h) over the corresponding room air control.

**Figure 3**: The effects of chronic hyperoxia on NRF2 target gene expression in AECs. Cells were exposed to either room air or hyperoxia for 36 or 48 h and then harvested for total RNA and protein. (a) \( HMOX1 \), \( GCLC \), and \( NQO1 \) mRNA expression. The values represent the mean ± SD of four independent experiments (\( n = 6 \)). * \( P < 0.05 \), RA versus hyperoxia. (b) Western blot analysis of whole-cell lysates using anti-\( HMOX1 \), anti-\( NQO1 \), or anti-\( GCLC \) antibodies. Membranes were stripped and reprobed with \( \beta \)-actin. Graph represents the relative band intensities of \( HMOX1 \), \( NQO1 \), or \( GCLC \), as quantified in Figure 2. Values represent the mean ± SD of four independent experiments (\( n = 4 \)). * \( P < 0.05 \), RA versus hyperoxia.
groups (Figure 3(b)), but the levels of each protein were not reflected by a corresponding mRNA abundance.

In order to examine the effects of acute and chronic hyperoxia on NRF2 nuclear accumulation, we exposed AECs to hyperoxia then prepared nuclear extracts and performed western blotting using an anti-NRF2 antibody. Hyperoxia increased the levels of nuclear NRF2 (Figure 4(a)) as early as 1 h (1.9-fold) after exposure, and the levels remained higher than those of the corresponding room air control group at 3 h (3.7-fold) and up to 12 h (4.4-fold). In response to chronic hyperoxia, NRF2 nuclear accumulation was higher at 36 h (4.6-fold) and 48 h (5.3-fold) than to room air (Figure 4(b)).

To determine whether the increased levels of HMOX1 and NQO1 expression are due to NRF2 binding, we performed ChIP assays analyzing the recruitment of NRF2 to endogenous HMOX1 and NQO1 promoters in cells exposed to acute and chronic hyperoxia. We used gene-specific primers flanking the critical AREs in each case (Figure 5(a)). These experiments revealed the recruitment of NRF2 to the HMOX1 promoter as early as 3 h; the levels returned to baseline at 6 h during acute hyperoxia (Figure 5(b), left panel). NRF2 binding to the NQO1 promoter was very low or undetectable under conditions of room air, and it rapidly increased as early as 3 h (13.5-fold) but returned to basal levels at 6 h (Figure 5(b), right panel). However, the binding of NRF2 to the NQO1 promoter rose again at 12 h of hyperoxia (22.4-fold) (Figure 5(b), left panel). An increased enrichment of NRF2 at the NQO1 promoter at the 12 h time point was also reflected in higher mRNA levels, but western blot analysis revealed no increase in NQO1 protein expression at the 12 h time point, perhaps because of a lag in mRNA translation.

During chronic hyperoxia, we found that the binding of NRF2 to the HMOX1 enhancer was significantly higher at 36 h (1.8-fold) and 48 h (2.4-fold) than in cells exposed to room air (Figure 5(c), left panel). Likewise, during chronic hyperoxia, the binding of NRF2 to the NQO1 promoter, although not higher at the 36 h time point, was significantly higher at 48 h than in cells exposed to room air (Figure 5(c), right panel). However, the level of NRF2 enrichment at the NQO1 promoter was considerably lower than in cells exposed to acute hyperoxia (Figure 5(b), right panel).
Figure 5: The effects of acute and chronic hyperoxia on the binding of NRF2 to HMOX1 and NQO1 promoters. Cells were exposed to room air or hyperoxia for 1 to 12 h or for 36 to 48 h; chromatin was cross-linked and immunoprecipitated with IgG or anti-NRF2 antibodies, and DNA was amplified using gene specific primers. (a) Scheme showing the positions of the ARE sites, forward and reverse primers of HMOX1 and NQO1 promoters used in ChIP assays. (b) NRF2 binding to the HMOX1 and NQO1 promoters in cells exposed to acute hyperoxia (1–12 h). (c) NRF2 binding to the HMOX1 and NQO1 promoters in cells exposed to chronic hyperoxia (36 h or 48 h). PCR products were analyzed on 2% agarose gel. Band intensities were quantified with Image J software. Input DNA was used as a control. Graph represents mean ± SD of five independent experiments (n = 5). Fold increase was calculated over their respective room air controls *P < 0.05, RA versus hyperoxia.
We next determined the transcriptional activity of the NQO1 promoter in cells exposed to acute and chronic hyperoxia. Cells were transiently transfected with the NQO1-Luc reporter construct and then exposed to hyperoxia. As shown in Figure 6, hyperoxia enhanced NQO1-Luc expression by 2.1- and 6.9-fold at 6 h and 12 h of exposure (Figure 6(a)), respectively, when compared to room air controls. We found that the Luc activity was also significantly higher (1.5-fold) in cells exposed to chronic hyperoxia for 36 h than in the corresponding room air control group (Figure 6(b)); this result is comparable to the 2.1-fold increase found in cells exposed to hyperoxia for 6 h. However, the magnitude of the promoter inducibility was considerably lower than the 6.9-fold increase observed in cells exposed to hyperoxia for 12 h.

4. Discussion

Exposure to hyperoxia for prolonged periods (chronic exposure) is known to induce oxidative stress, mainly as a result of a redox imbalance caused by excessive accumulation of reactive oxygen species, which initially causes cellular damage and ultimately cell death [23]. NRF2-induced expression of antioxidative and cytoprotective genes is crucial to maintaining redox homeostasis during exposure to toxicants and injurious insults. We have previously shown that mice lacking Nrf2 are highly susceptible to hyperoxia-induced lung injury and lung epithelial cell death in vivo and in vitro [8, 14], suggesting that prolonged exposure to hyperoxia promotes lung epithelial cell injury and death by impairing NRF2 activation and subsequently inhibiting its induction of downstream target genes. However, in the present study, we demonstrate that the enhanced expression levels of putative NRF2 target genes in lung epithelial cells exposed to chronic hyperoxia are of a higher magnitude than those observed under acute hyperoxia. Moreover, we observed that the recruitment of nuclear NRF2 to the promoters of antioxidant genes (e.g., HMOX1 and NQO1) in cells exposed to chronic hyperoxia. Thus, it appears that NRF2-mediated gene expression is not globally or largely compromised, but it induced to mount a cytoprotective response to preserve redox homeostasis, thereby helping to maintain cell survival or preventing lung epithelial cell death during chronic hyperoxia.

NRF2 is mainly localized to the cytoplasm in its native state, and its nuclear accumulation in response to stressful insults is critical for antioxidant gene induction [24, 25]. The nucleocytoplasmic shuttling of NRF2 appears to vary according to the inducer and/or cell type and is regulated by multiple complex mechanisms [24, 25]. For example, various protein kinases, such as PKC [26, 27], ERK1/2 [28], and AKT [29], activated by pro-oxidant exposure, are also known to facilitate and enhance the accumulation of NRF2 in the nucleus. After the signal-induced dissociation from its cytoplasmic inhibitor Keap1, importins facilitate the nuclear translocation of NRF2 [30]. Phosphorylation of NRF2 in the nucleus promotes its nuclear exclusion and subsequent Keap1-mediated degradation [31, 32]. Thus, deregulation of NRF2 nuclear accumulation following chronic stressful stimuli, such as hyperoxia, is generally connected with cellular injury and death. However, our present results indicate that this is not the case. We found higher levels of NRF2 in the nucleus of cells during chronic hyperoxia than in room air-exposed cells, and the levels were comparable to those of...
cells exposed to acute hyperoxia, suggesting that the various effector pathways that facilitate nuclear accumulation of NRF2 and its DNA binding are functional during conditions of hyperoxia-induced lung epithelial cell death.

NRF2 alone is insufficient to bind to the ARE. Its heterodimerization with other b-ZIP transcription factors, such as JUN and the small MAF family of proteins, is also required [33–36]. The magnitude and duration of the antioxidant gene expression can be dictated by both cooperative and combinatorial interactions that occur between NRF2 and the MAF/JUN family of proteins. Thus, the impairment of NRF2 binding to the DNA might affect its transactivational activity, despite elevated levels of NRF2 in the nucleus. Because the levels of NRF2-dependent target gene expression are almost higher under chronic hyperoxia than during exposure to acute hyperoxia, it appears that hyperoxia does not alter these interactions or diminish the lung antioxidant capacity, leading to a dysfunctional cellular response. Members of the Jun family of proteins, c-Jun and Jun-D, can dimerize with Nrf2 and upregulate antioxidant gene expression [37, 38]. For example, mouse embryonic fibroblasts lacking c-Jun or Jun-D demonstrate decreased levels of antioxidant enzymes and enhanced oxidative stress [33–36]. Likewise, c-Myc upregulates the expression of cytoprotective genes in response to stressful stimuli [39]. Thus, it is possible that these proteins act in a cooperative or synergistic manner with NRF2 to potentiate ARE-mediated gene transcription during chronic hyperoxia.

We have previously reported the binding of Nrf2 to AREs in murine alveolar type-II like epithelial cells following acute hyperoxia [14, 28]. In the present study, ChIP assays revealed the binding of NRF2 to its target gene promoters (NQO1 and HMOX1) in cells exposed to chronic hyperoxia (48 h), suggesting that chronic hyperoxia does not compromise NRF2 binding to antioxidant promoters. Although the magnitude of the NRF2 binding at the HMOX1 promoter was similar under acute (at 3 h) and chronic (at 36 h and 48 h) hyperoxic conditions (Figure 5), the enrichment of NRF2 at the NQO1 promoter appeared to be variable and biphasic in response to hyperoxia. There was a 13-fold increase in the binding of NRF2 to the NQO1 promoter at 3 h, and the binding returned to basal levels at 6 h after hyperoxia. However, the NRF2 binding increased significantly to greater levels at 12 h than at 3 h after hyperoxia. During chronic hyperoxia, a significant increase in NRF2 binding at the NQO1 promoter was detected at 48 h, but not at 36 h, when compared to room air controls. It is possible that the association of NRF2 with its partners is dynamic or that its negative regulators, such as BACH1 [40] and Fra-1 [41], limit the availability of NRF2 or its partners to form complexes with NRF2, making it differentially bind to the AREs in a gene-promoter context-dependent manner during acute and chronic hyperoxia. The relevance and mechanisms of such differential NRF2 binding to the NQO1 promoter during hyperoxia-induced cell death warrants a separate investigation.

It is noteworthy that the lower NRF2 binding at the endogenous NQO1 promoter was reflected in diminished promoter activation by chronic hyperoxia in our transient transfection assays; however, NQO1 mRNA expression levels in cells exposed to chronic hyperoxia were nearly comparable to those observed in cells exposed to acute hyperoxia. We assume that the posttranscriptional regulation of NQO1 explains the discordance between diminished promoter activity and increased mRNA expression of NQO1 in chronic hyperoxia. It should also be noted that reporter analyses utilize a short fragment of the promoter, unlike the native gene promoter, and this difference may also explain some of the differences. Also, we would like to point out the lack of a direct correlation between mRNA and protein expression. For example, the increased protein abundance of HMOX1 and other genes analyzed by western blot analysis was ∼10-fold less than the corresponding mRNA expression levels in cells exposed to either acute or chronic hyperoxia. It is unclear whether this result reflects error and noise in the real-time PCR and immunoblot analysis and/or variations in protein synthesis and degradation [42].

Previously, it has been shown that the exposure to oxidants (such as high doses of H2O2) leads to decreased NRF2 levels and a consequent suppression of the antioxidant response pathway, culminating in cellular injury and death [43, 44]. However, we found that nuclear NRF2 protein levels in cells exposed to chronic hyperoxia are not reduced when compared to room air-exposed cells or to cells exposed to acute hyperoxia (Figure 4), ruling out such a possibility. The ability of hyperoxia to induce cell death despite the presence of high levels of antioxidant gene expression is somewhat surprising. It is important to note that we have only analyzed the expression of a subset of the NRF2-target genes (GCLC, HMOX1, and NQO1) that are markedly induced during chronic hyperoxia. Previously, by global gene expression profiling and primary lung type alveolar II epithelial cultures from Nrf2-null and wild-type mice, we have shown that Nrf2 regulates the expression of several genes involved in antioxidative and cytoprotective responses as well as cell proliferation and survival [14, 45], Global mapping ChIP-binding assays and mRNA expression profiling in Keap1-null or Nrf2-null mouse embryonic fibroblasts revealed that Nrf2 binds and regulates the expression of ∼500 genes involved in cell proliferation and stress response [46]. It is possible that some of these gene products that dampen the initiation and execution of cell death pathways are not being induced by NRF2 during chronic hyperoxia, despite its nuclear presence; we cannot rule out this possibility. Recently, Taguchi et al. have demonstrated that an increased level of NRF2 accumulation promotes liver damage in autophagy-deficient mice [47]. Whether high levels of nuclear NRF2 have any role in mediating cellular injury during chronic hyperoxic setting remains to be investigated.

In summary, we have demonstrated that both NRF2 nuclear enrichment and upregulation of antioxidative gene expression occur in cells exposed to chronic hyperoxia. ChIP assays revealed that the magnitude of the NRF2 binding at the antioxidant gene promoters is dynamic, variable, and biphasic in response to hyperoxia exposure, suggesting that NRF2-mediated signaling is not globally or largely compromised.
Rather, it continues to be functional during chronic hyperoxia that causes the death of lung epithelial cells.

**Conflict of Interests**

All the authors declare no conflict of interests.

**Acknowledgments**

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**References**


Research Article

The Influence of Nrf2 on Cardiac Responses to Environmental Stressors

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Nrf2 protects the lung from adverse responses to oxidants, including 100% oxygen (hyperoxia) and airborne pollutants like particulate matter (PM) exposure, but the role of Nrf2 on heart rate (HR) and heart rate variability (HRV) responses is not known. We hypothesized that genetic disruption of Nrf2 would exacerbate murine HR and HRV responses to severe hyperoxia or moderate PM exposures. Nrf2−/− and Nrf2+/+ mice were instrumented for continuous ECG recording to calculate HR and HRV (low frequency (LF), high frequency (HF), and total power (TP)). Mice were then either exposed to hyperoxia for up to 72 hrs or aspirated with ultrafine PM (UF-PM). Compared to respective controls, UF-PM induced significantly greater effects on HR (P < 0.001) and HF HRV (P < 0.001) in Nrf2−/− mice compared to Nrf2+/+ mice. Nrf2−/− mice tolerated hyperoxia significantly less than Nrf2+/+ mice (~22 hrs; P < 0.001). Reductions in HR, LF, HF, and TP HRV were also significantly greater in Nrf2−/− compared to Nrf2+/+ mice (P < 0.01). Results demonstrate that Nrf2 deletion increases susceptibility to change in HR and HRV responses to environmental stressors and suggest potential therapeutic strategies to prevent cardiovascular alterations.

1. Introduction

The deleterious effects of environmental exposures and associated oxidative stress on the cardiopulmonary system are well established and present one of the most significant public health problems [1]. Diseases and disorders of the cardiopulmonary system associated with an enhanced oxidant load include, but are not limited to, inflammatory lung diseases (e.g., acute respiratory distress syndrome [2] and bronchopulmonary dysplasia [3, 4]) and a host of cardiovascular (CV) diseases (e.g., atherosclerosis [5, 6], hypertension [7], and heart failure [8]).

Exposure to oxidants can exacerbate the pathogenesis of these diseases by further increasing oxidative stress and in some cases overwhelm antioxidant defenses. Inflammatory lung disease and post-resuscitation from cardiac arrest are frequently treated with oxygen therapy (hyperoxia), which can cause significant lung injury [9], adverse cardiac responses [10], and death if exposure is sufficiently long, even in young healthy laboratory animals.

However, not all oxidants such as air pollution produce overt outcomes, but they are no less problematic in terms of public health because exposure is frequent, wide spread, and exacerbated by other influential factors such as age and
preexisting disease. One prominent example is exposure to particulate matter (PM). PM is a diverse composition of metals and inorganic matter, the constituents of which are dependent on the source, geographic region, and particle aerodynamic diameter which have been reviewed in detail [11]. Exposure to PM is known to induce pulmonary [12–14] and cardiovascular [15, 16] responses, which have been associated with increases in hospital admissions and premature mortality (for review [17]), especially in those with preexisting cardiopulmonary disease. Direct and indirect pathways for PM-induced effects on cardiovascular function have been proposed ([18, 19] for review). Indirect effects include lung exposure derived influences on the cardiovascular system via alterations in nervous system function [20, 21], thus altering heart rate variability (HRV) [22–24] and systemic [25] and/or vascular inflammation [26]. Direct PM effects on cardiovascular function have been associated with infiltration of PM, especially PM with an aerodynamic diameter of <0.1 μm (UF-PM) [27, 28]. Subsequent effects include vascular dysfunction [29, 30] and increased oxidant burden [31–33].

Resistance to oxidant stress relies upon effective antioxidant defenses including enzymes NAD(P)H:quione oxidoreductase 1 (NQO1), superoxide dismutase (SOD), glutathione peroxidases, and heme oxygenase-1 (HO-1). These and other phase II enzyme genes contain promoter antioxidant response elements (AREs) which bind to a heterodimer containing a small Maf protein and nuclear factor-erythroid 2-(NF-E2-) related factor 2 (Nrf2), a member of the Cap ‘n’ Collar family of transcription factors. Although the role of Nrf2 in cardiovascular diseases is complex (refer to a review by R. Howden in the current issue), it has been implicated in resistance against lung injury induced by oxidant exposure [34–36].

Recently, significant adverse changes in cardiac function were reported in mice during exposure to hyperoxia [10], a well-established murine model for acute lung injury and inflammatory lung disease [37, 38], and results suggested a genetic component to cardiac responses. Furthermore, several studies have reported cardiovascular responses to PM exposure, especially heart rate variability (HRV), but genetic factors leading to susceptibility are poorly defined (for review [39]).

Changes in HR and HRV are accepted as indicators for increases in cardiovascular risk, including in response to oxidative stress [10, 40–42]. The purpose of this study was to test the hypothesis that Nrf2 protects against the cardiac responses (HR and HRV) to hyperoxia or UF-PM exposure and improve understanding of the widespread importance of Nrf2 activity in resistance to oxidative stress.

2. Materials and Methods

2.1. Animals and Survival Surgery. Male ICR/sv129: Nrf2+/− and ICR/sv129: Nrf2+/* (wild-type littermates) mice were obtained from a colony maintained at NIEHS, and were originally developed at Tsukuba University [43]. Mice n = 8–16 (per strain; 20–30 g; 8–12 weeks of age) were housed individually in standard polycarbonate cages with a 12:12 hours light-dark cycle. Food (AIN-76A) and water were provided ad libitum. Animals were handled in accordance with The National Institutes of Health Humane Care and Use of Laboratory Animals guidelines. The study protocol was reviewed and approved by the National Institute of Environmental Health Science Animal Care and Use Committee.

Mice were anesthetized with inhaled isoflurane (1.5–2% in oxygen) with buprenorphine (0.1 mg/Kg) given for analgesia. Following a midline dorsal cutaneous incision (3 cm), a subcutaneous tissue pocket was made with a blunt instrument, into which an ETA-F20 ECG transmitter (DSI; Arden Hills, MN, USA) was placed. The positive and negative ECG leads were sutured over the left superficial gluteus and right trapezius muscles, respectively. All incisions were closed using wound clips and animals recovered for five days.

2.2. Hyperoxia and Ultrafine Particulate Matter (UF-PM) Exposure. Prior to any exposure, mice were housed in individual whole body plethysmographs (Buxco Electronics, Wilmington, NC, USA) and allowed at least 30 minutes to become quiescent before recording 20 minutes of baseline ECG. Mice of each genotype were randomly assigned to the following groups: group 1, UF-PM exposure by aspiration (n = 4 per strain; normoxia); group 2, saline exposure by aspiration under normoxic conditions (n = 4 per strain; normoxia); and group 3, hyperoxia exposure (n = 8 per strain; no UF-PM or saline exposure). The number of mice exposed to UF-PM (group 1) was lower because the particles were in limited supply.

UF-PM (aerodynamic diameter <0.1 μm) was collected at the University of North Carolina at Chapel Hill in 2002 [44]. Mice were anesthetized by isoflurane and exposed by aspiration to 100 μg UF-PM suspended in 50 μL of sterile 0.9% saline (group 1) or 50 μL of sterile 0.9% saline only (group 2). Saline/UF-PM suspension was vortexed immediately prior to dosing each animal. Within 10–15 min of UF-PM exposure, mice were housed in whole body plethysmographs (for consistency with hyperoxia exposure procedures below) for 48 hr of continuous ECG data recording.

Group 3 mice were exposed to 100% oxygen using individual whole body plethysmographs as exposure chambers. The oxygen was delivered from a liquid oxygen tank, warmed to room temperature, and sufficiently humidified. ECG was recorded continuously, while mice were exposed to hyperoxia for a maximum of 72 hr, until moribund or when HR declined to ∼250 bpm. These endpoints were chosen based on previous studies of prolonged hyperoxia exposure of inbred mice [10].

R-R interval and HR data were calculated from the ECG records using specialist ECG pattern recognition software (Ponemah, v4.8-SP4). We calculated HRV using a Lomb periodogram as described previously [45]. The frequency ranges used were 0.2–1.5 Hz (low frequency; LF) and 1.5–50 Hz (high frequency; HF), and a summation of the LF and HF was used to represent total power (TP).

2.3. Statistical Analysis. Group mean baseline phenotypic values (HR and HRV) for each genotype were calculated,
3. Results

3.1. HR and HRV Responses to UF-PM. No significant differences in group mean baseline HR, LF, HF, or TP were detected between $\text{Nrf2}^{-/-}$ and $\text{Nrf2}^{+/+}$ (519.8 ± 18.3 versus 482.8 ± 3.4 bpm; 1.14 ± 0.17 versus 1.51 ± 0.16 ms$^2$/Hz; 0.94 ± 0.17 versus 0.75 ± 0.09 ms$^2$/Hz; and 2.08 ± 0.18 versus 2.26 ± 0.22 ms$^2$/Hz, resp.; Figures 1(a)–1(d)). However, compared to saline, a significant overall increased effect of UF-PM exposure on HR responses was found in $\text{Nrf2}^{-/-}$ mice (48 hr mean difference 21.26 bpm; $P < 0.001$; Figure 1(a) and Table 1) but not in $\text{Nrf2}^{+/+}$ mice. Moreover, HR responses were significantly greater in $\text{Nrf2}^{-/-}$ compared to $\text{Nrf2}^{+/+}$ mice treated with UF-PM (48 hr mean difference 24.26 bpm; $P < 0.001$).

A significant overall reduction effect of UF-PM treatment on LF HRV responses (48 hr mean difference 0.02 ms$^2$/Hz; $P = 0.048$; Figure 1(b) and Table 1) was also found, but it was not dependent on genotype. However, multiple significant effects on HF HRV were detected (Figure 1(c) and Table 1). Interestingly, an overall significantly increased HF HRV was found within $\text{Nrf2}^{-/-}$ mice treated with UF-PM versus saline (48 hr mean difference 0.37 ms$^2$/Hz; $P < 0.001$), but not within $\text{Nrf2}^{+/+}$ mice (Table 1). Moreover, overall genotype effects were found for saline and UF-PM treatment groups (48 hr mean difference 0.15 ms$^2$/Hz; $P = 0.019$ and 48 hr mean difference 0.37 ms$^2$/Hz; $P < 0.001$, resp.; Table 1). However, it is important to note that the studentized range distribution ($q$ value) was more than three times higher when

<table>
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<th>HR responses to UF-PM or saline</th>
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<tr>
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<tr>
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<td>0.048</td>
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<td>Comparison: treatment UF-PM versus saline</td>
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<td>0.002</td>
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<tr>
<td>Comparison: treatment within $\text{Nrf2}^{-/-}$ UF-PM versus saline</td>
<td>0.37 ms$^2$/Hz</td>
<td>UF-PM &gt; saline</td>
<td>9.04</td>
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<td>Comparison: genotype within UF-PM $\text{Nrf2}^{-/-}$ versus $\text{Nrf2}^{+/+}$</td>
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<td>Comparison: genotype within UF-PM $\text{Nrf2}^{-/-}$ versus $\text{Nrf2}^{+/+}$ TP HRV responses to UF-PM or saline</td>
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<td>Comparison: genotype within UF-PM $\text{Nrf2}^{-/-}$ versus $\text{Nrf2}^{+/+}$</td>
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<td>$\text{Nrf2}^{+/+}$ &gt; $\text{Nrf2}^{-/-}$</td>
<td>4.06</td>
<td>0.004</td>
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<tr>
<td>Comparison: genotype within UF-PM $\text{Nrf2}^{-/-}$ versus $\text{Nrf2}^{+/+}$</td>
<td>0.34 ms$^2$/Hz</td>
<td>$\text{Nrf2}^{-/-}$ &gt; $\text{Nrf2}^{+/+}$</td>
<td>7.93</td>
<td>&lt;0.001</td>
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and differences were assessed independently using a one-way ANOVA (alpha level was set at 0.05). HR and HRV responses (hourly means ± SEM) to hyperoxia (genotype X time) were assessed independently using a two-way ANOVA with SNK post hoc test for pairwise comparisons up to the common length of exposure time between $\text{Nrf2}^{-/-}$ and $\text{Nrf2}^{+/+}$ groups (45 hr exposure; alpha level was set at 0.05). Differences in HR and HRV phenotypes between baseline and hyperoxia (hourly means ± SEM) were assessed for each genotype using a one-way ANOVA (alpha level was set at 0.05). To assess changes in HR and HRV in response to hyperoxia in the $\text{Nrf2}^{+/+}$ mice (i.e., a longer exposure time compared to $\text{Nrf2}^{-/-}$) from baseline, a one-way ANOVA ($P < 0.05$) was used. HR and HRV responses to UF-PM exposure (genotype X treatment X time) were assessed independently using a three-way ANOVA with Students-Neuman-Keuls post hoc test for pairwise comparisons (alpha level was set at 0.05).
Figure 1: (a) Heart rate (HR, bpm) responses in \(Nrf2^{-/-}\) and \(Nrf2^{+/+}\) mice following aspiration of either ultrafine particulate matter (UF-PM, <0.1 \(\mu\)m) in saline or saline alone. Significant overall effects between treatment and genotype were found \((P < 0.05; \text{Table 1})\). (b) Low frequency heart rate variability (HRV, ms\(^2\)/Hz) responses in \(Nrf2^{-/-}\) and \(Nrf2^{+/+}\) mice following aspiration of either ultrafine particulate matter (UF-PM, <0.1 \(\mu\)m) in saline or saline alone. Significant overall effects between treatments only were found \((P < 0.05; \text{Table 1})\). (c) High frequency (HF) heart rate variability (HRV, ms\(^2\)/Hz) responses in \(Nrf2^{-/-}\) and \(Nrf2^{+/+}\) mice following aspiration of either ultrafine particulate matter (UF-PM, <0.1 \(\mu\)m) in saline or saline alone. Significant overall effects between treatment and genotype were found \((P < 0.05; \text{Table 1})\). (d) Low frequency (LF) heart rate variability (HRV, ms\(^2\)/Hz) responses in \(Nrf2^{-/-}\) and \(Nrf2^{+/+}\) mice following aspiration of either ultrafine particulate matter (UF-PM, <0.1 \(\mu\)m) in saline or saline alone. Significant overall effects between treatment and genotype were found \((P < 0.05; \text{Table 1})\). Group means ± SEM are presented \((n = 4/\text{group})\).

Comparing \(Nrf2^{+/+}\) and \(Nrf2^{-/-}\) treatment groups \((q = 2.40 \text{ and } 9.04, \text{ resp.})\) and more than twice as high for \(Nrf2^{+/+}\) versus \(Nrf2^{-/-}\) within UF-PM or saline \((q = 3.32 \text{ and } 8.06 \text{ resp.; Table 1})\), suggesting a greater effect in \(Nrf2^{-/-}\) mice when treated with UF-PM. Moreover, the interactions for HF HRV between genotypes within each treatment group were opposing. Overall, HF HRV was higher in \(Nrf2^{+/+}\) versus \(Nrf2^{-/-}\) mice following saline treatment, but HF HRV was higher in \(Nrf2^{-/-}\) versus \(Nrf2^{+/+}\) mice following UF-PM treatment (Table 1). Specific time points at which these differences occurred were undetectable, perhaps due to the high degree of variability in the \(Nrf2^{-/-}\) UF-PM treated group.

TP HRV is the sum of HF and LF HRV, and multiple overall effects were found (Figure 1d and Table 1), but specific time points at which these differences occurred were not detectable. Overall differences in TP HRV responses
between UF-PM and saline treatment were found within Nrf2\textsuperscript{+/+} and Nrf2\textsuperscript{−/−} groups (48 hr mean difference for Nrf2\textsuperscript{+/+}, 0.17 ms\textsuperscript{2}/Hz, $P = 0.007$; 48 hr mean difference for Nrf2\textsuperscript{−/−}, 0.35 ms\textsuperscript{2}/Hz, $P < 0.001$). Moreover, Nrf2\textsuperscript{−/−} and Nrf2\textsuperscript{+/+} groups were different from each other with respect to TP HRV responses irrespective of treatment (48 hr mean difference after saline, 0.19 ms\textsuperscript{2}/Hz, $P = 0.004$; 48 hr mean difference after UF-PM, 0.34 ms\textsuperscript{2}/Hz, $P < 0.001$). However, the $q$ value was approximately twice as high when comparing Nrf2\textsuperscript{+/+} and Nrf2\textsuperscript{−/−} treatment groups ($q = 3.78$ and 8.22, resp.) and Nrf2\textsuperscript{+/+} and Nrf2\textsuperscript{−/−} within UF-PM or saline ($q = 4.06$ and 7.93, resp.), again suggesting a greater effect in Nrf2\textsuperscript{−/−} mice treated with UF-PM, although this effect was primarily influenced by HF HRV responses as the interactions were similar (Table 1). Despite the significant differences in HR and HRV responses between treatment and genotype, we were not able to detect specific posttreatment time points where these differences lie.

### 3.2. HR and HRV Responses to Hypoxia

In mice used for hyperoxia exposures, no significant differences in group mean (±SEM) baseline HR, LF, HF, or TP were detected between Nrf2\textsuperscript{−/−} and Nrf2\textsuperscript{+/+} (483.2 ± 21.2 versus 486.6 ± 12.3 bpm; 1.17 ± 0.18 versus 1.35 ± 0.10 ms\textsuperscript{2}/Hz; 1.25 ± 0.18 versus 1.12 ± 0.14 ms\textsuperscript{2}/Hz; 2.42 ± 0.17 versus 2.47 ± 0.17 ms\textsuperscript{2}/Hz, resp.; Figure 2).

Group mean (±SEM) HR of Nrf2\textsuperscript{−/−} mice reduced to below 250 bpm in significantly less time compared to Nrf2\textsuperscript{+/+} mice (determined by the first hr at which individual mouse HR was less than 250 bpm was detected; 41.6 ± 1.9 versus 64.0 ± 2.9 hours; $P < 0.001$; Figure 2). Prolonged hypoxia caused highly significant and precipitous reductions in HR after a period of normal circadian variation, which was genotype dependent. Compared to respective genotype mean baseline values, HR reduced significantly in Nrf2\textsuperscript{−/−} mice after 34 hrs hyperoxia (group mean difference 178.2 bpm; $P < 0.001$) and continued to decline until exposure terminated at 45 hrs (group mean difference 234.4 bpm; $P < 0.001$; Figure 3(a)). In Nrf2\textsuperscript{+/+} mice, the decline in HR compared to baseline was not significant until 54 hrs hyperoxia (group mean difference 147.8 bpm; $P < 0.001$; Figure 3) and 20 hrs after a significant group mean HR reduction in Nrf2\textsuperscript{−/−} mice. HR continued to decline in Nrf2\textsuperscript{−/−} mice until exposure terminated at 70 hr (group mean difference 236.4 bpm; $P < 0.001$; Figure 3(a)).

LF HRV was significantly reduced in Nrf2\textsuperscript{−/−} mice compared to Nrf2\textsuperscript{+/+} mice after 40 hrs hyperoxia (group mean difference 0.63 ms\textsuperscript{2}/Hz; $P = 0.01$; Figure 3(b)) and continued to decline until the Nrf2\textsuperscript{−/−} mice were euthanized. No significant changes in LF HRV were detected in the Nrf2\textsuperscript{+/+} mice during hyperoxia. Within each genotype, no significant effect of hyperoxia on HF HRV was found, except after 35 and 36 hrs of hyperoxia when HF HRV was significantly reduced in Nrf2\textsuperscript{−/−} mice compared to Nrf2\textsuperscript{+/+} mice (group mean difference 0.71 ms\textsuperscript{2}/Hz; $P < 0.001$ and group mean difference 0.81 ms\textsuperscript{2}/Hz; $P < 0.001$; Figure 3(c)). Thereafter, no differences in mean HF HRV were found between genotypes. Because TP HRV is the sum of LF and HF HRV, it was not surprising to find a significant overall genotype effect during hyperoxia exposure (group mean difference 0.24 ms\textsuperscript{2}/Hz; $P < 0.001$; Figure 3(d)). Mean TP HRV in Nrf2\textsuperscript{−/−} mice was significantly lower compared to Nrf2\textsuperscript{+/+} mice from 43 hr exposure (group mean difference 0.92 ms\textsuperscript{2}/Hz; $P = 0.03$) to the end of exposure in Nrf2\textsuperscript{−/−} mice (45 hrs).

### 4. Discussion

Factors contributing to oxidative stress are widely accepted as important to the pathogenesis of cardiopulmonary diseases. Examples include inflammatory lung diseases, exposure to oxidant air pollution, and a wide range of clinical scenarios that require oxygen therapy with high fraction of inspired oxygen (FiO\textsubscript{2}; for example, acute respiratory distress syndrome and postmyocardial infarction patients). Understanding susceptibility mechanisms for severe oxidant stresses (such as advanced cardiopulmonary disease or high FiO\textsubscript{2}) or less severe changes in oxidant burden (such as air pollution exposure) is a primary public health concern. Importantly, overlap in responsible mechanisms between oxidative stress inducing exposures could partially explain reported extremes in susceptibility or resistance to adverse reactions. A prominent example is the negative effect of pre-existing cardiopulmonary disease on susceptibility to adverse cardiac responses to oxidative stress and poor responses to further oxidant burden induced by oxygen therapy, all of which may operate through the same or similar mechanisms.

In this study, we found that Nrf2 was important in cardiac responses to a severe (hyperoxia) and moderate (UF-PM) oxidant stress. A central role for Nrf2 in resistance to hyperoxia-induced lung injury has been described in detail [34, 35], and Nrf2 appears to be also important in epithelial cell response to particle exposure [46], especially when
Figure 3: (a) Hourly mean heart rate (HR, bpm) responses in $\text{Nrf}2^{-/-}$ and $\text{Nrf}2^{+/+}$ mice during hyperoxia (100% oxygen) exposure. Heart rates in $\text{Nrf}2^{-/-}$ mice were significantly reduced from baseline from 34 hr until the end of exposure ($P < 0.05$). Heart rates in $\text{Nrf}2^{+/+}$ mice were significantly reduced from baseline from 54 hr until the end of exposure ($P < 0.05$). (b) Hourly mean low frequency (LF) heart rate variability (HRV, (ms$^2$/Hz) responses in $\text{Nrf}2^{-/-}$ and $\text{Nrf}2^{+/+}$ during hyperoxia (100% oxygen) exposure. LF HRV reduced in $\text{Nrf}2^{-/-}$ versus $\text{Nrf}2^{+/+}$ after 40 hr of exposure ($P < 0.05$). LF HRV in $\text{Nrf}2^{+/+}$ mice did not change significantly ($P > 0.05$). (c) Hourly mean high frequency (HF) heart rate variability (HRV, ms$^2$/Hz) responses in $\text{Nrf}2^{-/-}$ and $\text{Nrf}2^{+/+}$ during hyperoxia (100% oxygen) exposure. HF HRV reduced significantly in $\text{Nrf}2^{-/-}$ versus $\text{Nrf}2^{+/+}$ at 35 and 36 hrs of exposure ($P < 0.05$). (d) Hourly mean total power heart rate variability (TP HRV, ms$^2$/Hz) responses in $\text{Nrf}2^{-/-}$ and $\text{Nrf}2^{+/+}$ during hyperoxia (100% oxygen) exposure. TP HRV reduced in $\text{Nrf}2^{-/-}$ versus $\text{Nrf}2^{+/+}$ after 43 hr to the end of the exposure ($P < 0.05$). Group means ± SEM are presented ($n = 8$/group).

combined with allergy and/or asthma [36]. Since interactions between the cardiovascular and pulmonary systems are well known, lung injury response to oxidative stress is likely to involve the heart. Moreover, an influence of oxidative stress on cardiac function has been demonstrated, especially during hypoxia [47], reperfusion injury [48], and in response to particle exposure [49]. Because Nrf2 is established as critically important in antioxidant defense, this suggests that oxidative stress was a common component to hyperoxia and UF-PM cardiac responses in this study.

HR responses to UF-PM exposure were statistically significant though not as severe as responses to hyperoxia (see Figures 1 and 3). Nonetheless, the changes elicited by UF-PM may have physiological relevance because the mice used were young and healthy and were otherwise not compromised. Targeted deletion of Nrf2 exacerbated the HR responses,
though the mechanism through which Nrf2 protects against the response remains unclear. It would also be of interest to determine whether interactions exist between Nrf2 and preexisting disease and/or age, both of which are important susceptibility factors associated with PM exposure [50–54].

Cardiovascular responses to particulate matter exposures have been investigated in detail ([55] for review). However, little is known about genetic susceptibility to particle exposure or which sectors of the population are most at risk. Because such a large percentage of the global population is exposed to particulate matter, this presents the potential for widespread adverse health outcomes and highlights the importance of understanding susceptibility. In this study, we found overall effects of Nrf2 deletion on cardiac responses to UF-PM that could act through similar mechanisms that become important in a compromised host, especially since pre-existing disease is an important factor in susceptibility to UF-PM exposure [52, 54, 56]. These effects may not have manifested in this experiment since all mice were otherwise healthy, and therefore subtle responses were produced.

Previously, we reported highly significant HR responses to hyperoxia that preceded changes in pulmonary function and lung injury [10], suggesting that cardiac responses to oxidative stress may predict impending adverse pulmonary events. In the present study, we found similar HR responses to hyperoxia, and Nrf2−/− mice were highly susceptible compared to Nrf2+/+ mice, reaching the HR end point of 250 bpm ~22 hr before Nrf2+/+ mice (Figure 2). Hyperoxia is known to cause significant lung injury, pulmonary edema and, at least in patients with acute respiratory distress syndrome, for which hyperoxia is a model, poor gas exchange leading to hypoxemia [57–59]. Although we were unable to measure blood gases during hyperoxia exposure, it is known that bradycardia can result from either hypoxemia and/or permissive hypercapnia, as a consequence of respiratory insufficiency [60–62], which may be associated with chemoreceptor activation and respiratory acidosis [60]. These mechanisms may be partially responsible for the severe bradycardia observed in this and previous studies [10] exposing mice to prolonged hyperoxia, which warrants further investigation. Decreases in HF HRV have been observed when mice were exposed to a hypoxia/hypercapnia combination, suggesting a role for autonomic nervous system (ANS) control of the heart under these conditions. In this study, we found opposing overall genotype effects (Nrf2−/− versus Nrf2+/+) for HRV phenotypes during hyperoxia or after UF-PM exposure (decreases during hyperoxia and increases following UF-PM). While these data suggest an interaction between Nrf2 and ANS function, the opposing effects of hyperoxia and UF-PM treatment on HRV are challenging to interpret because the correlation between changes in HRV and ANS tone is currently a matter of debate (for review [63]). Nonetheless, our data do suggest a disturbance in autonomic regulation of cardiac function during hyperoxia and after UF-PM treatment that was modulated by Nrf2. These HRV changes may therefore have important implications for susceptibility to adverse cardiac outcomes in response to oxidant exposure.

However, since hypoxia and hypercapnia are unlikely to result from UF-PM exposure, Nrf2 may act through different mechanisms compared to hyperoxia exposure. For example, Nrf2 has been implicated in defense against cadmium-induced oxidative stress in the olfactory bulb of zebrafish [64]. Interestingly, human olfactory bulb stimulation is associated with changes in HRV [65], and UF particles have been shown to translocate from the lung to the olfactory bulb of rats [66]. Taken together, it is possible that changes in HR and HRV following UF-PM exposure in this study were partially mediated through UF-PM-induced oxidative stress effects on the olfactory bulb.

While speculative, a contributory mechanism for the observed HR and HRV responses to hyperoxia could be associated with the candidate gene thrombospondin, type I, domain containing 4 (ThSD4 or AdAMTS6) [10]. Adamts6 has been reported to bind directly to fibrillin-1 (Fbn-1), initiating widespread extracellular matrix (ECM) assembly, including the myocardium [67]. Fbn-1 mediates bone morphogenetic protein-induced expression of important ECM collagens. Interestingly, absence of Fbn-1 is associated with Marfan’s syndrome [68], and over expression leads to myocardial fibrosis [69]. Moreover, changes in the myocardial ECM is associated with the development of diastolic dysfunction in heart failure, even in the short term, possibly through the renin-angiotensin-aldosterone system ([70] for review). Regulation of these genes have been linked to Nrf2 expression levels during hyperoxia exposure in mice [71], which may explain part of the cardiac responses observed here during hyperoxia or following UF-PM exposures. Further work is required to determine the importance of changes in ECM proteins in cardiac responses to oxidative stress.

5. Conclusions

In this study, we found that severe (hyperoxia) and moderate (UF-PM) environmental oxidant stressors caused HR and HRV responses in the mouse, and targeted deletion of Nrf2 significantly augmented the detrimental responses to these environmental oxidants. The magnitude of cardiac functional responses may have been proportional to the degree of oxidant burden during hyperoxia or after UF-PM aspiration. Understanding the mechanisms by which the myocardium defends against these stressors is critical for identifying individuals at risk, and we provide evidence that Nrf2 may be an important determinant in defense against severe and moderate oxidative stress.

Conflict of Interests

The authors confirm that no conflict of interests exists in relation to this paper.

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Curcumin Protects Human Keratinocytes against Inorganic Arsenite-Induced Acute Cytotoxicity through an NRF2-Dependent Mechanism

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Human exposure to inorganic arsenic leads to various dermal disorders, including hyperkeratosis and skin cancer. Curcumin is demonstrated to induce remarkable antioxidant activity in a variety of cells and tissues. The present study aimed at identifying curcumin as a potent activator of nuclear factor erythroid 2-related factor 2 (NRF2) and demonstrating its protective effect against inorganic arsenite- (iAs\textsuperscript{3+}) induced cytotoxicity in human keratinocytes. We found that curcumin led to nuclear accumulation of NRF2 protein and increased the expression of antioxidant response element- (ARE-) regulated genes in HaCaT keratinocytes in concentration- and time-dependent manners. High concentration of curcumin (20 \( \mu \)M) also increased protein expression of long isoforms of NRF1. Treatment with low concentrations of curcumin (2.5 or 5 \( \mu \)M) effectively increased the viability and survival of HaCaT cells against iAs\textsuperscript{3+}-induced cytotoxicity as assessed by the MTT assay and flow cytometry and also attenuated iAs\textsuperscript{3+}-induced expression of cleaved caspase-3 and cleaved PARP protein. Selective knockdown of NRF2 or KEAP1 by lentiviral shRNAs significantly diminished the cytoprotection conferred by curcumin, suggesting that the protection against iAs\textsuperscript{3+}-induced cytotoxicity is dependent on the activation of NRF2. Our results provided a proof of the concept of using curcumin to activate the NRF2 pathway to alleviate arsenic-induced dermal damage.

1. Introduction

Arsenic is a natural element ubiquitous in the environment. Chronic human exposure to inorganic arsenic (iAs) induces various skin lesions, including Bowen’s disease, hyperkeratosis, and skin cancers [1–4]. Our previous studies reveal that oxidative stress occurs in response to inorganic arsenite (iAs\textsuperscript{3+}) exposure [5–8], which may partly account for the dermal toxicity of iAs\textsuperscript{3+}, including hyperkeratosis and carcinogenesis.

Nuclear factor erythroid 2-related factors (NRFs) are a family of transcription factors that regulate the cellular adaptive response to oxidative stress through the cis-regulating antioxidant response element (ARE). Many ARE-dependent genes are important in maintaining the cellular redox homeostasis and limit oxidative damage. Under normal conditions, NRF1 is targeted to the endoplasmic reticulum [9], whereas NRF2, constitutively expressed at a low level, is primarily in the cytoplasm and mainly controlled by the Kelch-like ECH-associated protein 1 (KEAP1) through ubiquitination and proteasomal degradation [10]. Upon oxidative stress, NRF2 and/or NRF1 dimerize with small MAF or other bZIP proteins in the nucleoplasm, and then the heterodimer binds to the AREs in the promoter regions of various detoxifying and antioxidative stress response genes, such as NADPH: quinone oxidoreductase 1 (NQO1), glutamate cysteine ligase catalytic (GCLC) and regulatory (GCLM) subunits, and heme oxygenase-1 (HMOX-1). Thus, NRF2...
and/or NRF1-mediated adaptive antioxidant response plays important roles against oxidative/electrophilic stress and in chemical detoxification. Our previous studies demonstrated that NRF2, NRF1, and KEAP1 contribute to the coordinated regulation of antioxidant and detoxification enzyme expression and protect cells from arsenic-induced apoptosis and cytotoxicity in human HaCaT cells [6–8, 11]. Therefore, enhancing the NRF2-dependent adaptive response through chemoprevention holds the promise of conferring protection against toxicity and carcinogenicity induced by iAs^{3+}.

Curcumin is a polyphenol natural product isolated from the rhizome of Curcuma longa. For centuries, curcumin has been used in some medicinal preparations or as a food-coloring agent. Extensive in vitro and in vivo studies demonstrated that curcumin has a number of biological and pharmacological activities, such as anti-inflammatory, antioxidant, antimutagenic, and anticarcinogenic activities [12–14]. The effects of curcumin have been extensively investigated in liver cells [15], human lymphocytes [16], endothelial cells [17], renal epithelial cells [18], astrocytes [19], and murine splenocytes [20]. The protective effect of curcumin owing to its antioxidant property by inducing NRF2-mediated antioxidant and detoxifying enzymes has been demonstrated [21, 22]. Numerous studies have provided evidence that curcumin protects against iAs^{3+}-exerted neurotoxicity, genotoxicity and DNA damage in vivo and in vitro [20, 23–27]. Thus, with its substantial antioxidant property, curcumin can combat the adverse effects of arsenic in a variety of experimental settings and epidemiological surveys.

However, the role of curcumin in regulating NRF2 and its target genes in human keratinocytes and whether curcumin protects against iAs^{3+}-induced cytotoxicity in these cells are not clear. In the present study, we confirmed curcumin as a potent NRF2 activator and investigated the NRF2-dependent protective role of curcumin against iAs^{3+}-induced cytotoxicity and apoptosis in human HaCaT cells. Our findings have important implications not only for understanding the role of curcumin against iAs^{3+}-induced cytotoxicity in human keratinocytes but also for developing preventive and/or corrective strategies against chronic arsenicosis, including arsenic-induced skin disorders.

2. Materials and Methods

2.1. Cell Culture and Experimental Reagents. HaCaT cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U penicillin/mL, and 100 μg streptomycin/mL, as described previously [6]. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. Culture media, FBS, and supplements were purchased from Invitrogen (Carlsbad, CA, USA). Sodium arsenite and curcumin were obtained from Sigma (St. Louis, MO, USA).

2.2. Lentiviral-Based shRNA Transduction. MISSION shRNA lentiviral particles were obtained from Sigma. Transduction of HaCaT cells with lentiviral-based shRNAs targeting NRF2 (SHVRS-NM_006164), KEAPI (SHVRS-NM_012289), or scrambled nontarget negative control (SHC002V) was performed and confirmed as described previously [7, 8]. Cells were maintained in medium containing 1.0 μg/mL of puromycin.

2.3. Antioxidant Response Element (ARE) Reporter Assay. Cignal Lenti ARE reporter transduction of HaCaT cells was performed as described previously [8]. Cells were grown to ~90% confluence and subcultured in medium containing 1.0 μg/mL of puromycin. The luciferase activity was measured by Luciferase Reporter Assay System (E1960, Promega, Madison, WI, USA) according to the manufacturer’s protocol. The luciferase activity was normalized to cell viability which was determined using a Non-Radioactive Cell-Proliferation Assay Kit (G5430, Promega).

2.4. Acute Cytotoxicity Assay. A minimum of 5 replicates of 10,000 cells per well were plated in 96-well plates and allowed to adhere to the plate for 24 hr, at which time the media were removed and the cells were treated with medium containing curcumin and/or iAs^{3+}. Cells were then incubated for indicated time and cell viability was determined using Non-Radioactive Cell-Proliferation Assay Kit as detailed previously [7, 8].

2.5. Western Blot Analysis. Protein isolation from whole-cell lysates and determination of protein concentration were conducted with BCA kit according to the manufacturer’s protocol (Beyotime, P0010, Shanghai, China). For immunoblot analysis, 50 μg protein was run on an 8% or 12% Tris-Glycine gel and blotted to PVDF membrane. The membrane was blocked in 5% nonfat milk at room temperature (RT) for 2 hr, then it was incubated with primary antibodies (Ab) at 4°C overnight followed by treatment with horseradish peroxidase-conjugated secondary Ab at RT for 2 hr. Protein expression was detected by Chemiluminescence Luminol Reagent (sc-2048, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunoblotting was performed by using Abs against the following antigens: NRF2 (1:500, sc-13032, Santa Cruz Biotechnology, Inc., Santa Cruz Biotechnology, Inc.), KEAPI (1:500, sc-15246, Santa Cruz Biotechnology, Inc.), HMOX-1 (1:500, sc-136960, Santa Cruz Biotechnology, Inc.), Cleaved caspase-3 (1:1000, #9664, Cell Signaling Technology, MA, USA), PARP (1:1000, #9542, Cell Signaling Technology), and β-actin (1:2000, A1978, Sigma).

2.6. Immunostaining. Fluorescence immunostaining was performed as described previously [6]. Briefly, cells were grown on glass cover slips in six-well plates for 48 hr. Then, cells were washed with PBS and fixed for 15 min at RT in 3% (v/v) formaldehyde. After being washed, in PBS, cells were permeabilized in 1% (v/v) Triton X-100 in PBS, washed and incubated with 10% goat serum (ZLI-9021, ZSGB-Bio, Beijing, China) in PBS for 1 hr at RT. Cells were first treated with NRF2 antibody overnight at 4°C and subsequently with goat anti-rabbit IgG-CFL 488 (sc-362262, Santa Cruz Biotechnology, Inc.) for 1 hr at RT. After being washed with PBS and incubated for 10 min with 1:500 fluorescein isothiocyanate-conjugated secondary Ab (FITC-Alexa Fluor 488, Life Technologies, Carlsbad, CA, USA) for 30 min at RT. Coverslips were mounted on glass slides with ProLong Gold Antifade Reagent (Invitrogen).
PBS, the cover slips were mounted with the Prolong Gold antifade reagent (P36930, Molecular Probes, Inc., Eugene, OR, USA) on microscope slides, and immunostaining was examined by using Leica DM4000 B Fluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany).

2.7. Quantitative Real-Time RT-PCR Analysis. Total RNA was isolated with TRizol (10926-028, Life Technologies) and then was subjected to cleanup by using RNase-Free DNase Set and RNeasy Mini kit (Qiagen, Valencia, CA, USA). Quantitative real-time RT-PCR was performed as described previously [7, 8]. SYBR Green PCR master mix was purchased from Applied Biosystems (Carlsbad, CA, USA). Primers (sequences are shown in Supplemental Material, Table 1) (Supplementary Material available online at http://dx.doi.org/10.1155/2013/412576) were designed by using Primer Express 4 (Applied Biosystems) and synthesized by MWG-BIOTECH Inc. (High Point, NC, USA). Real-time fluorescence detection was carried out by using an ABI PRISM 7900 HT Fast Real-Time PCR System (Applied Biosystems).

2.8. Cell Death Assessment by Flow Cytometry. HaCaT cells were seeded in a six-well plate and grown to approximately 70% confluence. Cells were treated with various concentrations of curcumin for a total of 24 hr. At the end of the 6th hr, iAs$^{3+}$ was added for the remaining 20 hr. Floating and attached cells were harvested for apoptosis analysis. Apoptotic and necrotic cells were analyzed by flow cytometry (Muse Cell Analyser, Merck Millipore, Billerica, MA, USA) by using the Muse Annexin V & Dead Cell Kit (MCH100105, Merck Millipore). For each sample, approximately 10,000 cells were examined each time and the percentage of apoptotic and necrotic cells was calculated from experiments run in triplicate by statistical analysis of the various dot plot using Muse 1.1.2 analysis software (Merck Millipore).

2.9. Statistical Analyses. All statistical analyses were performed by using Graphpad Prism 5 (GraphPad Software, San Diego, CA, USA), with $P < 0.05$ taken as significant. Data are expressed as mean ± SD. Statistical analyses to evaluate the time- and concentration-dependent effects of curcumin exposure on gene expression and cell viability were performed by using two-way ANOVA with Bonferroni post hoc testing. Statistical analyses to evaluate the protective effect of curcumin on iAs$^{3+}$-induced cytotoxicity were carried out by using one-way ANOVA with Tukey’s multiple comparison test.

3. Results

3.1. Cytotoxicity and ARE-Luciferase Activity Induced by Curcumin. After HaCaT cells were exposed to various concentrations of curcumin for 24 hr, curcumin at 1.25–5 μM significantly increased the viability of HaCaT cells compared to control, while there was a concentration-dependent decrease in cell viability at 10 μM or higher. The LC50 of curcumin for 24 hr exposure was 27.33 ± 1.53 μM (Figure 1(a), left panel).

To evaluate the potential activation of the antioxidant response pathway by curcumin in HaCaT cells, cells stably transduced with the ARE-luciferase reporter were used. These cells were responsive to noncytotoxic concentration (40 μM) of NRF2 activator tBHQ-induced ARE activation in a time-dependent fashion (Figure 1(a), right panel, and Figure 1(b), left panel), confirming that the cells are responsive to NRF2 activation. As shown in Figure 1(b) (middle and right panels), curcumin concentration and time dependently increased the activity of ARE-luciferase reporter in HaCaT cells.

3.2. Curcumin Increased NRF2 Protein Expression and Induced the Adaptive Antioxidant Response. In response to a 6 hr exposure to curcumin, the protein expression of NRF2 was increased in a concentration-dependent manner (Figure 2(a)). In response to 5 μM curcumin treatment, NRF2 protein was elevated quickly and peaked 2–6 hr (Figure 2(b)). These results confirmed that curcumin is a potent NRF2 activator in human HaCaT cells. Interestingly, protein expression of NRF1 was elevated only at a higher concentration of curcumin (20 μM) (Figure 2(a), suggesting that NRF1 can probably be activated only at more toxic conditions. Cell immunostaining showed that NRF2 was mainly localized in the cytoplasm in untreated cells (Figure 2(c), left panel) but accumulated in the nucleus after exposure to curcumin for 6 hr (Figure 2(c), right panel).

Since curcumin augmented the ARE activity of the luciferase reporter (Figure 1(b)), we next sought to confirm the result with endogenous ARE-dependent genes. As expected, mRNAs of NQO1, HMOX1, GCLC, and GCLM were induced significantly by curcumin in a concentration- and time-dependent manner (Figures 3(a) and 3(b), bottom panels). The mRNA expression of NRF2 and NRF1 decreased slightly at a high concentration of curcumin and did not change significantly over time (Figures 3(a) and 3(b), upper panels), suggesting that NRF2 and NRF1 were primarily posttranscriptionally regulated. Interestingly, the mRNA expression of KEAP1 was also induced by curcumin, suggesting a potential feedback from NRF2 to KEAP1. Our results demonstrate that curcumin is able to induce the NRF2 pathway and its target genes.

3.3. Curcumin Protected against iAs$^{3+}$-Induced Cytotoxicity. To determine the protective effect of curcumin on iAs$^{3+}$-induced cytotoxicity, noncytotoxic concentrations of curcumin (2.5 and 5 μM, Figure 1(a)) were used. As shown in Figure 4, HaCaT cells were pretreated with 2.5 μM or 5 μM curcumin for 6 hr. Subsequently, the cells were exposed to 30 μM of iAs$^{3+}$ for 20 hr in the continued presence of curcumin (Figure 4(a)), after which cell viability and apoptosis were measured. Compared with untreated cells, treatment with curcumin caused a significant increase in cell viability in response to iAs$^{3+}$ (Figure 4(b)). In addition, flow cytometry measurement with Annexin V-FITC and PI double staining showed that exposing cells to 30 μM iAs$^{3+}$ for...
Figure 1: Curcumin activates ARE in HaCaT cells. (a) Cytotoxicity of curcumin and tBHQ. HaCaT cells were exposed to different concentrations of curcumin or tBHQ for 24 hr, and then cell viability was measured by MTT assay. Data are expressed as mean ± SD. \( n = 6 \). *\( P < 0.05 \) versus vehicle (medium). (b) Curcumin activates ARE in a concentration- and time-dependent manner. Cultured HaCaT cells stably transduced with the ARE-luciferase reporter were exposed to 40 \( \mu \)M tBHQ for indicated period of time as a positive control (left panel); then ARE activation was detected in the condition of different concentrations of curcumin for 6 hr (middle panel) or 2.5 and 5 \( \mu \)M of curcumin for indicated period of time (right panel). Values shown are mean ± SD. \( n = 6 \). *\( P < 0.05 \) versus vehicle (medium).

3.4. The Protective Effect of Curcumin on iAs\(^{3+}\)-Induced Cytotoxicity and Apoptosis Is Dependent on NRF2 Activation. We proposed that the protection against the cytotoxicity of iAs\(^{3+}\) by curcumin in human HaCaT cells is owing to the activation of NRF2. To study the mechanism, the effects of curcumin on iAs\(^{3+}\)-induced cytotoxicity in HaCaT cells with stable knock-down (KD) of NRF2 or KEAP1 were examined. The silencing efficiency of the constructs was confirmed by immunoblot under basal and curcumin-treated condition (Figure 5(a)), at the same time, the protein level of HMOX-1, an NRF2-specific downstream gene, was also detected (Figure 5(a)). As expected, the protection by curcumin against iAs\(^{3+}\) was obvious in scramble (SCR) cells (Figure 5(b), left panel). In NRF2-KD cells, treatment with 5 \( \mu \)M curcumin offered no protection against iAs\(^{3+}\) as compared with cells exposed to iAs\(^{3+}\) alone (Figure 5(b), midpanel). Interestingly, curcumin did not offer further protection against iAs\(^{3+}\) in KEAPI-KD cells either (Figure 5(b), right panel). This is likely because in these cells NRF2 was already fully activated due to the lacking of KEAPI, and thus maximal protection against iAs\(^{3+}\) was already in place, as indicated by the dramatic right-ward shift.
of the response curve in untreated cells. Therefore, treatment with curcumin did not provide additionally activated NRF2 and hence no further protection. The fraction of apoptotic cells, as revealed by flow cytometry (Figure 5(c)) and cleaved caspase-3/cleaved PARP expression (Figure 5(d)), showed no difference between curcumin-treated and untreated cells with either NRF2-KD or KEAP1-KD. Our data demonstrate that the cytoprotection provided by curcumin requires activation of the NRF2 pathway.

4. Discussion

NRF2 plays a pivotal role in directly regulating many antioxidant and detoxification enzyme genes via AREs in gene promoters. The important role of NRF2 in chemoprevention and cellular defense has been clearly demonstrated in NRF2-null mice which are susceptible to both oxidative and carcinogenic insults [28–31]. Accumulating evidence from both animal models and human epidemiological studies has shown that many naturally occurring phytochemicals, including sulforaphane, epigallocatechin-3-gallate (EGCG), curcumin, and oridonin, possess chemopreventive potential by inducing NRF2-mediated antioxidant/detoxification enzymes [32–34].

Curcumin has been used for chemoprevention and treatment of various skin lesions, such as scleroderma, psoriasis, skin cancer, and wound healing [35–37]. It has been shown that curcumin activated NRF2 in several cell types [21, 32, 38] and exerted a cytoprotective effect through transcriptional induction of phase II enzymes, such as glutathione transferase, NQO1, HMOX-1, GCLC, and GCLM in certain human cancers, skin lesions, and neurodegenerative diseases [39, 40]. However, whether curcumin is a potent NRF2/1 activator in human HaCaT cells has not been demonstrated previously. Our present study indicates that curcumin induced NRF2 protein nuclear accumulation in a time- and concentration-dependent manner, which is unlikely to be attributed to an increase in mRNA expression, confirming a previous report [41]. Though curcumin disrupted the NRF2-KEAP1 complex, leading to increased NRF2 occupancy of AREs [42], or indirectly stimulated the phosphorylation of NRF2 at serine and/or threonine residues which may facilitate its nuclear accumulation [21], the exact mechanism by which curcumin activates NRF2 needs further investigation. Furthermore, research using relative high concentrations of curcumin (10–25 µM) on cultured cells showed that curcumin upregulated phase II enzymes especially HMOX-1 [41, 43], while, in our experiment, curcumin was found to readily induce NQO1, HMOX-1, GCLC, and GCLM genes at even lower concentrations.

Curcumin has been found to affect the structure and function of cellular membrane, mimic typical events occurring during apoptosis [44], and induce apoptosis of epidermal cells at the concentration of 12.5 or 25 µM [41]. When HaCaT cells were treated in combination with UV or radiation, they

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**Figure 2:** Effects of curcumin on protein expression of NRF2 and NRF1 in HaCaT cells (a) and (b). Representative images of western blot. HaCaT cells were exposed to indicate concentrations of curcumin for 6 hr (a) or 5 µM curcumin for the indicated period of time (b). Whole-cell lysates (50 µg protein) were separated on 8% Tris-Glycine gels and detected using anti-NRF2 or anti-NRF1. β-Actin was used as a loading control. Vehicle, medium. (c) Immunofluorescence staining of NRF2. HaCaT cells were treated with vehicle (left) or 5 µM curcumin (right) for 6 hr.
Figure 3: mRNA expression of NRF2, NRF1, KEAP1, and some ARE-dependent genes induced by curcumin in HaCaT cells. (a) Concentration response of curcumin-induced gene expression. Cells were exposed to different concentrations of curcumin for 6 hr. (b) Time course of gene expression induced by 5 μM curcumin. The number in parentheses after each gene name is the Ct (cross-threshold) value of that gene in HaCaT cells treated with vehicle (medium). Values are mean ± SD. n = 3. *P < 0.05 versus vehicle.
showed increased apoptosis [45, 46]. In our studies, acute exposure to curcumin at low concentrations has no effect on the apoptotic rate, while it increased viability against iAs^{3+}-induced cytotoxicity.

Arsenic, a ubiquitous environmental element, causes dermal toxicity [4, 47]. Our previous studies [6–8] demonstrated that the NRF2 and NRF1 signaling pathways can be activated by iAs^{3+} in human HaCaT cells, suggesting that NRF2 and NRF1 may be involved in the pathogenesis of arsenic-induced skin cancer and hyperkeratosis. Furthermore, numerous studies show that the NRF2-mediated stress response program is activated in early tumor development, and oncogene activities are coupled with NRF2 activation, thereby providing malignant cells a survival and growth advantage.
However, activating the NRF2-dependent protective pathway has also proved to be beneficial in reducing arsenic-induced toxicity in human bladder urothelial cells [51]. Our previous study showed that stable knockdown of NRF2 using shRNA rendered human HaCaT cells more sensitive to iAs3+-induced cell death [8], suggesting a potential usage of NRF2 activators for therapeutic and dietary interventions against adverse effects of arsenic. The paradoxical health effects of NRF2 activated by a specific chemical agent were mainly determined by the balance between the induction of the NRF2 defense response and the otherwise adverse outcomes elicited by the agent. Therefore, the agent used for cytoprotection should be preferred at low concentrations without eliciting tangible cytotoxicity. In the present study, treatment with curcumin counteracted iAs3+-induced cell damage through activating NRF2, as demonstrated by MTT, apoptosis, and apoptotic-executive protein expression assays. Our cell-based study supports the notion that curcumin can be used as a chemopreventive agent. Low-concentration curcumin specifically targets NRF2-induced cellular antioxidant defense and has an important role in maintaining homeostasis in epidermis [52]. Although the finding that treatment with curcumin led to the activation of NRF2 and protected HaCaT cells against the acute cytotoxicity of iAs3+ is consistent with the result in hepatocytes with sulforaphane [53], the suppression of oxidative stress and/or reduction of cellular accumulation of arsenic in HaCaT cells needs further investigation to illustrate the underlying mechanisms.

Figure 5: The protective effect of curcumin on iAs3+-induced cytotoxicity and apoptosis is dependent on NRF2 activation in HaCaT cells. Protocols for curcumin treatment and arsenic exposure are the same as Figure 4(a). (a) The protein level of NRF2, KEAP1, and HMOX-1 under basal and curcumin-treated condition in scramble, NRF2-KD, and KEAP1-KD cells. Cells were treated with vehicle or 20 μM curcumin for 6 hr. Whole-cell lysates were separated on 4–12% Tris-Glycine gels. Vehicle, medium. (b) The effect of curcumin treatment on iAs3+-induced cytotoxicity in Scramble, NRF2-KD, and KEAP1-KD cells. Apoptotic cells were determined by flow cytometry. Annexin V-positive cells were quantified as apoptotic cells. (c) Effect of curcumin treatment on iAs3+-induced apoptosis in NRF2-KD and KEAP1-KD cells. Apoptotic cells were determined by flow cytometry. Annexin V-positive cells were quantified as apoptotic cells. (d) Immuno blotting of cleaved caspase-3, PARP, and cleaved PARP. Vehicle, medium; vehicle + iAs3+, cells exposed to 30 μM of iAs3+ for 20 hr; Cur + iAs3+, cells treated with 5 μM curcumin for 26 hr and exposed to iAs3+ for 20 hr. Whole-cell lysates were used for analysis and β-actin was used as a loading control.
At nontoxic concentrations, curcumin had no effect on NRF1 protein expression; thus, the protective effect of curcumin in these concentrations is mainly NRF2 dependent. NRF1 is an essential gene during development [54], and the 120-kD isoform of NRF1 is glycosylated and located in the ER [55], which mediates antioxidant defense response against arsenic-induced cytotoxicity in human keratinocytes [7]. Our present results reveal that the protein expression of long isoforms of NRF1 is accumulated by high concentrations of curcumin, which is consistent with the distinctive role of NRF1; that is, NRF1 may offer protection in more severe stress conditions or provide additional protection when the antioxidant capacity provided by NRF2 is exhausted. This work provides a proof of concept of using curcumin to activate the NRF2 pathway to alleviate arsenic-induced damage and suggests that its chemopreventive potential requires optimization of dose.

5. Conclusion

Curcumin functions as a chemopreventive compound at low concentrations against arsenic-induced damage, which is mediated through activating the NRF2 cytoprotective pathway.

Abbreviation

Ab: Antibody
AREs: Antioxidant response elements
CASP-3: Caspase-3
Cur: Curcumin
DMEM: Dulbecco's modified Eagle's medium
ECL: Enhanced chemiluminescence
EGCG: Epigallocatechin-3-gallate
FBS: Fetal bovine serum
FITC: Fluorescein-4-isothiocyanate
GCLC: Glutamate cysteine ligase catalytic subunits
GCLM: Glutamate cysteine ligase regulatory subunits
HMOX-1: Heme oxygenase-1
\textit{iAs}^{3+}: Arsenite
KD: Knockdown
KEAP1: Kelch-like ECH-associated protein 1
MTT: 3-(4,5)-Dimethylthiazol-2-yl)-3,5-di-phenyltetrazoliumromide
NQO1: NADPHquione oxidoreductase 1
NRF1: Nuclear factor erythroid 2-related factor 1
NRF2: Nuclear factor erythroid 2-related factor 2
RT: Room temperature
RT-PCR: Reverse transcription-polymerase chain reaction
SCR: Scramble
tBHQ: tert-butylhydroquinone
Veh: Vehicle.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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References


Research Article

Hepatic Gene Expression Profiling in Nrf2 Knockout Mice after Long-Term High-Fat Diet-Induced Obesity

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Introduction. The transcription factor NFE2-related factor 2 (Nrf2) is a central regulator of antioxidant and detoxification gene expression in response to electrophilic or oxidative stress. Nrf2 has recently been shown to cross-talk with metabolic pathways, and its gene deletion protected mice from high-fat-diet-(HFD-) induced obesity and insulin resistance. This study aimed to identify potential Nrf2-regulated genes of metabolic interest by comparing gene expression profiles of livers of wild-type (WT) versus Nrf2 knockout (Nrf2-KO) mice after a long-term HFD. Methods. WT and Nrf2-KO mice were fed an HFD for 180 days; total RNA was prepared from liver and used for microarray analysis and quantitative real-time RT-PCR (qRT-PCR). Results. The microarray analysis identified 601 genes that were differentially expressed between WT and Nrf2-KO mice after long-term HFD. Selected genes, including ones known to be involved in metabolic regulation, were prioritized for verification by qRT-PCR: Cyp7a1 and Fabp5 were significantly overexpressed in Nrf2-KO mice; in contrast, Car, Cyp2b10, Lipocalin 13, Aquaporin 8, Cbr3, Me1, and Nqo1 were significantly underexpressed in Nrf2-KO mice. Conclusion. Transcriptome profiling after HFD-induced obesity confirms that Nrf2 is implicated in liver metabolic gene networks. The specific genes identified here may provide insights into Nrf2-dependent mechanisms of metabolic regulation.

1. Introduction

Obesity, type 2 diabetes, and the metabolic syndrome are multifactorial diseases [1] that are considered an epidemic in westernized societies [2]; by increasing the risk of cardiovascular events, cancer, and other diseases, they have detrimental effects on life expectancy and quality [3, 4]. Although knowledge on the pathophysiology of obesity and diabetes is expanding, the identification of new molecular pathways involved in these disorders is necessary to better understand their pathogenesis and to identify potential drug targets. A transcription factor that has recently been implicated in obesity and metabolic dysregulation is Nrf2 (NFE2-related factor 2), encoded by NFE2L2 (nuclear erythroid factor 2-like 2) [5].

Nrf2 is a transcription factor of the “cap n’collar” family that has a central role in maintaining cellular homeostasis in response to oxidative and electrophilic stress [6–9]. Under basal conditions Nrf2 is localized mainly in the cytoplasm where it binds to the Kelch-like ECH-associating protein (Keap1) and is thereby targeted for ubiquitination and proteasomal degradation. Upon exposure to oxidative and electrophilic stress, Nrf2 escapes Keap1-mediated degradation and accumulates in the nucleus where it binds to cis elements
in the regulatory domains (antioxidant response elements, AREs) of antioxidant and detoxification genes, inducing their expression [10].

Nrf2 has been described to have a protective function against a number of pathologies that are caused or aggravated by oxidative stress such as cancer, pulmonary disease, and neurodegenerative or inflammatory conditions [11, 12]. Recently, a role of Nrf2 in obesity has also been discovered. Using mainly the Nrf2 knockout (Nrf2-KO) mice as a model, it has been shown by our group and by others that deletion of Nfe2l2 protected mice from diet-induced obesity and insulin resistance [13–16]. In these studies, a variety of diet types has been used: high-fat diet with 60 kcal% fat [13, 16], high-fat diet with 41 kcal% fat [14], and high-fat western diet with 39.7 kcal% fat [15]. The exact mechanisms underlying the protective effect of Nrf2 deletion in high-fat diet-induced obesity remain to be elucidated. However, there is evidence that the cross-talk of Nrf2 with other metabolic factors such as peroxisome proliferator-activated receptor gamma (PPARγ) [14] or fibroblast growth factor 21 (FGF21) [13] may, at least partially, explain this phenotype. In a recent study, we described the phenotypic comparison of WT versus Nrf2-KO mice under high-fat diet (HFD, 60 kcal% fat) or a control diet (standard diet, St.D.) for 180 days [13]. Briefly, under St.D. no difference was observed in body weight gain, glucose tolerance, or insulin tolerance between the two genotypes. While under HFD, both genotypes initially gained weight at about the same rate, the Nrf2-KO mice reached a plateau earlier than WT, and after about 90 days on HFD weighed significantly lower than WT (about 15% lower). Already after 30 days on HFD, the Nrf2-KO mice were significantly more glucose tolerant than WT, and after 180 days they were also significantly more insulin sensitive (as evidenced by intraperitoneal (i.p.) glucose tolerance test and i.p. insulin tolerance test) [13].

Gene expression profiling studies in Nrf2-KO mice under metabolic stress have not yet been reported. The present study used microarray analysis to investigate hepatic genes and gene networks that are regulated directly or indirectly by Nrf2 in mice on a long-term (180 days) HFD regimen.

2. Materials and Methods

2.1. Mice. All animal procedures were approved by the institutional review board of the University of Patras Medical School and were in accordance with E.C. Directive 86/609/EEC. C57BL6/J Nrf2+/- mice, originally developed by Professor M. Yamamoto, were obtained from RIKEN BRC (Tsukuba, Japan). Wild type (WT) and Nrf2-KO mice were generated by mating Nrf2+/- male and female mice; the offspring were genotyped as previously described [17]. Male WT and Nrf2-KO mice (9-10 weeks old) were fed ad libitum an St.D. (10 kcal% fat) or an HFD (60 kcal% fat, Research Diets, New Brunswick, NJ) for 180 days (n = 8 for each group). Mice were housed in the animal facility of the University of Patras Medical School in temperature-, light-, and humidity-controlled rooms with a 12 h light/dark cycle.

2.2. Liver RNA Isolation. Liver was excised from mice and was submerged immediately in RNA later solution (Ambion, Foster City, CA). Total RNA was isolated from liver samples from WT or Nrf2-KO mice using the TRIzol reagent (Life Technologies, Carlsbad, CA), following the manufacturer’s instructions, and further purified using the RNeasy mini-kit (Qiagen, Hilden, Germany). RNA yield and quality were determined with a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Montchanin, DE) and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). For microarray analysis, pooled RNA from either WT or Nrf2-KO mice was used. For qRT-PCR purposes, RNA from individual liver samples was used to generate cDNA.

2.3. Microarray Experimentation and Analysis. The microarray experiments were performed using total pooled RNA from liver samples of 8 WT or 8 Nrf2-KO mice fed an HFD for 180 days. Four technical replicates were used for each genotype. Microarray experiments were carried out as one-color hybridizations on murine 4plex arrays from Agilent. The Agilent Whole Mouse Genome Microarray 4x44K slides were used (8 slides in total), each slide including 39430 probes. The labelling reaction of total RNA was performed using the Low Input Quick Amp Labelling Kit (Agilent) using 100 ng of total RNA as starting material, according to the manufacturer’s instructions. cRNA synthesis was regarded successful provided that ≥1.65 μg of cRNA with a Cy3-incorporation rate ≥8.0 pmol/μg cRNA were synthesized. Fragmentation and hybridization of cRNA was performed as follows: 1.65 μg of Cy3-labelled cRNA were fragmented according to the manufacturer’s instructions, and 1.425 μg of fragmented cRNA were hybridized. The hybridization was performed at 65°C for 17 h in an Agilent hybridization oven. Agilent arrays were then washed, scanned, and processed according to the supplier’s protocol. After scanning at 5 μm resolution with a DNA microarray laser scanner (Agilent), features were extracted with image analysis tool version A.8.3.1 using default protocols and settings (Agilent). Primary data analysis was performed using Agilent’s Feature Extraction Software (version 10.7.3.1). ATLAS Biolabs (Berlin, Germany) performed labelling and hybridization of samples as well as generation of the primary data.

The raw microarray data were initially background corrected, normalized using quantile normalization, and further log2 transformed. Significantly up- or downregulated genes were identified using Significance Analysis of Microarrays (SAM) in the software platform MeV 4.8 (TM4 Microarray Software Suite) [18, 19]. SAM assigns a score to each gene on the basis of a change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than an adjustable threshold, SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance (the false discovery rate, FDR). Analysis parameters (Delta) were set to result in FDR ≤ 1% (a stringent criterion).

2.4. GEO Accession Numbers. Microarray data discussed in this publication are MIAME compliant and have been
2.6. Ingenuity Pathway Analysis. Differentially expressed genes for a category was set at 2. Gene expression omnibus deposits in NCBI gene expression omnibus with the following accession number: GSE33575 (GSM830131 through GSM830138).

2.5. Gene Ontology (GO) and Enrichment Analysis. Gene ontology (GO) analysis is helpful for the deduction of conclusions from microarray data. GO is a database with curated annotations for known genes, that is, gene biological processes, molecular functions, and cellular components. GO analysis was performed, using the Genesis 1.7.2 software and the Webgestalt toolkit (http://bioinfo.vanderbilt.edu/webgestalt/), as previously reported [20, 21]. The hypergeometric test with Bonferroni correction was used for enrichment evaluation analysis. The R function adjP was used in order to adjust the nominal P values of the large number of categories at the same time. The significance level for the adjusted P value was set at 0.01, and the minimum number of genes for a category was set at 2.

2.6. Ingenuity Pathway Analysis. Differentially expressed genes (DEGs) were investigated for network interrelation by ingenuity pathway analysis (IPA) software (Ingenuity Systems, Redwood City, CA). IPA scans the set of input genes to identify networks by using the ingenuity pathway knowledge base for interactions between identified "focus genes." In this study, the liver DEGs between WT and Nrf2-KO mice and hypothetical interacting genes (stored in the knowledge base in IPA software) were used to generate a set of networks with a maximum network size of 35 genes/proteins. Networks are displayed graphically as genes/gene products (nodes) and the biological relationships between the nodes (edges). All edges are from canonical information stored in the ingenuity pathways knowledge base. In addition, IPA computes a score for each network according to the fit of the user's set of significant genes. The score indicates the likelihood that the focus genes in a network from ingenuity's knowledge base are found together due to random chance. A score of 3, as the cutoff for identifying gene networks, indicates that there is only a 1/1000 chance that the focus genes shown in a network are due to random chance; therefore, a score ≥3 indicates a 99.9% confidence level.

2.7. Quantitative Real-Time PCR. Total RNA from individual liver samples was used for cDNA synthesis after a DNase digestion step (Turbo DNase, Life Technologies) so as to prevent genomic DNA contamination. cDNA was synthesized using the superscript first-strand synthesis system (Life Technologies), and quantitative real-time PCRs were performed in triplicate 20 μL reaction volumes on a StepOne Plus Instrument (Applied Biosystems, Foster City, CA) using Fast SYBR Green Master Mix (Applied Biosystems). Relative mRNA levels were calculated by the comparative threshold cycle method using TBP (TATA-binding protein) as the housekeeping gene. PCR efficiency was determined from a standard curve, and the Pfaffl method was used to calculate fold changes [22]. The correct size of the PCR products was confirmed by electrophoresis on a 2.5% agarose gel stained with ethidium bromide. Purity of the amplified products was assessed by melting curve analysis using the StepOne Software version 2.1 (Applied Biosystems). The primers used for Cyp7a1 (cytochrome P450, family 7, subfamily A, polypeptide 1), Fadb5 (fatty acid binding protein 5), Car (constitutive androstane receptor), Cyp2b10 (cytochrome P450, family 2, subfamily B, polypeptide 10), Lipocalin 13, Aquaporin 8, Chr3 (carbonyl reductase 3), Mel (malic enzyme 1), and Nqo1 (NADPH dehydrogenase quinone 1) were obtained from the PrimerBank (Center for Computational and Integrative Biology, Harvard Medical School, Massachusetts, USA) [23–25]. All primer sequences are shown in Table S1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2013/340731).

2.8. Statistical Analysis. In microarrays, normality of the data distribution was checked by the Kolmogorov-Smirnov test. Differences in gene expression levels between WT and Nrf2-KO mice in liver were explored using SAM and the t-test. Numerical values were expressed as the mean ± standard deviation (SD). Statistical significance was set at the 95% confidence level (P < 0.05), and the fold change cutoff was set at 2. For statistical analysis of qRT-PCR data, one-way ANOVA followed by Tukey’s test was used; qRT-PCR data were expressed as the mean ± SD. The number of biological or technical replicates used is described in the corresponding results. Statistical significance was set at the 95% confidence level (P < 0.05). The statistical package GraphPad Prism was used for calculations (GraphPad Software, La Jolla, CA).

3. Results

3.1. Differentially Expressed Liver Genes between Nrf2-KO and WT Mice Fed an HFD for 180 Days. SAM analysis, based on strict statistical criteria (fold change >2; median FDR < 0.01; 90th percentile FDR = 0.32), identified 601 liver differentially expressed genes (DEGs) between Nrf2-KO and WT mice after 180 days on HFD. Of these genes, 428 were significantly overexpressed (Table S1) and 173 were significantly underexpressed (Table S3) in Nrf2-KO versus WT mice. The 601 DEGs were clustered using a two-dimensional hierarchical clustering with Euclidean distance. Figure S1 depicts the heatmap of the genes that were over- (Figure S1A) or underexpressed (Figure S1B) in Nrf2-KO versus WT mice.

3.2. Gene Ontology (GO) Analysis of Differentially Expressed Genes. To obtain insights into the functions of the 601 DEGs, gene ontology (GO) analysis was performed. The main processes that these genes are involved in are categorized as follows: (1) immune response; (2) inflammatory response; (3) carbohydrate and pattern binding; (4) G protein and chemokine receptor binding; (5) glutathione transferase activity; (6) peptidase inhibitor activity; (7) cell surface, plasma membrane, and extracellular region genes; and (8) ion homeostasis. Table 1 lists the differentially expressed genes implicated in each of the aforementioned functions.
Table 1: Differentially expressed genes implicated in the enriched gene ontology processes.

<table>
<thead>
<tr>
<th>Biological process—defense response—GO:0006952</th>
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<td>Nupr1</td>
<td>Nuclear protein 1</td>
</tr>
<tr>
<td>Aoah</td>
<td>Acyloxyacyl hydrolase</td>
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<tr>
<td>Cd28</td>
<td>CD28 antigen</td>
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<tr>
<td>C8b</td>
<td>Complement component 8, beta polypeptide</td>
</tr>
<tr>
<td>Lat</td>
<td>Linker for activation of T cells</td>
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<tr>
<td>Calca</td>
<td>Calcitonin/calcitonin-related polypeptide, alpha</td>
</tr>
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<td>Prg2</td>
<td>Proteoglycan 2, bone marrow</td>
</tr>
<tr>
<td>Orm3</td>
<td>Orosomucoid 3</td>
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<td>Clec2h</td>
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<tr>
<td>Camp</td>
<td>Cathelicidin antimicrobial peptide</td>
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<td>Wdcl2</td>
<td>WAP four-disulfide core domain 12</td>
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<td>Chi3l3</td>
<td>Chitinase 3-like 3</td>
</tr>
<tr>
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<td>CD24a antigen</td>
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<td>WAP four-disulfide core domain 15B</td>
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<tr>
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<td>Preproenkephalin</td>
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<td>Hepcidin antimicrobial peptide</td>
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<td>Hamp2</td>
<td>Hepcidin antimicrobial peptide 2</td>
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<tr>
<td>Cxcl9</td>
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<td>Adora1</td>
<td>Adenosine A1 receptor</td>
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<td>Myeloperoxidase</td>
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<td>Cxcl9</td>
<td>Chemokine (C-X-C motif) ligand 9</td>
</tr>
<tr>
<td>Serpina3g</td>
<td>Serine (or cysteine) peptidase inhibitor, clade A, member 3G</td>
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<td>Cd8b1</td>
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</tr>
<tr>
<td>Thy1</td>
<td>Thymus cell antigen 1, theta</td>
</tr>
<tr>
<td>Ltb</td>
<td>Lymphotoxin B</td>
</tr>
<tr>
<td>Eomes</td>
<td>Eomesodermin homolog (Xenopus laevis)</td>
</tr>
<tr>
<td>Cd28</td>
<td>CD28 antigen</td>
</tr>
<tr>
<td>Igj</td>
<td>Immunoglobulin joining chain</td>
</tr>
<tr>
<td>H2-Aa</td>
<td>Histocompatibility 2, class II antigen A, alpha</td>
</tr>
<tr>
<td>Themis</td>
<td>Thymocyte selection associated</td>
</tr>
<tr>
<td>C8b</td>
<td>Complement component 8, beta polypeptide</td>
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<td>Nuclear protein 1</td>
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<td>Linker for activation of T cells</td>
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<tr>
<td>Prg2</td>
<td>Proteoglycan 2, bone marrow</td>
</tr>
<tr>
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<td>CD24a antigen</td>
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<tr>
<td>Btl</td>
<td>B and T lymphocyte associated</td>
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</table>

Table 1: Continued.

<p>| Cfd | Complement factor D (adipsin) |
| Ccl2 | Chemokine (C-C motif) ligand 12 |
| Bcl2a1d | B-cell leukemia/lymphoma 2 related protein A1d |
| Sh2d1a | SH2 domain protein 1A |
| Ccl4 | Chemokine (C-C motif) ligand 4 |
| Mpo | Myeloperoxidase |
| Ccl8 | Chemokine (C-C motif) ligand 8 |
| Cxcl9 | Chemokine (C-X-C motif) ligand 9 |
| Serpina3g | Serine (or cysteine) peptidase inhibitor, clade A, member 3G |
| Cd3d | CD3 antigen, delta polypeptide |</p>
<table>
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<th>Biological process—inflammatory response—GO:0006954</th>
<th>Biological process—response to wounding—GO:0009611</th>
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<tr>
<td>adj $P = 0.0010$ Nupr1 Nuclear protein 1 Aoah Acyloxyacyl hydrolase Cd28 CD28 antigen C8b Complement component 8, beta polypeptide Lat Linker for activation of T cells Calca Calcitonin/calcitonin-related polypeptide, alpha Orm3 Orosomucoid 3 Chi3l3 Chitinase 3-like 3 Ccl5 Chemokine (C-C motif) ligand 5 Cd24a CD24a antigen Cfd Complement factor D (adipsin) Cc12 Chemokine (C-C motif) ligand 12 Ccl4 Chemokine (C-C motif) ligand 4 Ccl8 Chemokine (C-C motif) ligand 8 Cxcl9 Chemokine (C-X-C motif) ligand 9</td>
<td>adj $P = 0.0054$ Slc1a3 Solute carrier family 1 (glial high affinity glutamate transporter), member 3 Nupr1 Nuclear protein 1 Aoah Acyloxyacyl hydrolase Cd28 CD28 antigen C8b Complement component 8, beta polypeptide Lat Linker for activation of T cells Calca Calcitonin/calcitonin-related polypeptide, alpha Tff1 Trefoil factor 1 Orm3 Orosomucoid 3 Chi3l3 Chitinase 3-like 3 Cd5 Chemokine (C-C motif) ligand 5 Cd24a CD24a antigen Cfd Complement factor D (adipsin) Cc12 Chemokine (C-C motif) ligand 12 Ccl4 Chemokine (C-C motif) ligand 4 Ccl8 Chemokine (C-C motif) ligand 8 Cxcl9 Chemokine (C-X-C motif) ligand 9 Adora1 Adenosine A1 receptor</td>
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<tr>
<td>Biological process—cellular di-, tri-valent inorganic cation homeostasis—GO:0030005</td>
<td>Biological process—di-, tri-valent inorganic cation homeostasis—GO:0055066</td>
</tr>
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<td>adj $P = 0.0067$ Lck Lymphocyte protein tyrosine kinase Egr1 Early growth response 1 Cd5 CD5 antigen Btl B and T lymphocyte associated Cd24a CD24a antigen Bcl2 B-cell leukemia/lymphoma 2 related protein Ail</td>
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**Cellular component—cell surface—GO:0009986**

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<td>CD8 antigen, alpha chain</td>
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**Cellular component—external side of plasma membrane—GO:0009897**

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**Cellular component—plasma membrane part—GO:0044459**

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**Cellular component—plasma membrane part—GO:0044459**

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**Cellular component—plasma membrane part—GO:0044459**

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Cellular component—extracellular region part—GO:0044421

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Cellular component—extracellular region part—GO:0044421

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Cellular component—extracellular region part—GO:0044421

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<td>Prominin 1</td>
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<tr>
<td>C8b</td>
<td>Complement component 8, beta polypeptide</td>
</tr>
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<td>Lat</td>
<td>Linker for activation of T cells</td>
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<td>Il17rb</td>
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3.3. qRT-PCR Verification of Microarray Results. For validation of the microarray data, a group of genes known to be directly or indirectly implicated in lipid or carbohydrate metabolism were selected for quantification with quantitative real-time RT-PCR (qRT-PCR). These genes were Cyp7a1 (cytochrome P450, family 7, subfamily A, polypeptide 1), which is the rate limiting enzyme in bile acid synthesis from cholesterol; Fabp5 (fatty acid binding protein 5); Car (Nr1I3) (constitutive androstane receptor); Cyp2b10 (cytochrome P450, family 2, subfamily B, polypeptide 10), which is a Car target; Lipocalin 13; Aquaporin 8; Chr3 (carboxyl reductase 3); and Mel (malic enzyme 1). Nqo1 (NAD(P)H dehydrogenase, quinone 1) was selected as a prototypical Nrf2 target gene. These genes were quantified not only in the liver of WT and Nrf2-KO mice under HFD for 180 days, but also in the liver of WT and Nrf2-KO mice under standard diet (St.D.) for the same time period. The relative gene expression levels are depicted in Figure 1. Cyp7a1 and Fabp5 were found to be overexpressed in the livers of Nrf2-KO mice after HFD feeding compared to WT mice, while the rest of the genes were underexpressed. There was excellent agreement between the microarray and the qRT-PCR data (Pearson correlation coefficient = 0.919; P value < 0.001) (Figure 2).

3.4. Canonical Pathways and Networks Impacted by Nrf2 under HFD Feeding. Ingenuity pathway analysis (IPA) was used to rank gene networks by order of consistency of the microarray results with relationships confirmed by previously published results. Figure S2 presents a network which comprises Nrf2 and was generated by the microarray data. Nrf2 and genes that are under-expressed in the Nrf2-KO mice are shown in green; genes that are over-expressed in the Nrf2-KO mice are shown in red. It is obvious that all of the under-expressed genes in this network have been described to be directly regulated by Nrf2: carboxylesterase 1g (Ces1g) [26]; glutathione S-transferase mu 5 (Gstm5) [27]; glutathione S-transferase alpha 5 (Gsta5) [28]; and NAD(P)H dehydrogenase quinone 1 (Nqo1) [29]. In contrast, none of the genes that are over-expressed in the Nrf2-KO mice is known to be directly regulated by Nrf2. These genes are calcitonin-related polypeptide beta (Calc b); collagen type V alpha 2 (Col5a2); cytochrome P450 family 2 subfamily C polypeptide 8 (Cyp2c8); growth factor independent 1 (Gfi1); H2-M2 histocompatibility 2, M region, locus 2 (H2-m2); interferon gamma (Ifng); solute carrier family 14 (urea transporter) member 1 (Slc14a1); solute carrier family 26 member 3 (Slc26a3); solute carrier family 9 (sodium hydrogen exchanger) member 3 (Slc9a3); serine peptidase inhibitor, Kazal type 4 (Spink4); sulfotransferase family 1E, estrogen preferring, member 1 (Sult1e1); and trefoil factor 1 (Tff1).

IPA analysis identified the statistically significant canonical pathways in the gene list. A corrected Fischer's exact test P value < 0.05 was used as the threshold of significance (Figure 3). The number of genes (n) that were differentially expressed in each canonical pathway is shown below along with the P value and the ratio. In the "xenobiotic metabolism signaling" pathway (n = 7; P value = 3.95E−06; ratio = 0.027), Cyp2c8 and Sult1e1 were overexpressed, whereas Gstm5, Ces1g, Gsta5, Nqo1, Nf2l2l2 (as expected), and sulfotransferase family cytosolic 2A dehydroepiandrosterone-prefering member 1 (Sult2a1) were under-expressed. In the "aryl hydrocarbon receptor signaling" pathway (n = 6; P value = 5.88E−06; ratio = 0.038), Tjfl and Fas ligand (Faslg) were over-expressed, whereas Gstm5, Gsta5, Nqo1, and Nf2l2l2 were underexpressed. In the "LPS/IL-1 mediated inhibition of RXR function" pathway (n = 5; P value = 5.49E−04; ratio = 0.022), Cyp2c8 and Sult1e1 were over-expressed, whereas Gstm5, Gsta5, and Sult2a1 were underexpressed. In the "metabolism of xenobiotics by cytochrome P450" pathway (n = 4; P value = 5.65E−04; ratio = 0.02), Cyp2c8 was over-expressed and Cyp2b13/Cyp2b9, Gstm5, and Gsta5 were underexpressed. In the "sulfur metabolism" pathway (n = 2; P value = 1.96E−03; ratio = 0.034), Sult1e1 was overexpressed, whereas Sult2a1 was underexpressed.

4. Discussion

The findings of our previous study that male Nrf2-KO mice were at least partially protected from HFD-induced (60 kcal% fat) obesity and were more insulin sensitive and more glucose tolerant compared to their WT counterparts [13] are consistent with previous reports using comparable but different treatment parameters (40 kcal% fat diet or modified high-fat-diets) [14–16]. The purpose of this study was to identify hepatic genes differentially expressed between WT and Nrf2-KO mice after long-term (180 days) high-fat-diet-(HFD-) induced obesity. Such information could provide insights into the recently appreciated implication of Nrf2 in the development of obesity and metabolic syndrome. To this end, microarray-based transcriptome analysis was performed, employing strict statistical criteria.

The microarray-based gene expression analysis in these mice generated a total of 601 genes that were differentially expressed between the two genotypes: 478 genes were overexpressed in the Nrf2-KO mice, and 173 were underexpressed. These genes are not only implicated in functions that are associated with homeostatic and/or physiological processes [11]. Among a total 601 differentially expressed genes (DEGs), a subset was selected for validation by qRT-PCR quantification based on their relevance to metabolic pathways. The metabolic pathways and the respective representative genes chosen were bile acid synthesis from cholesterol (Cyp7a1), free fatty acid binding and transport (Fabp5), glucose metabolism (Lipocalin 13), glycerol transport (Aquaporin 8),
fatty acid biosynthesis (Me1), and energy homeostasis (Car and its target gene Cyp2b10). Nqo1 mRNA levels were quantified as Nqo1 is considered a prototypical Nrf2 target gene.

The qRT-PCR-based mRNA quantification of specific genes of metabolic interest that were either over-expressed (Cyp7a1 and Fabp5) or under-expressed (Car, Cyp2b10, Lipocalin 13, Aquaporin 8, Cbr3, Me1, and Nqo1) in Nrf2-KO mice compared to WT under HFD revealed potential candidate genes that may be implicated in the development of the different metabolic phenotype of Nrf2-KO mice. As shown in Figure 1, the differential expression of some of these genes was also evident under the St.D. regimen (with the exception of Aquaporin 8, Lipocalin 13, and Cbr3), indicating that these genes may be regulated by Nrf2 under basal conditions as well. HFD for 180 days increased the expression of these genes (with the exception of Cyp2b10 in both genotypes and of Nqo1 in the Nrf2-KO mice), and the fold difference between the two genotypes was generally accentuated. This observation may indicate that the possible regulation (direct or indirect) of these genes by Nrf2 becomes more prominent under stress.
partially protect them from obesity [39]. To clarify these molecular mechanisms, future experiments should involve investigations, such as cell culture studies with manipulation of Nrf2 levels/activity, and measurement of Car levels/activity, are necessary to clarify the mechanisms that underlie the possible regulation of Car by Nrf2.

Cyp7a1 is the rate-limiting enzyme in bile acid synthesis from cholesterol. In agreement with previous studies [36], we show that Cyp7a1 mRNA levels increased significantly in both genotypes after HFD feeding (Figure 1). The Cyp7a1 mRNA levels also differed between the two genotypes, with the Nrf2-KO mice showing higher levels under St.D. (about 60% higher) and much higher levels under HFD (about 120% higher) compared to WT. In a previous study, a short-term (30 days) HFD did not accentuate the basal difference between the two genotypes [36], probably because an HFD feeding for a shorter period exposed the animals to lower metabolic and oxidative stress, such that the differences caused by Nrf2 deletion were not as pronounced. Moreover, given that small heterodimer partner (Shp) represses Cyp7a1 expression [37], and Nrf2 induces Shp gene expression [38], a reasonable hypothesis could be that Nrf2 represses Cyp7a1 expression through Shp. This repression of Cyp7a1 expression is abrogated by Nrf2 deletion, leading to increased levels of Cyp7a1 in Nrf2-KO mice compared to WT, which may partially protect them from obesity [39]. To clarify these molecular mechanisms, future experiments should involve Shp and Nrf2 single and double KO mice.

Fabp5 is a member of the fatty acid-binding proteins which binds free fatty acids and regulates lipid metabolism and transport; it was first identified as being upregulated in psoriasis tissue [40]. In this study, Fabp5 was increased after HFD feeding in both genotypes (Figure 1), which is in agreement with previous studies that have shown strong up-regulation of Fabp5 by western-type diet or HFD [41, 42]. Fabp5 also exhibited higher mRNA levels in Nrf2-KO mice under either St.D. or HFD; a study using proteomic analysis showed similar results [43]. The specific physiological significance of Fabp5 elevation in Nrf2-KO mice remains to be elucidated. Further experiments with Nrf2 over-expression or silencing and with concurrent measurement of Nrf2 levels in hepatocytes are warranted to elucidate the possible regulation of Fabp5 by Nrf2.

Car (Nrl13), initially characterized as a sensor of xenobiotics that regulates responses to toxicants [44], has recently been implicated in the control of energy and metabolism [45]. Car has been ascribed a function as an antiobesity receptor, because treatment of mice with a Car agonist partially prevented HFD-induced obesity in mice, and partially reversed obesity in mice that were already obese [46, 47]. In the present study, mRNA levels of Car, along with those of its primary target gene, Cyp2b10 [48], were lower in Nrf2-KO mice compared to WT under either St.D. or HFD (Figure 1). In this case, the lower expression of Car cannot justify the ameliorated metabolic phenotype of Nrf2-KO compared to WT after long-term HFD. Nevertheless, the observation that Car and Cyp2b10 mRNA levels are lower in Nrf2-KO mice than WT is in accordance with previous studies [49, 50]. Car mRNA was found to be increased in both genotypes after the HFD regimen. But Cyp2b10 that is considered a Car target gene does not follow the same trend. This may indicate a difference in the mRNA turnover of Cyp2b10 that may or may not be reflected in the protein levels. Further investigations, such as cell culture studies with manipulation of Nrf2 levels/activity and measurement of Car levels/activity, are necessary to clarify the mechanisms that underlie the possible regulation of Car by Nrf2.

Lipocalin 13 is a lipocalin family member involved in glucose metabolism, and its deficiency is associated with obesity [51]. Herein, lipocalin 13 liver mRNA levels were found to be lower in Nrf2-KO mice than in WT after long-term HFD; no difference was observed between the two genotypes under St.D. (Figure 1). As the existing data on the role of lipocalin 13 in obesity are scarce, this differential lipocalin 13 mRNA expression between the two genotypes after the HFD feeding for 180 days warrants further elucidation.

Aquaporin family members are mainly water channels, but some of them have also been found to transport glycerol and to be involved in the development of obesity [52]. Aquaporin 8 is expressed in liver [53], and in the present experimental model it exhibited lower mRNA expression in the liver of Nrf2-KO mice compared to WT after HFD. No difference was found between the two genotypes under St.D., but aquaporin 8 was markedly induced in both genotypes after HFD with its levels being lower in the Nrf2-KO mice (Figure 1). As aquaporins may be implicated in the transport of glycerol (a product of the catabolism of triacylglycerols), a
possible indirect regulation of aquaporin 8 by Nrf2 may be of metabolic interest. Cbr3 catalyzes the reduction of carbonyl compounds (highly reactive lipid aldehydes) to the corresponding alcohols (inactive compounds) [54]. A recent clinical study [55] showed that a genetic variation in Cbr3 gene in humans correlates with type 2 diabetes and this effect can potentially be attributed to the catalysis of the conversion of prostaglandin E2 to prostaglandin F2α. In the present study, Cbr3 liver mRNA levels were about 5 times lower in Nrf2-KO mice after HFD compared to WT. Although WT mice tended to have greater Cbr3 mRNA levels under standard diet, this difference was not statistically significant. Given that recent studies have shown that the Cbr3 promoter comprises antioxidant response element (ARE) sequences that are recognized by Nrf2 to induce Cbr3 expression [50, 56, 57], it would be interesting to test whether the Nrf2-regulated Cbr3 expression can contribute to the observed phenotype in the Nrf2-KO mice after HFD feeding.

Me1 is an enzyme that generates NADPH for fatty acid biosynthesis. In this study, Me1 showed decreased mRNA levels in Nrf2-KO mice under St.D. or HFD; this is consistent with previous gene expression profiling studies that describe Me1 as a Nrf2-dependent gene [58–60]. It has been previously shown that Nrf2 can redirect glucose and glutamine to anabolic pathways in cancer cells, and Me-1 is implicated in these pathways [60]; however, these results in cancer cells cannot necessarily be safely extrapolated to nontransformed hepatocytes [61].

A limitation of this study is that microarray analysis was performed only in mice under HFD and not also on standard diet (St.D.). Thus, it is not possible to delineate among the 601 DEGs those genes that are differentially expressed irrespective of the treatment versus those that demonstrate diet-induced differential expression, except for the subset of genes that were validated by qRT-PCR, which analyzed gene expression in mice under both St.D. and HFD. Another limitation of this study is that the hepatic gene expression in this whole body knock-out model may be affected indirectly by endocrine factors that are secreted from other tissues (e.g., adipose tissue, muscle) that are also deficient in Nrf2. Therefore, some of the differentially expressed genes we detect in the liver may be affected by extrahepatic factors. The use of a liver-specific knock-out model could resolve this issue.

5. Conclusions

In conclusion, the current study showed that Nrf2 deletion significantly altered the hepatic gene expression profile after long-term HFD, yielding a set of 601 DEGs that can be the focus of further studies on the role of Nrf2 in obesity. The majority of these DEGs are involved in pathways relevant to the defense against oxidative and electrophilic stress, already known to be regulated by Nrf2. However, certain genes such as Cyp7a1, Fabp5, Car, Cbr3, and Me1 have specific metabolic effects and appear to be directly or indirectly regulated by Nrf2; these genes may be implicated in the less obese and more insulin sensitive metabolic phenotype of the Nrf2-KO mice. Novel mechanistic understanding and therapeutic interventions for obesity/metabolic syndrome are needed.
may arise from the elucidation of the cross-talk of Nrf2 with metabolic pathways regulated by these genes.

**Conflict of Interests**

All the authors declare that they have no conflict of interests.

**Authors’ Contribution**

Dionysios V. Chartoumpekis and Panos G. Ziros contributed equally to this work. Dionysios V. Chartoumpekis, Panos G. Ziros, Gerasimos P. Sykiotis, and Ioannis G. Habeos conceived and designed the experiments. Dionysios V. Chartoumpekis, Panos G. Ziros, Ralista P. Iskrenova, and Ioannis G. Habeos performed the experiments. Apostolos Zaravinos, Dionysios V. Chartoumpekis, and Ioannis G. Habeos analyzed data. Apostolos Zaravinos, Agathoklis I. Psyrogiannis, and Venetsana E. Kyriaizopoulou contributed to these experiments with the reagents, materials, and analysis tools. Dionysios V. Chartoumpekis, Apostolos Zaravinos, Agathoklis I. Psyrogiannis, and Ioannis G. Habeos wrote the paper.

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Research Article

NRF2-ome: An Integrated Web Resource to Discover Protein Interaction and Regulatory Networks of NRF2

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NRF2 is the master transcriptional regulator of oxidative and xenobiotic stress responses. NRF2 has important roles in carcinogenesis, inflammation, and neurodegenerative diseases. We developed an online resource, NRF2-ome, to provide an integrated and systems-level database for NRF2. The database contains manually curated and predicted interactions of NRF2 as well as data from external interaction databases. We integrated NRF2 interactome with NRF2 target genes, NRF2 regulating TFs, and miRNAs. We connected NRF2-ome to signaling pathways to allow mapping upstream NRF2 regulatory components that could directly or indirectly influence NRF2 activity totaling 35,967 protein-protein and signaling interactions. The user-friendly website allows researchers without computational background to search, browse, and download the database. The database can be downloaded in SQL, CSV, BioPAX, SBML, PSI-MI, and in a Cytoscape CYS file formats. We illustrated the applicability of the website by suggesting a posttranscriptional negative feedback of NRF2 by MAFG protein and raised the possibility of a connection between NRF2 and the JAK/STAT pathway through STAT1 and STAT3. NRF2-ome can also be used as an evaluation tool to help researchers and drug developers to understand the hidden regulatory mechanisms in the complex network of NRF2.

1. Introduction

NRF2 (NF-E2-related factor 2, NFE2L2) transcription factor is the master controller of oxidative and xenobiotic stress responses [1]. Function of NRF2 influences oxidative stress-related physiologic and pathologic processes such as carcinogenesis, inflammation, and neurodegenerative diseases. We developed an online resource, NRF2-ome, to provide an integrated and systems-level database for NRF2. The database contains manually curated and predicted interactions of NRF2 as well as data from external interaction databases. We integrated NRF2 interactome with NRF2 target genes, NRF2 regulating TFs, and miRNAs. We connected NRF2-ome to signaling pathways to allow mapping upstream NRF2 regulatory components that could directly or indirectly influence NRF2 activity totaling 35,967 protein-protein and signaling interactions. The user-friendly website allows researchers without computational background to search, browse, and download the database. The database can be downloaded in SQL, CSV, BioPAX, SBML, PSI-MI, and in a Cytoscape CYS file formats. We illustrated the applicability of the website by suggesting a posttranscriptional negative feedback of NRF2 by MAFG protein and raised the possibility of a connection between NRF2 and the JAK/STAT pathway through STAT1 and STAT3. NRF2-ome can also be used as an evaluation tool to help researchers and drug developers to understand the hidden regulatory mechanisms in the complex network of NRF2.

While there are nearly 3000 articles for the keyword “NRF2” in PubMed (as of January 2013), major network resources (BioGRID, MINT, STRING, HPRD and InnateDB) contain only a few dozen NRF2 interactors. Prompted by the lack of a systems-level NRF2-related resource, we recently collected the literature information on NRF2 interacting proteins and regulated genes, as well as predicted novel NRF2 interactors and regulators [5]. We also imported NRF2 regulating transcription factors (TFs) and miRNAs from major network resources. The NRF2 interactome and regulome datasets allowed us to examine fine-tuning autoregulatory loops of NRF2 and to identify multifunctional proteins interacting with NRF2 [5]. However, the former dataset [5] is stored in more than 16 separate datasheet tables, containing minimal information on the proteins (only the short name of a protein with one UniProt ID) and no links to web resources or other datasheets. This format makes further analysis difficult for several reasons. (1) Search in the datasheets requires the canonical short name of a protein though each protein has
several synonyms. (2) The separate datasheets neither allow any global search for a given protein, nor provide a uniform data collection about an NRF2 interactor. (3) Only experts with computational background could visualize and perform network analysis with the datasets. Importantly, the formerly published datasheets [5] contain mostly NRF2-centric (i.e., star-like) interactions and regulatory information and lack important cross-regulatory connections between NRF2 interactors and other TFs or signaling proteins that are indirectly connected to NRF2.

Here, we report the development of a user-friendly web resource to analyze systems-level data on NRF2, which we hope will provide a help for researchers working with NRF2 or NRF2-related processes. Going beyond the creation of a database based on the datasheets on NRF2 interactome and regulome [5], we extended this database with further protein-protein and regulatory information. We also included signaling pathway data from SignaLink 2, a signaling network resource we previously developed [6, 7]. This process extended the number of protein-protein interactions from the previously published [5] 311 to 13,053 and the regulatory connections from 8,833 to 22,095. Finally, we created a novel website (http://nrf2.elte.hu/) to provide an easy-to-use graphical interface allowing users to browse or download the NRF2-ome resource.

2. Materials and Methods

2.1. Compilation of NRF2-ome Resource. The NRF2-ome contains a manually curated core interactome, which was extended with further protein-protein interactions (PPIs) as well as with transcriptional and posttranscriptional regulatory components and interactions. In the following, we list the compilation process of the NRF2-ome resource (Figure 1).

The starting point of NRF2-ome was a set of 112 interactions between 84 NRF2 interactor proteins that we previously developed by manual curation of the literature [5]. All these interactions were found in human cells and further information about the interactions (e.g., direction, and the literature reference) were already listed. To extend this information, we used experimentally verified interactions from PPI databases and in silico predictions to create the network of first neighbors of NRF2. We used the following PPI databases: InnateDB [8], HPRD [9], and BioGRID [10]. InnateDB and HPRD contain mostly curated interaction data from small- and medium-scale studies, while BioGRID also contains high-throughput studies. For additional enrichment of the NRF2-ome resource, we predicted novel interactions based on protein structure data. We used domain composition data from PFAM and domain-domain interactions data from DOMINE [11, 12] to predict undirected PPIs based on domain-domain interactions. To predict directed PPIs, we used domain-motif-interactions, retrieved from the ELM Server [13]. We used only those domain-motif-based PPIs for which ELM’s Structure Filter cut-off value was above the default.

Next, we integrated regulatory information for NRF2 and for the already included first neighbors of NRF2. To identify TFs that regulate NRF2 or its first neighbors, we imported TF-target gene interactions from the following databases: ABS, ENCODE, HTRIdb, JASPAR, and ORegAnno [14–18]. To list the target genes of NRF2, we used the previously curated data of the literature [5], as well as integrated data from InnateDB [8], two ChIP-Seq profiling studies [19, 20], and JASPAR-based predictions [8]. If an interaction was found in multiple resources or methods, then we listed all versions, offering a comprehensive view. We also integrated posttranscriptional regulatory interactions (i.e., miRNAs that can regulate NRF2 or its first neighbors) by manual curation and from miRBase, TarBase, Miranda, TargetScan, and miRecords resources [21–25]. To list those TFs that can regulate NRF2-regulating miRNAs, we used data on the transcriptional regulation of miRNAs from ENCODE, PutMir, and ‘TransMir resources [18, 26, 27].

Finally, we used SignaLink 2, a signaling network resource we recently developed [6, 7], to connect all of the already included proteins to signaling pathways. SignaLink 2 contains 7 major signaling pathways: RTK (receptor tyrosine kinase), TGF-β (transforming growth factor beta), WNT/Wingless, Hedgehog, JAK/STAT, Notch, and NHR (nuclear hormone receptor) [6]. Integration of signaling pathways to NRF2-ome allows the mapping upstream components of NRF2 and other TFs, which regulate NRF2 or its first neighbors. As a final step, we used BioGRID [10] and HPRD [9] resources again to include further PPIs known between all the inserted components (e.g., PPIs between TFs, or between signaling pathway components and NRF2 interactors).

2.2. Database Implementation and Structure. NRF2-ome stores data in a MySQL database, which is connected to the webpage by an interface written in PHP. On the client side, the webpage uses jQuery to offer a high interactivity. It loads data asynchronously by small http requests, making possible to efficiently browse through hundreds of interactions. Data can be exported and downloaded in various formats: CSV, BioPAX, PSI-MI TAB, PSI-MI XML, SBML, and Cytoscape’s CYS format. The download page offers several options to customize the network to be the downloaded; user can select desired interaction types (e.g., PPIs and transcriptional regulation) and filter the interactions by sources. There is an option to separate experimentally verified and predicted interactions. The customized network files are generated after the selection, by an export module running in the background, implemented in Python. This process can take few minutes. Then, for each download, the database generates a URL, where users can access the data for 14 days. Optionally, users can provide their e-mail addresses to which files smaller than 10 MB will be e-mailed. The whole dataset is also available as a standard SQL dump, so that any complex query or modification can be applied using SQL statements.

The core of NRF2-ome database is the interaction table. In the interaction table source and target fields are integers pointing to the primary keys of protein or mirna tables. The layer field denotes the type of the interaction, and its value determines whether the source or the target refer to a protein or miRNA. The meanings of the values in the layer
The NRF2-ome database contains interactors and regulators of NRF2, their interactions to NRF2, and physical as well as regulatory interactions between them. Altogether, the NRF2-ome database contains 7,777 proteins and 35,967 interactions (Figure 3). From the 7,777 proteins, 227 are directly interacting with NRF2, 45 are TFs directly regulating NRF2, while 165 TFs are regulating miRNAs capable to downregulate NRF2. 7,252 proteins in the NRF2-ome database are encoded by genes regulated by NRF2. Interestingly, there are only 108 proteins that are both interactors and target genes of NRF2. We also integrated signaling pathway data and found that from the 7,777 proteins, 591 are involved in signaling. As a more
indirect connection, we found 51 signaling pathway proteins interacting with NRF2, 163 pathway proteins binding to NRF2 interactors and 8 pathway proteins affecting TFs regulating NRF2. We note that the NRF2-ome database also contains pathway connections through second neighbor interactors.

There are four types of interactions in the NRF2-ome database: (1) protein-protein interactions; (2) TF-target gene regulatory connections; (3) miRNA-mRNA regulatory interactions; (4) TF-miRNA regulatory connections. Figure 3(b) shows the number of interactions by types in the NRF2-ome database. For all four categories, NRF2-ome contains both experimentally verified and predicted connections. As NRF2-ome distinguishes between these two evidence types and also interactions are stored with their original sources, users can examine interactions separately according to their requirements.

3.2. The NRF2-ome Website. We developed a user-friendly web interface for NRF2-ome that can be accessed at http://nrf2.elte.hu/. The aim of the website is twofold. (1) It gives an interactive opportunity to browse the NRF2-ome database and use hyperlinks to other web resources and Pubmed abstracts. (2) It provides an easy-to-use download interface, where users without computational background can select data from the content of the NRF2-ome database. The search field available on the main page autocompletes the queried terms to facilitate users' search. The search engine of NRF2-ome understands many database IDs, accession numbers, and protein names based on the mapping table of UniProt [28]. If the search term is ambiguous, users can manually select the needed item from a list.

If the search is successful, a datasheet of the selected protein or miRNA will be shown (Figures 4(a)–4(d)). The header of the protein datasheet shows the full name, gene name, Ensembl protein ID, and UniProtKB AC of the protein. Below the header, an interactive interaction list is shown to present the first neighbor interactors of the protein. All details of an interaction can be examined here, including the interaction properties (direct/indirect, directed/undirected, stimulatory/inhibitory, and predicted/experimentally verified) and the Pubmed links to the external source from where we integrated the given interaction, and Pubmed links to the original paper used as a reference. At the bottom of the page, we present a "Pathway connections" section. Here, users can examine connections of the given protein and members of a signaling pathway interactively selected by the user. We distinguish between upstream and downstream pathway interactors (i.e., interactors affecting the given protein or interactors affected by the given protein, resp.).

On the right side of the protein datasheet page, an interactive network image is presented, using the Cytoscape Web embedded flash application [29]. Clicking on the nodes or edges brings the user to the datasheet page of the selected protein, miRNA, or interaction. The network view can be enlarged to full screen mode. By default, only manually curated and integrated PPIs are shown. Further interactions can be turned on by the user; however, in case of too many nodes and edges, the limitations of the flash application may make the visual experience uncomfortable. In this case, the webpage gives a warning message before visualizing the network and recommends the possibility to download a custom network using the download option of the webpage and visualize the network offline.

In case of the NRF2 protein, the website provides a different view because of the high number of interactions (Figure 4(e)). At the datasheet page of NRF2, the interaction types and the network image are shown separately, and the interaction lists are loaded only in small portions to optimize
the interactive visual experience. Only in the datasset page of NRF2 we list regulatory loops. Regulatory loops contain two or three nodes that form positive or negative feedbacks to regulate NRF2. The shorter type of these loops consists a protein interacting with NRF2, or a TF of NRF2, which is regulated reciprocally by NRF2 at the transcriptional level. NRF2-ome contains 24 and 12 such proteins and TFs, respectively. A more loose type of regulatory loops involves miRNAs that repress the translation of NRF2 and a TF regulating the transcription of that miRNA and regulated by NRF2. In NRF2-ome there are 385 such regulatory loops, involving 61 miRNAs and 82 TFs.

The download option is accessible under the "download" menu of the webpage. The user interface offers an easy way to select the desired parts of the database. The entire database is available as a MySQL dump file. Alternatively, for less-experienced users, we developed a BioMART-like customizable download page, where users can easily select interaction types and file format of the download. A general switch is also available to exclude all predicted interactions. The customized subnetworks can be downloaded in various formats: CSV, BioPAX, SBML, PSI-MI tab or PSI-MI XML, and in a Cytoscape CYS file.

3.3. Applications of the NRF2-ome Website. We present three examples to illustrate the applicability of the NRF2-ome website. We show how regulatory loops and pathway connections can point out important novel information about NRF2. Both approaches require the integrated transcriptional and signaling data present in NRF2-ome.

To illustrate the functionality of NRF2 regulatory loops, we investigated the list of miRNA-containing regulatory loops. These loops are formed by NRF2, a target gene of NRF2 that functions as a TF, which regulates a miRNA capable to downregulate the translation of NRF2. First, we selected only those loops, where the effect of the miRNA was experimentally verified. By this, we narrowed the list of miRNAs from 63 to four miRNAs (miR-27a, miR-28, miR-93, and miR-144). These four miRNAs form 20 identical loops with 14 different TFs. We selected the MAFG transcription factor as an example because it was predicted to regulate the expression of two from the four miRNAs (miR-93, and miR-144). As NRF2 has been found be induced by viral infection [32], we suppressed or merged in the Uniprot resource. Thus, there is a slight decrease in the number of target genes in NRF2-ome, but this decrease is coupled with more reliable IDs.

As we integrated signaling pathway data to NRF2-ome, users can examine the pathway annotations and signaling connections of known and predicted NRF2 interactors or regulators. For this analysis, we selected the JAK/STAT pathway as it has high functional overlap with NRF2. To predict and explain pathway connection between the JAK/STAT pathway and NRF2, we selected and examined two JAK/STAT pathway members: STAT1, a predicted TF of NRF2, and STAT3, a potential interactor of NRF2.

STAT1 is activated in response to interferons (IFN alpha, beta, and gamma) upon viral infection and inflammation [32]. As NRF2 has been found be induced by viral infection and IFN gamma [33, 34], and NRF2 deficient (−/−) mice

![Figure 3: Number of components and interactors in the NRF2-ome database.](image)
Figure 4: Combined snapshots of the NRF2-ome web resource. (a)-(d) The protein datasheet page of KEAP1. (a) The header contains the full name, gene name, UniProt ID, and Ensembl ENSP ID of KEAP1. (b) The interaction list of KEAP1 shows the interactions by types and also the “Pathway connections” section, which shows one- or two-step-long connections to signaling pathway member proteins. Detailed information on the interactions can be accessed by clicking on the triangles appearing below each interaction. (c) The slide-down box contains sources, references, and confidence scores of the interactions. (d) The protein datasheet page also contains an interactive visualization of the network of KEAP1. (e) NRF2 has a special datasheet page at the website, which makes interaction browsing easier for the users. On the bottom of NRF2 datasheet, page users can choose the interaction types to be shown as well as the regulatory loops involving NRF2. Here, all arrows symbolizing interactions are clickable, to get the same information about the interaction as on the protein datasheet. We show the details of the regulatory loop involving MAFG and mir-144 as an example.
had more severe injuries upon viral exposure [35], we may hypothesize that STAT1 as a TF can be an important regulator in the antiviral and anti-inflammatory role of NRF2. Further experimental validation should determine the role of STAT1 in the expression of NRF2 upon viral infection.

Another member of the JAK/STAT pathway, STAT3 was predicted to interact with NRF2 based on domain-motif interactions. Though we found no publication that could validate STAT3-NRF2 interaction, we found several pieces of indirect, functional evidence, which support this interaction. Both STAT3 and NRF2 have anti-inflammatory and anti-apoptotic functions upon ethanol exposure [36, 37]. In liver cells, ethanol-induced inflammation and apoptosis are repressed by globular adiponectin that increases the production of heme-oxygenase 1 (HO-1) through NRF2 and the IL-10/STAT3 pathway [38, 39]. As HO-1 can also be induced in an NRF2-independent way [38], future experiments will be needed to clarify the role of IL-10 and STAT3 in NRF2-dependent HO-1 induction. In addition, experimental approaches could validate that IL-10 indeed influences the activation of NRF2. It is intriguing to speculate that STAT3 and NRF2 may cooperate to serve as anti-inflammatory and anti-apoptotic TFs upon ethanol exposure.

3.4. Discussion. In this work, we significantly extended our previously developed NRF2 interactome and regulome dataset [5] and developed it to an easy-to-use systems-level resource. The current database provides 214 manually curated and predicted NRF2 interactors as well as includes interactors and regulators from several external resources covering nearly four times more information than its preliminary version published earlier [5]. The integrated and unified database structure allows the users to search data from different sources in a single resource. Thus, it does not require prior bioinformatics knowledge on the prediction tools and on the structures of the different databases. NRF2-ome also serves as an integrated resource, where protein interaction and regulatory and signaling data can be analyzed simultaneously. The included manual curation was performed in early 2012, and it will be updated regularly (in each 2 to 3 years). In addition, as data in the integrated external resources will probably be extended in the future, we designed the database building scripts that the database can be easily updated from the same resources. We plan to update data from external resources every year.

The NRF2-ome website that we developed to serve as a graphical interface to the NRF2-ome database (http://nrf2.elte.hu/) provides a user-friendly environment to interactively search, browse, or download the database. The UniProt-based, automatic mapping function of the search module allows users to search with different names or database IDs of a protein. On the website, network data on proteins directly or indirectly related to NRF2 can be easily accessed. Hyperlinks to further resources and literature references facilitate the interactive and fast exploration of protein functions. As a unique feature, we show signaling pathway connections to the proteins, including NRF2, other TFs, or first neighbors of NRF2. This option allows mapping upstream components that could directly or indirectly influence NRF2 activity. For NRF2, a specific website function is available, where users can browse regulatory loops containing possible feedback mechanisms that could up- or downregulate the expression of NRF2. After or instead of browsing the website, users can download the NRF2-ome database as a whole or a user-specified part of it. We provide a user-friendly download page allowing researchers without computational background to select and filter the NRF2-ome database. As the download is possible in CSV and Cytoscape formats, users without advanced bioinformatics knowledge can instantly examine and visualize the data of their interest.

As with most bioinformatics resources, the NRF2-ome has several limitations. Many of the predicted regulatory loops may not be functional or may function only under very specific circumstances. Therefore, users should keep in mind that experimental validation is needed to confirm the function of a predicted feedback loop. Due to the high complexity and centrality of NRF2 regulation, it is also possible that in most cases the effect of one miRNA is negligibly low to the overall expression of NRF2. As we have limited information about the effect of most of the transcriptional events (i.e., whether the TF has a stimulatory or inhibitory effect), in most of the cases we cannot distinguish between positive and negative feedbacks.

Keeping in mind the limitations listed before, the NRF2-ome is capable to provide both simple predictions (i.e., list predicted PPIs or regulatory interactions), as well as complex predictions involving TFs, miRNAs, or signaling pathways. To illustrate the applicability of NRF2-ome, we presented examples about MAFG protein containing regulatory loops and raised the possibility of a connection between NRF2 and the JAK/STAT pathway through STAT1 or STAT3. All three examples are predictions; thus, further experimental validation is needed to confirm them. The NRF2-ome can also be used as an evaluation tool to help researchers explain a given expression pattern. A combination of the NRF2-ome network and user-made expression datasets with the Cytoscape application could help to uncover hidden regulatory mechanisms.

NRF2-ome could also be used as a resource for network pharmacology. The medical importance of NRF2 is coming from its involvement in oxidative stress, inflammation, and many age-related diseases, including cancer, neurodegenerative diseases, and diabetes [40, 41]. The NRF2 activators are potential therapies for oxidative stress, inflammation, and chemoprevention [40]. Activation of NRF2 in healthy cells could delay or prevent the onset of some forms of human cancers [41], but its constitutive activation is responsible for acquired chemo-resistance in tumor cells [42, 43]. Therefore, investigating the complex interaction and regulatory and signaling network that influence the activation of NRF2 could facilitate novel pharmacological attempts. The NRF2-related regulatory loops and pathway connections listed in NRF2-ome could help the evaluation of drug development failures and guide developers to target proteins with clear effect on NRF2. We hope that NRF2-ome will serve as resource for such attempts and help researchers to identify drug targets that can specifically modify the activity of NRF2.
4. Conclusions

We have compiled a network resource, which contains a total of 7,777 manually curated, integrated, and predicted interaction data of NRF2, its first neighbor interactors, its target genes, regulating TFs, and miRNAs, as well as signaling pathways regulating NRF2. The user-friendly website (http://nrf2.elte.hu/) allows researchers without computational background to search, browse, and download the database. NRF2-ome contains integrated information on regulatory loops and pathway connections of NRF2 and its interactors. NRF2-ome is able to provide interesting predictions to be tested experimentally as well as to help researchers to evaluate experimental data or drug treatment outcomes.

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References


Review Article

Nrf2 and Cardiovascular Defense

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The cardiovascular system is susceptible to a group of diseases that are responsible for a larger proportion of morbidity and mortality than any other disease. Many cardiovascular diseases are associated with a failure of defenses against oxidative stress-induced cellular damage and/or death, leading to organ dysfunction. The pleiotropic transcription factor, nuclear factor-erythroid (NF-E) 2-related factor 2 (Nrf2), regulates the expression of antioxidant enzymes and proteins through the antioxidant response element. Nrf2 is an important component in antioxidant defenses in cardiovascular diseases such as atherosclerosis, hypertension, and heart failure. Nrf2 is also involved in protection against oxidant stress during the processes of ischemia-reperfusion injury and aging. However, evidence suggests that Nrf2 activity does not always lead to a positive outcome and may accelerate the pathogenesis of some cardiovascular diseases (e.g., atherosclerosis). The precise conditions under which Nrf2 acts to attenuate or stimulate cardiovascular disease processes are unclear. Further studies on the cellular environments related to cardiovascular diseases that influence Nrf2 pathways are required before Nrf2 can be considered a therapeutic target for the treatment of cardiovascular diseases.

1. Introduction

Cardiovascular diseases contribute more to morbidity and mortality than any other group of diseases in the developed world [1]. Oxidative stress is an important component in the pathogenesis of many cardiovascular disorders, including atherosclerosis [2, 3], hypertension [4], heart failure [5], and ischemia/reperfusion injury [6–8]. Sources of potentially damaging reactive oxygen species (ROS) leading to oxidative stress have been extensively reviewed (e.g. [9–11]) and include, but are not limited to, mitochondrial electron transport chain inefficiencies, NADPH oxidase and ubiquitous xanthine oxidase activity, and metallic ions released during cell lysis. This suggests that the activation of antioxidant defenses has an important role in reducing oxidant-induced cellular damage. However, cellular damage or death can still result, leading to organ dysfunction, when cellular antioxidant defenses are overwhelmed by excess ROS production [12].

A well-established and critical component to cellular antioxidant defense mechanisms is expression of direct ROS scavenging enzymes, phase II detoxification enzymes, and other detoxification proteins bearing antioxidant response elements (AREs) in their promoter regions. A principal regulator of the ARE is the highly conserved transcription factor nuclear factor-erythroid (NF-E) 2-related factor 2 (NRF2 for human, Nrf2 for mouse and rat), which is a member of the Cap “n” collar family of transcription factors (more details on Nrf2 are provided elsewhere in this special issue). Nrf2 induces transcriptional activation of a number of ARE-bearing antioxidants, including NAD(P)H dehydrogenase (quinone 1) (NQO1), superoxide dismutases (SODs), and glutathione peroxidases (GPx). Many of the Nrf2 regulated enzymes are essential in the pathogenesis of cardiovascular diseases [13]. However, there exists evidence for both beneficial and detrimental effects of Nrf2 activation in the cardiovascular system. Further investigation is required to better understand the range of interactions between Nrf2 and the cardiovascular system, which could have profound effects on the pathogenesis of cardiovascular diseases. Therefore, the purpose of this review is to discuss the current evidence for a role of Nrf2 in a selection of prominent cardiovascular pathologies.

2. Nrf2 and Atherosclerosis

Several vascular disease processes are associated with oxidative stress, and therefore suboptimal antioxidant defenses
may increase patient risk and accelerate disease progression [14]. Atherosclerosis is an inflammatory disease [15] characterized by endothelial infiltration and accumulation of oxidized low-density lipoproteins (LDLs), physical damage to the endothelium (e.g. turbulent blood flow, hypertension, and/or toxins from cigarette smoking), and/or infection (e.g. HIV). This process leads to atherosclerotic lesions, compromised blood vessel diameter, and increased risk of ischemia, which is a major concern especially in the myocardium.

Interestingly, evidence suggests that susceptibility to atheroma formation is not uniform throughout the vascular system. Several studies suggest that shear stress generated by oscillatory, nonunidirectional, and turbulent blood flow, for example at bifurcations or points of vessel branching, results in atheroma-prone regions [16, 17], increasing the risk of atherosclerosis development. Conversely, atheromas are less likely to form in vascular regions only exposed to unidirectional laminar blood flow [16, 18]. It is well established that laminar vascular wall shear stress stimulates the release of nitric oxide (NO), known for its protective role against atherosclerosis (see [19] for review). However, when blood flow becomes oscillatory (e.g. high flow rates, stenosis, or vessel branching), shear stress on the vascular wall is inconsistent. This reduces NO production and increases superoxide release, which leads to enhanced oxidative stress and atherosclerosis progression [20]. Therefore, laminar versus oscillatory blood flow may be responsible for the apparent confusion regarding the role of Nrf2 in the pathogenesis of atherosclerosis. It is becoming clear that laminar blood flow promotes antiatherogenic activation of Nrf2, and oscillatory blood flow suppresses Nrf2 activation, creating a proatherogenic environment [21, 22]. However, specific blood flow characteristics, in relation to atherosclerosis progression, should be investigated in greater detail to improve the understanding of the interaction between blood flow, Nrf2, and atherosclerosis susceptibility.

3. Nrf2 as an Antiatherogenic Factor

It is becoming increasingly apparent that Nrf2 is important to vascular integrity and long-term endothelial function, for example, sustained release of NO and protection from apoptosis [23–29]. Conversely, specific changes in vascular physiology that are related to Nrf2 can lead to increased susceptibility to atheroma development, such as increases in oxidative stress leading to oxidation of LDLs, reduced NO production, and increased levels of superoxide [20].

One important stage of atherosclerotic plaque formation is a well-established endothelial infiltration by macrophages and foam cell formation following macrophage absorption of accumulated LDLs. In mice, Nrf2 is an important component in this process, since macrophages exposed to oxidized LDLs (oxLDL) increased Nrf2 expression in response, which indirectly protected macrophages from oxLDL-mediated injury via phase II antioxidant enzyme activity [30]. Moreover, absence of Nrf2 in high fat diet myeloid-derived macrophages [31] or LDL receptor deficient myeloid-derived macrophages [32] increased foam cell formation and atherosclerosis progression, further suggesting that Nrf2 is important in resistance to atherosclerosis.

Increases in Nrf2 expression at this stage of atherosclerosis development is significant because of downstream effects on heme oxygenase-1 (HO-1), which produces antiatherogenic reductions foam cell formation [33–36]. Moreover, atherosclerosis was accelerated in HO-1 absent/apolipoprotein E-deficient (ApoE−/−) mice [37]. The ApoE−/− mouse strain is a well-established model for atherosclerosis [38, 39]. In addition to its antioxidant properties, HO-1 protects against inflammation in vascular tissue [33, 40, 41] and has been reported to act in an atheroprotective manner through this mechanism [42, 43]. Since oxidative stress and inflammation are known to be important at all stages of atherosclerosis development [2, 44], these data suggest a central role for HO-1 in atherosclerosis pathophysiology.

In mice, HO-1 has also been reported to suppress atherosclerotic lesion formation by reducing oxLDL-induced transmigration of monocytes, and the reverse was found when HO-1 was inhibited [34]. In addition to oxLDL activated increases in HO-1 expression through Nrf2, other Nrf2 downstream targets appear to play a role in this process, like glutathione-cysteine ligase modifying subunit and NQO1, both of which have been associated with protection against atherosclerosis [26]. In adolescents, low serum glutathione was an independent risk factor for parental coronary heart disease risk [45]. Moreover, low GPx levels, for which glutathione is a cofactor, combined with low high-density lipoprotein levels may be partly responsible for increased atherosclerosis-related mortality rates in humans [46]. Nrf2 was identified as an important regulator of GPx in mice [47], and therefore taken together, these data demonstrate that Nrf2 is an important component in protection against the pathogenesis of atherosclerosis.

HO-1 may also offer protection from atherosclerosis-related morbidity and/or mortality at more advanced stages of the disease by promoting atherosclerotic plaque stability. It has been suggested that matrix metalloproteinase 9 (MMP9) levels are linked to plaque destabilization [48, 49], which are important events in acute constriction of vessel blood flow and sudden cardiac or cerebral events. Interestingly, an atheroprotective role for HO-1 may be partially associated with MMP9 suppression to maintain or improve plaque stability [50], potentially avoiding an acute, life-threatening coronary or cerebral event. These data present convincing evidence for the importance of Nrf2 and its downstream targets in protection against atherosclerotic plaque formation or stability.

In addition to atherosclerotic processes, Nrf2 expression may also be induced by extrinsic factors, leading to protection against the disease. For example, activation of Nrf2 by dosing mice with the cruciferous vegetable extract sulforaphane had an anti-inflammatory effect on atherosusceptible endothelial cells [51], although the effect of the sulforaphane dose used in their study on Nrf2 expression was not assessed. Therefore, it is possible that in this case, Nrf2 was not activated and sulforaphane induced other anti-inflammatory factors.
However, it should be noted that sulforaphane has been reported to induce expression of antioxidant enzymes regulated by Nrf2 [52–54]. Furthermore, increased Nrf2 message and nuclear NRF2 were found with sulforaphane-treated mice in a model of respiratory syncytial virus disease [55].

4. Nrf2 as a Proatherogenic Factor

Interestingly, Nrf2 has been reported to be proatherogenic in an elegant study comparing atherosclerotic plaque formation in ApoE−/− mice that were either Nrf2 sufficient or deficient, combined with either a 10- or 20-week high fat diet [56]. ApoE−/−Nrf2 2+/− mice developed significantly less aortic plaque area and loss of vessel wall elasticity compared to ApoE−/−Nrf2 2+/- mice, which was reported to occur in a sex-dependent manner [57]. Moreover, this effect appeared to be partly dependent on diet, which increases the urgency for diet modification in the general population, especially in low socioeconomic regions where diet-related susceptibility to atherosclerosis, among other cardiovascular diseases, is known to be higher [58]. However, the study of Barajas et al. suggests independent actions of Nrf2 and HO-1 in atherosclerosis. In ApoE−/− mice, Nrf2 deletion resulted in atherosclerosis suppression [57], but with HO-1 deletion the atherosclerosis was accelerated [37]. Considering the regulation of HO-1 by Nrf2, this illustrates the current confusion regarding the role of Nrf2 in atherosclerosis.

The paradox between compromised antioxidant defenses in Nrf2 2−/− mice and lower aortic atheroma area could be associated with a reduction in macrophage uptake of oxLDL and foam cell formation, as previously discussed. The scavenger protein CD36 regulates macrophage uptake of oxLDL, and normal oxLDL-induced increases in CD36 expression were not found in Nrf2 2−/− macrophages [59]. This suggests that in atherosclerosis development, inhibition of macrophage uptake of oxLDL is more important than antioxidant capacity, both of which are regulated by Nrf2. This highlights the current confusion regarding the role of Nrf2 in atherosclerosis development. In the previous section, discussion of Nrf2 as an antiatherogenic factor involved increases in Nrf2 expression leading to lower foam cell formation in the presence of HO-1 [33, 35, 36, 60]. However, these opposing influences of Nrf2 on atherosclerosis development operated via different mechanisms and the degree of interindividual atherosclerosis patient difference in the presence of one mechanism or the other are not known but may be critical in understanding the progression of this disease.

In addition to Nrf2-mediated upregulation of scavenger proteins promoting atherosclerosis progression, other factors regulated by Nrf2 may add to a proatherosclerotic effect. Activating transcription factor 4 (ATF4), known to control vascular endothelial growth factor, stimulates plaque formation by recruiting monocytes to the atherogenic region [61, 62]. Recently, crosstalk between Nrf2 and ATF4 was demonstrated in endothelial cells [63], further suggesting a proatherogenic effect of Nrf2. However, it is beyond the scope of this review to discuss all factors that interact with Nrf2 in the pathogenesis of atherosclerosis, principally because Nrf2 is a highly influential gene, especially when enhanced oxidative stress is present. For example, Nrf2 is known to interact with other well-established pro-atherogenic factors, including vascular cell adhesion molecule 1 [64], NQO1 [65], and interleukin-1 [66]. However, it should be noted that NQO1 has also been reported as both an anti- and proatherogenic factor and therefore improved the understanding of the role of NQO1 in atherosclerosis susceptibility that may lead to clarification of Nrf2 influences on this process. Nonetheless, it is possible through multiple mechanisms that Nrf2 produces competing effects on the pathophysiology of atherosclerosis, which highlights the complexity of this disease.

5. Nrf2 and Ischemia-Reperfusion Injury

It has long been recognized that compromised blood flow and cellular perfusion leading to ischemia has major injurious effects on the organ in question. Prominent examples include stroke, myocardial infarction, and organ transplantation. Therefore, reestablishment of blood flow as quickly as possible is a primary clinical goal in ischemia. However, an acute restoration of blood flow to an ischemic region can lead to an enhanced degree of injury as a consequence of oxidative stress compared to the initial period of ischemia.

The myocardium is particularly vulnerable to ischemic injury, because oxygen uptake at any given time during cellular perfusion is around 80%, and therefore cardiac myocytes are unable to significantly increase percent oxygen uptake from arterial blood when blood flow is severely compromised by vascular constriction (e.g. atherosclerotic plaque thrombosis or vasospasm, leading to ischemia). When blood flow is restored however, a substantial inflammatory response is induced [67], significantly increasing oxidative stress, which can overwhelm antioxidant defenses resulting in cardiac dysfunction from cell damage or death. This process makes Nrf2 an important candidate for resistance to ischemia-reperfusion injury, but there is little information about its role in this situation.

Nonetheless, in rat cardiac H9c2 cells, simulated ischemia reperfusion (10 hrs hypoxia, followed by 16 hrs normoxia) resulted in a significant increase in intracellular ROS levels. Under the same conditions, H9c2 cells were treated with the phase II antioxidant enzyme inducer D3T, which was accompanied by a significant reduction in intracellular ROS levels. In these cells, increases in Nrf2 mRNA and protein were found, suggesting that Nrf2 may be important in controlling intracellular ROS levels following ischemia reperfusion [68]. Conversely, in rat hearts, 30 minutes of left anterior descending coronary artery occlusion resulted in a reduction in Nrf2 nuclear protein, which was prevented by ischemic preconditioning of the myocardium [69]. This finding is very important as it suggests that in order for Nrf2 to initiate antioxidant defenses against reperfusion-induced oxidative stress, the length of the prior ischemic phase may be a critical factor. Early rescue from ischemia may attenuate Nrf2 responses to oxidative stress upon reperfusion, reducing protection from reperfusion-induced oxidative stress. Alternatively, ischemic preconditioning may act as an “early warning” signal and activate Nrf2 prior to a prolonged...
ischemic event. Acute activation of \textit{Nrf2} has been shown as cardioprotective following ischemia reperfusion. When mice were treated with hydrogen sulfate [70] or 4-hydroxy-2-nonenal [71] to activate \textit{Nrf2} prior to cardiac ischemia reperfusion, reduced infarct size \textit{in vivo} or improved recovery time in Langendorff-perfused mouse hearts were observed respectively.

6. \textit{Nrf2} and Hypertension

While oxidative stress and hypertension appear to be related, a "chicken or the egg" scenario means that it is not clear if oxidative stress is a contributing factor to hypertension or if hypertension induces oxidative stress, even though both are likely the case. There are some convincing arguments for the latter [72], although it is possible that oxidative stress caused by preexisting disease (e.g. diabetes) could be a catalyst for hypertension [73]. Certainly, increased levels of ROS in renin-angiotensin-induced hypertension have been established [74, 75].

NADPH oxidases (NOX for human, Nox for mouse) are a significant source of ROS in cardiovascular diseases, including angiotensin II-dependent hypertension [74, 76, 77]. A number of Nox isoforms are emerging as important components in the pathophysiology of hypertension in their interaction with \textit{Nrf2}. Nox1, expressed by vascular smooth muscle cells, has been reported to stimulate an increase in ROS levels during an angiotensin II-mediated pressor response [78]. Moreover, activation of \textit{Nrf2} by Nox1 has been found in response to intermittent hypoxia [79], suggesting a mechanism to attenuate oxidative stress through increases in \textit{Nrf2} expression. Vascular endothelial cells express Nox2, and increases in NOX2 levels have recently been associated with angiotensin II-mediated hypertension, endothelial dysfunction, and vascular remodeling [80]. These data suggest that increases in Nox are an important mechanism for resistance to oxidative stress in hypertension mediated by angiotensin II dysfunction.

\textit{Nrf2} may also be important in blood pressure regulation through an alternative and interesting mechanism. \textit{Nrf2} induces expression of HO-1, which has hypotensive effects when upregulated in spontaneously hypertensive rats [81–83]. HO-1 is also implicit in the production of carbon monoxide (CO), in the breakdown of heme into CO, iron, and bilirubin. CO has direct vasodilatory effects [84], which appear to be independent of NO [85]. CO also inhibits the production of endothelin [86], a powerful vasoconstrictor, which is likely an important component of CO effects on vascular tone, regulated by HO-1, the expression of which is induced by \textit{Nrf2}. Moreover, a number of studies have shown reduced blood pressure in response to increases in HO/CO pathway activity in spontaneously hypertensive rats [87–89]. While speculative, these data suggest that \textit{Nrf2} may be important in blood pressure regulation in a capacity other than its part in antioxidant defenses.

However, the potential role for \textit{Nrf2} regulation of HO-1 in blood pressure control is not well defined and may only become important under specific conditions of oxidative stress, like exposure to lipopolysaccharide [90]. Moreover, there were no differences in basal blood pressure between \textit{Nrf2}−/− and wild-type (WT) mice [91]. Li et al. also reported no significant differences between \textit{Nrf2}−/− and WT mice in angiotensin II induced blood pressure elevation. However, the 16 mmHg greater response in systolic blood pressure in WT mice should not go unnoticed from a clinical perspective, suggesting a potential inhibition of hypertensive responses to angiotensin II in \textit{Nrf2}−/− mice.

\textit{Nrf2} expression was upregulated in deoxycorticosterone acetate (DOCA)-salt-induced hypertension in rats. This response, which was enhanced by concomitant epicatechin treatment (\textit{Nrf2} inducer), attenuated the hypertensive response [92]. However, it seems likely that this was due to increases in oxidative stress in association with hypertension, rather than a direct effect of \textit{Nrf2} on blood pressure regulation.

It is clear that \textit{Nrf2} is important, either directly or indirectly, in blood pressure regulation under specific biological environments (e.g. hypertension). However, the circumstances in which \textit{Nrf2} influences blood pressure must first be described in detail before \textit{Nrf2} can be considered as a target for blood pressure therapy in the clinical setting.

7. \textit{Nrf2} and Heart Failure

Increased oxidative stress in the diseased myocardium is a well-established phenomenon. Therefore, the potential for \textit{Nrf2} being an important factor in either prevention or slowing of pathophysiologic processes in the myocardium is high. In relation to heart failure, ROS impair cardiac function [93] and increase susceptibility to arrhythmia [94] by a direct toxic effect of increased necrosis and apoptosis [95].

Several \textit{Nrf2} downstream target genes have been associated with protection against abnormal myocardial remodeling in response to hypertension, including HO-1 [96, 97], SOD [98], and GPx [99]. Unfortunately, the role of \textit{Nrf2} in heart failure, while likely on the evidence, has not received significant attention. However, some evidence suggests that \textit{Nrf2} is protective against pathological myocardial hypertrophy and heart failure. In a mouse model of pressure overload by transverse aortic constriction, \textit{Nrf2} overexpression attenuated ROS production and hypertrophic growth in cardiomyocytes, and cardiac fibroblasts [100]. This protective effect of \textit{Nrf2} in myocardial remodeling and heart failure may be mediated through Nox4 [101], which is known to be an important regulator of reduction-oxidation (redox) signaling in many cell types including cardiomyocytes and is a major source of mitochondrial oxidative stress during pressure overload [102]. Furthermore, recent studies have demonstrated cardioprotective activation of \textit{Nrf2} by the Krebs cycle intermediate fumarate [103] and triterpenoids [104–106], suggesting potentially useful treatments with fumarate derivatives or triterpenoids in patients suffering from pathological levels of oxidative stress.

However, while acute activation of \textit{Nrf2} is cardioprotective [70, 71, 107], there is accumulating evidence that chronic activation of \textit{Nrf2} may be harmful to cardiac function [108, 109] leading to pathophysiological processes and heart failure. Chronic activation of \textit{Nrf2} has been reported in
association with the concept of “reductive stress” in the murine cardiac hypertrophy and heart failure model of human αB-crystallin overexpression [108]. In this model, constitutive activation of Nrf2 has been reported due to an excess of the reducing equivalents, reduced GSH and NADPH. Therefore, more information about the dynamics of acute versus chronic Nrf2 activation is required before useful treatment strategies taking advantage of this mechanism can be developed.

Moreover, there are potentially important comorbidity effects that are associated with increases in oxidative stress in the myocardium that point to Nrf2 playing a protective role. For example, cardiac myocyte insulin resistance is a key component to diabetes-induced cardiac dysfunction, and oxidative stress can exacerbate this scenario. Nrf2 expression was suppressed in diabetic mice with cardiomyopathy in late stage disease [110]. In the same study, oxidative stress in cardiomyocytes (HL-1 cells) led to depressed Nrf2 expression, extracellular signal-related kinase (ERK) activation, and a lower glucose metabolism.

This novel interaction between diabetes-related cardiomyopathy and Nrf2 could provide insight into individual susceptibility to diabetic complications in the cardiovascular system and therefore should be investigated carefully. The principle reason for this need is the current confusion regarding the influence of ERK during cellular stress. ERK signaling is important in the activation of Nrf2 in response to oxidative stress [111]. Several studies reported a protective effect of oxidative stress-induced ERK activation [112–117], which is not surprising considering its importance in Nrf2 signaling. Conversely, other studies found that oxidative stress-induced upregulation of ERK was a contributing factor to apoptosis [115, 118, 119]. The precise mechanism by which ERK stimulates or prevents apoptosis is not clear. However, considering the important role of ERK in normal cell division [120], ERK-induced apoptosis has been suggested to be a mechanism to prevent uncontrolled cell proliferation under certain conditions of oxidative stress (e.g. cancer) [121], which could have negative implications when oxidative stress (e.g. hyperoxia) is not accompanied by aberrant cell proliferation.

8. Nrf2, Age, and Cardiovascular Disease

The Nrf2–Keap1 pathway is a critical element to redox homeostasis in the myocardium [109, 122]. With age, expression of several Nrf2 downstream targets declined in rats [123], and age-related arterial Nrf2 dysfunction in Macaca mulatta [124] and rats [125] has been reported. Since about 75% of cardiovascular disease associated deaths occur in people over the age of 65 years [126] and the mean global population age is increasing, this presents a significant public health concern. Especially important to consider is the large number of diseases associated with oxidative stress, not least cardiovascular diseases.

However, it may be possible to resist the reduction in Nrf2 activity associated with the aging process. In young mice, expression of myocardial Nrf2 and several downstream antioxidant target genes have been demonstrated to increase significantly after treadmill exercise comprising 90 minutes per day for 2 days [122]. Moreover, age-related reductions in Nrf2 transcriptional activity in the myocardium were reversed in mice subjected to the same treadmill exercise or following 6 weeks of moderate treadmill exercise training [127]. Exercise-induced increases in Nrf2 activity were accompanied by increased levels of antioxidants, like NQO1, HO-1, and Gpx1 with corresponding attenuation of ROS status in both young and aged mice [127]. These data highlight the potential importance of habitual exercise to maintain Nrf2 function in an aging population.

Two important methods by which Nrf2 activity can be maintained or restored in the myocardium during the aging process are apparent in the literature. First, as is frequently reported, habitual exercise is beneficial for reducing risk of a host of diseases, not least cardiovascular disease and therefore represents a simple, nonpharmacological intervention to protect against age-related Nrf2 dysfunction. Second, there is a significant amount of evidence suggesting Nrf2 as a useful therapeutic target to treat oxidative stress related diseases.

9. Models for Assessing Cardiopulmonary Responses to Oxidative Stress in Rodents

Exposure of mice to high concentrations of oxygen in air (hyperoxia) for prolonged periods (3–5 days) induces significant oxidative damage to the lung similar to important diseases like acute lung injury (ALI), the more severe acute respiratory distress syndrome (ARDS), and bronchopulmonary dysplasia (BPD). Moreover, significant effects of this model on the cardiovascular system are becoming apparent. In many studies, mice or rats were group housed in exposure chambers (e.g. [128–132]), or standard rodent cages were placed together in large exposure chambers (e.g. [133, 134]). This has been a successful and cost-effective method for exposing multiple animals simultaneously to investigate responses and adaptations in many biological systems, but principally in the lung. Prolonged exposure of mice to hyperoxia leads to acute alveolar inflammation and pulmonary epithelial and endothelial barrier necrosis leading to pulmonary edema and progressively compromised pulmonary gas exchange [135], which has significant effects on cardiac function [136].

Cardiovascular and pulmonary function in conscious, freely moving rodents can be monitored in real time. Electrocardiogram, blood pressure, electroencephalogram, body temperature, and activity level waveforms can be recorded continuously in animals using implantable radio-telemetry transmitters, and pulmonary function can be recorded using whole body plethysmography. Recently, combination of these methods was used to investigate the genetic component to cardiopulmonary function in a wide range of commercially available strains of mice [137]. In order for these systems to work correctly, mice must be singly housed, and therefore previous exposure chamber arrangements with group housed animals would not be appropriate for radio-telemetry or whole body plethysmography. Whole body plethysmographs have been used as hyperoxia exposure chambers (as well as a wide range of inhalants) using mice implanted with ECG telemeters, creating a model for assessing continuous cardiopulmonary responses to hyperoxia (also possible for other
species, e.g. rats or rabbits) [136]. This model is a very useful tool for assessing cardiopulmonary responses to hyperoxia-induced oxidative stress and was successfully implemented to investigate the role of Nrf2 in cardiopulmonary responses to oxidants, included in this issue (refer to Howden et al.).

10. Conclusions

Nrf2 is a key component to cellular redox homeostasis in the attenuation of oxidative stress-associated pathological processes. In the cardiovascular system, patients with insufficient NRF2 levels in multiple tissues are likely susceptible to several adverse components of disease development. If Nrf2 expression is insufficient to protect against hypertension, then Nrf2 is likely insufficient to protect against the resultant oxidative stress, atherosclerosis, and heart failure, highlighting the urgency for investigating Nrf2 further as a potential therapeutic target. Alternatively, there is evidence for increases in Nrf2 activity being detrimental to disease resistance and/or accelerating pathogenesis in cardiovascular diseases.

For example, while activating Nrf2 shortly before initiating ischemia reperfusion may be beneficial in terms of cardiac function outcome, there is some evidence that chronic activation of Nrf2 could be detrimental to cardiac function. Therefore, further work is required to understand the role for Nrf2 in cardiovascular pathogenesis before Nrf2 can be seriously considered as a therapeutic target for treatment of cardiovascular diseases. This is especially important when considering the increasing prevalence of multiple comorbidities in aging populations [138].

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References


Review Article
The Involvement of NRF2 in Lung Cancer

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Nuclear factor, erythroid-derived 2, like 2 (NRF2) is a key regulator of antioxidants and cellular stress responses. The role of NRF2 in pulmonary neoplasia, a diverse disease for which few biomarkers exist, is complicated and appears to depend on several main factors including the existence of activating mutations in NRF2 and/or loss of function mutations in KEAP1 and the stage of carcinogenesis studied, particularly in the mouse models tested. Therapeutic strategies for lung cancer targeting NRF2 have observed mixed results, both anti- and protumorigenic effects; however, these differences seem to reflect the mutation status of NRF2 or KEAP1. In this paper, we will discuss the studies on human NRF2 and the mechanisms proposed, several mouse models using various mice deficient in NRF2, as well as xenograft models, and the chemotherapeutic strategies using the NRF2 pathway.

1. Introduction
Lung cancer mortality rates are the highest among all cancers worldwide [1, 2]. Although smoking rates have decreased in the US, many countries have observed few changes in smoking habits, and 10–20% of lung cancer patients are nonsmokers [1, 3, 4]. Thus, understanding and identifying novel pathways for therapeutic targets is a primary goal in research on pulmonary neoplasms. Non-small-cell lung carcinoma (NSCLC) has the highest incidence rates and most studies focus on its specific subtypes, squamous cell carcinoma or adenocarcinoma (AC), although there are several other subtypes under the NSCLC heading [2]. NSCLC is also the most common among smokers as well as the only lung cancer found in nonsmokers [5]. NSCLC develops in the central bronchi in squamous cell carcinoma (SCC) and in the bronchioles and alveoli in adenocarcinoma (AC). Small-cell lung carcinoma (SCLC) accounts for ~20% of lung cancer and is almost exclusively associated with a smoking etiology [5]. SCLC tumors are centrally located in bronchi and express neuroendocrine markers [5]. While some lung tumor subtypes, such as SCC, have early precursor lesions, most have few early biomarkers for detection [5]. We refer the readers to reviews on lung cancer for more on the etiology [5–7].

This paper and special issue of this journal will focus on a molecule called nuclear factor, erythroid-derived 2, like 2 (NRF2), a master transcription factor that regulates antioxidant response element- (ARE-) mediated expression of antioxidant enzymes and cytoprotective proteins [8]. Oxygen is essential for the survival of all aerobic organisms and its metabolism results in partially reduced oxygen byproducts collectively known as reactive oxygen species (ROS) [9, 10]. Excess ROS causes oxidative damage to cellular DNA, lipids, and proteins; genetic changes and/or epigenetic alterations can lead to the dysregulation of oncogenes and tumor suppressor genes, ultimately contributing to the pathogenesis of cancer [11, 12]. To alleviate this oxidative stress, there are several antioxidative stress responses, many regulated by NRF2. NRF2 expression is abundant in tissues where detoxification reactions occur, including the lung [9], and under normal physiological conditions it interacts with its own negative regulator, Kelch-like ECH-associated protein 1 (KEAP1) [13]. KEAP1 is a cytoplasmic, cysteine-rich, actin-bound protein that sequesters NRF2 in the cytoplasm and directs it to CUL3 E3 ligase for ubiquitylation and subsequent degradation by the proteasome [9, 10, 13]. In times of oxidative stress, selected KEAP1 cysteines become oxidized leading to a disruption of the KEAP1-NRF2 complex and the release of the NRF2
peptide. NRF2 then translocates to the nucleus to transcribe genes encoding various antioxidant proteins and metabolic enzymes collectively known as phase II detoxifying enzymes [10]. Alternative pathways for NRF2 activation are through the phosphorylation of NRF2 by protein kinase C (PKC) or RNA-dependent protein kinase R- (PKR-) like endoplasmic reticulum kinase (PERK), resulting in the release of NRF2 from KEAP1 [14–16].

The role of NRF2 and KEAP1 in cancer development has been highly controversial and has led to many theories including NRF2 as an oncogene, or its manipulation by an oncogene, specifically in the lung [13, 17–20]. It is clear that the findings in lung cancer differ from those observed in most other organ systems, or even other pulmonary diseases, such as emphysema [21], hyperoxia [22], and respiratory syncytial virus [23], where disease symptoms significantly worsen in the absence of NRF2. Thus, the mechanisms driving these tumorigenic responses appear unique to tumor development. However, some studies examining activating mutations in NRF2 or loss of function of KEAP1 in human cancers, such as esophagus, skin, and ovarian cancers, did find one or more of these mutations altering the NRF2 pathway, which suggests protumorigenic involvement [24, 25] in these extrapulmonary tissues. We will only discuss NRF2 in the context of lung cancer in this paper, but many other mouse models, including colon, bladder, liver, and mammary, have demonstrated that a lack of NRF2 increases the potential for carcinogenesis [26–29]; this varies greatly in pulmonary neoplasias depending on the model tested. We will first discuss the human studies that have been done including the polymorphisms identified and their proposed effects, mouse models for lung cancer and NRF2, and the chemotherapeutic targets that use NRF2 in either a protumorigenic or antitumorigenic manner in lung.

2. Human NRF2 Studies

The lung is an organ of high surface area that is intimately associated with the central compartment to facilitate gas diffusion. Therefore, it is a seminal point of exposure to environmental toxins such as cigarette smoke, ozone, particulates, and exhaust emissions such as polyaromatic hydrocarbons and peroxyacetyl nitrate [30]. Such toxins have been implicated in the incidence of lung cancer and linked to increased burden of ROS in human tissues [31], as well as the upregulation of antioxidant-selective genes [32]. The growing tumor and its microenvironment are an additional source of ROS from accelerated mitochondrial function required for rapid cell growth and division in the proliferative phase [33]. Activating or stabilizing modifications of NRF2 increase its nuclear translocation in response to hypoxia or ROS in A549 cells (AC cells), and it has been suggested that this is a cell survival mechanism [34, 35]. In addition, analyses of tumor tissue from a variety of cancers, including lung, display overexpression of the phase II antioxidant enzymes regulated by NRF2, such as glutathione-S-transferase (GST) and NADP(H): quinone oxidoreductase 1 (NQO1), which are both known to facilitate the elimination of reactive, oxidized metabolites [30, 36]. While studies indicate that there is ample evidence to support the involvement of NRF2 in cancer biology [37–40], the dominant focus in human research are somatic mutations in NRF2 and/or its repressor protein KEAP1, that confer either enhanced tumor escape from apoptosis or resistance to a variety of cancer chemotherapeutics [41]. Interestingly, gain of function mutations in NRF2 are more closely associated with chronic smoking while loss of function KEAP1 mutations are not [40, 42, 43]. For further discussion on cigarette smoking and NRF2, we refer the readers to Muller and Hengstermann, 2012 [42].

2.1. Studies Examining the Effects of NRF2 Mutations. One group of somatic mutations identified specifically in AC and SCC lung tumors occurs within the Neh2 domain of the NRF2 gene [43]. This domain contains a bZIP region fused to a cap n’ collar (CNC) region and governs both the ability of NRF2 to dimerize with MAF proteins as well as DNA binding to ARE regions in the target genes [44, 45]. Of particular significance are mutations within the DLG (amino acid 27–32) and ETGE (amino acid 77–82) regions of the domain, as they affect the binding affinity to the KELCH domain of KEAP1 and inhibit redox-sensitive repression by KEAP1 that normally controls the basal levels of NRF2 expression [41, 46]. The inability to maintain NRF2 protein expression at or below a basal level has been directly linked to poor prognosis in clinical patients diagnosed with either lung AC or SCC [40, 43, 47]. Investigations in human cancer cell lines have linked elevated NRF2 expression with resistance to specific anticancer chemotherapeutics [48–50]. An in vitro study of the human cancer cell lines A549, NCI-H292 (mucopidermoid cells), and RERF-LC-Ai (SCC cells) (ranked from highest to lowest constitutive expression of NRF2) demonstrated that resistance to cisplatin was proportional to NRF2 expression [51]. Expressions of antioxidant enzymes, phase II metabolic enzymes, and drug efflux pumps in these cell lines were also elevated in proportion to NRF2 and sensitive to siRNA knockdown of NRF2. The NRF2 siRNA knockdown also profoundly inhibited the cellular proliferation of the A549 cells. Similar findings were observed in a study of human carbonyl reductase 3 (CBR3) and its regulation by NRF2 [52]. This study utilized human AC cell line A549, SW–480 (colon), HT-29 (colon), and the hepatocellular HepG2 cell line. In all lines, the magnitude of NRF2 expression reflected the degree of CBR3 induction/expression, but siRNA knockdown of NRF2 in A549 cells reduced the levels of CBR3 to just 30% of control. Multidrug resistance protein 3 (MDR3), which has been linked to drug resistance in NSCLC, is also known to be directly induced by NRF2 in both NSCLC tumor tissue and immortalized ATCC cell lines (DU-145 prostate; H1666, H1650, and A549 (AC); H358-unspecified NSCLC) [53]. The end result is that NRF2 levels alone have become a prognostication factor for patient treatment decisions in non-small-cell lung cancer [54, 55].

2.2. Studies on the Effects Elicited from Alterations in KEAP1

Due to the presence of six KELCH regions in the binding
domain of NRF2, a loss of NRF2 inhibition by KEAPI should also occur as a result of mutations in the KELCH binding region of the KEAPI protein itself. A study of Japanese lung cancer patients (AC, SCC, large cell carcinomas (LCCs), and SCLC) documented these KEAPI mutations and identified them as a source of constitutive expression of MDR proteins, phase II enzymes as well as specific cisplatin resistance in cultured lung AC cells [56]. Genetic studies of human lung tumors (AC, SCC) further substantiate this, and some suggest that tumor types may have distinct patterns of KEAPI muta-
tion frequency [57, 58]. In a study meant to further describe the translocation of NRF2 from KEAPI to the nucleus, it was found that the NSCLC cell lines A549 and H460 (LCC) (both KEAPI mutants) had constitutively high MDR3 levels. The authors hypothesized that the induction of MDR3 might be purely due to increased nuclear translocation of unbound NRF2 independent of the KEAPI mutation. However, the levels of MDR3 in these cell lines failed to superinduce when treated with 4-hydroxy-2-nonenal, which was shown to enhance the nuclear translocation of NRF2 in the human bronchial epithelial cell line (HBE1) and in a NSCLC cell line H358 (bronchoalveolar carcinoma), which lack the KEAPI mutation [59]. The cytoprotective benefits of these multi-
drug resistance mechanisms are mixed. A recent study in H358 demonstrated an aryl hydrocarbon receptor (AhR): NRF2 coinduction of multiresistance protein 4 (MRP4) actually lowers DNA-adduct formation in cells exposed to either benzo[a]pyrene (B(a)P) or 2,3,7,8-tetrachlorodibenzo-
p-dioxin (TCDD) via cellular efflux of the inactive metabo-
lites [60]. However, the same combination of AhR and NRF2 is enhanced by the exposure of lung cancer cells to cigarette smoke condensate, triggering upregulation of the xenobiotic pump ABCG2 and resistance to chemotherapeutics [61, 62]. Thus, enhanced expression of MDR proteins through NRF2 can elicit both pro- and antitumorigenic responses.

While the nature of NRF2 mutations involves direct DNA substitutions that result in a loss of binding affinity to the KELCH region, the KEAPI mutations create gross structural alterations and stearic hindrance to the formation of the NRF2: KEAPI complex. In addition, KEAPI mutations have the potential for epigenetic inactivation. Methylation of the KEAPI promoter region was observed with a frequency of up to 47% in tissues from NSCLC patients (both AC and SCC), with somatic mutations in only 15% and loss of heterozygosity in 21%—no methylation was detected in normal controls [63]. Similar epigenetic findings have been reported for a prostate cancer cell line (DU-145) and associated with resistance to a

2.3. Downstream Mechanisms. While the KEAPI mutation often increases ARE gene targets indirectly by increasing the availability of NRF2, it has also been shown to directly amplify induction of PPARy and confer chemoresistance [65]. KEAPI mutations can also directly interfere with BCL2 degradation to enhance cellular escape from apoptosis [66]. Thus, the KEAPI mutation itself can offer a significant survival adaptation to cancerous and precancerous cells. The combination of the NRF2 and KEAPI mutations is particularly advantageous. For example, in studies in A549 cells with a mutant KEAPI insertion, not only does the KEAPI mutation interfere with BCL2 degradation, but loss of NRF2: KEAPI dimerization leads to direct induction of BCL2 by NRF2, increasing the amount of available BCL2 for apoptotic escape [67]. These cells displayed increased resistance to etoposides and either UV or gamma irradiation, characteristics that were lost by siRNA knockdown of either NRF2 or BCL2.

The current state of knowledge regarding NRF2 and KEAPI in human lung cancer suggests that both apoptotic escape and resistance to anticancer treatments are responsible for the poor prognosis associated with elevated NRF2 levels and KEAPI dysfunction. Interestingly, if one examines the frequency of common mutations in human lung tumor tissue, for both SCLC and NSCLC (p53, RB, BCL2, etc.), these are found to occur at a rate exceeding 50% in both types [68]. A genomic study of SCC reports a NRF2/KEAPI mutation frequency of 34% in 178 tumors from affected individuals [69]. A recent review further calculates the frequency of KEAPI and NRF2 mutations for SCLC and NSCLC samples at approximately 25% overall and proposes that the acquisition of ARE-driven enhancement of tumor survival is likely to be the result of an insult that drives tumor promotion or progression rather than the initiation phase [41].

3. Animal Studies Assessing the Role of NRF2 in Lung Carcinogenesis

Animal studies on the NRF2 pathway have used Nrf2-deficient mice on multiple backgrounds, Keap1-deficient mice, xenograft models, metastasis models, as well as Nrf2-deficient mice crossed with a K-RasG12D mouse. We will discuss all of these models herein. The tumorigenic potential of human activating NRF2 mutations in an altered HEK293 cell line expressing mutant NRF2 (T80R and L30F, gain-of-function mutations in cancer) [43] was examined and found to induce tumorigenesis in vivo using xenografts in immunodeficient mice [70]. The xenografts consisted of mutant NRF2-induced tumors that were poorly differentiated with many microvessels, NQO1 production (downstream of NRF2), and an occasional metastasis to the liver [70]. Additionally, the mutated NRF2 was dependent on the mTOR pathway, identified using mTOR inhibitors. Certain heterozygous mutations in KEAPI, previously identified in human lung tumors, were also found to have a dominant negative effect on wild-type KEAPI, using an in vivo system in transgenic mice [71]. Specifically, KEAPIG400C or KEAPIG564C coexpressed with the WT KEAPI in mice (mixed background, 129Sv/J, C57BL/6, and ICR) resulted in significant hyperactivation of genes downstream of NRF2, such as Nqo1, supporting a dominant-negative effect of mutant KEAPI [71]. Thus, while these studies are not lung-specific, they demonstrate that NRF2 and KEAPI mutations identified in human lung cancer have functional effects in animal models.
In a primary lung cancer model using urethane, a well-established model [72, 73] to induce tumors, Nrf2-deficient mice (−/−) were significantly less susceptible to tumor development than the Nrf2+/+ mice (WT; BALB background strain) [37]. However, Nrf2−/− mice had increased hyperpermeability, inflammatory cell infiltrates, including monocytes, macrophages, and lymphocytes, and elevated myeloperoxidase, that was suggestive of increased numbers of PMNs, compared to the Nrf2+/+ mice 11 wks following urethane. Significant reductions in the early adenomatous lesions in the Nrf2−/− mice were also observed 12 wks following urethane with concomitant increases in apoptotic cells, compared to the wild-type mice [37]. Thus, the cell death pathways involved in apoptosis and necrosis, such as significant increases in LDH in the Nrf2−/− mice compared to Nrf2+/+ mice, support the hypothesis that the urethane-initiated epithelial cells, such as the type II alveolar pneumocyte or bronchiolar Clara cell, both progenitor cells for lung AC [74], were more susceptible to cell death in the mice lacking Nrf2. The Nrf2+/+ mice, therefore, have both a growth advantage and increased cytoprotection for tumorigenesis.

A transcriptome study was also performed to determine the differences between strains (Nrf2−/− and Nrf2+/+) involved in these responses to urethane at an early and late time point. At the 12 wk time point, Nrf2-modulated genes involved glutathione metabolism, cell-cell signaling, oxidative stress, and immune responses. In the more advanced stage, the Nrf2-dependent genes associated with cell cycle/proliferation and cell death, correlating in direction and magnitude with the increased death of initiated cells in the Nrf2−/− mice. At 22 wks, PMNs were also significantly increased in the tumor-bearing lungs of Nrf2+/+ mice compared to Nrf2−/− mice, as well as the chemokine Cxcl1 (KC) [37]. Altogether, these studies demonstrate that in a primary mouse lung cancer model, Nrf2 promotes survival properties and supports the human studies demonstrating resistance to anticancer drugs as well as increased malignancy. Interestingly, when an additional primary tumor model (MCA/BHT) was used with these strains, no differences were observed, suggesting Nrf2 protection may be both carcinogen and stage dependent [37]. In addition, because urethane is not considered a mimic of cigarette smoke, although it is a component of cigarettes [75], the underlying mechanisms of lung cancer may be different.

As described earlier, ROS are often involved in initiating disease states (i.e., cancer), and thus the concept that reduction of ROS may lead to increased carcinogenesis is against the normal doctrine [76]. However, studies demonstrated that when the oncogenes K-RasG12D or B-RafV619E (mutated, activated) were expressed in vitro in murine NIH3T3 or mouse embryonic fibroblasts (MEFs), ROS levels as measured by 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dGuo), and free hydrogen peroxide or superoxide, were all subsequently decreased [77]. In contrast, Nrf2 activity and its downstream genes were elevated and the ratio of reduced-to-oxidized glutathione increased (GSH/GSSH) [77]. These oncogenic pathways (K-Ras and B-Raf) were found to signal through MEK, ERK MAP Kinase, and the AP-1 transcription factors, Jun and Fos, finally inducing Nrf2, which led to the antioxidant responses observed [77]. These in vitro findings were then validated in several in vivo mice models, including those for lung and pancreatic cancer. Nrf2−/− and Nrf2+/+ mice were bred to the K-RasG12D mice (B6/129/SJL background strain) in lung tumorigenesis studies which demonstrated that the Nrf2−/− mouse had a significant reduction in K-RasG12D-initiated lung tumors compared to WT mice [77]. These mice also had reduced Ki67 staining indicating less proliferative activity as well as overall decreases in adenomas, adenomatous alveolar hyperplasias and bronchiolar hyperplasias [77]. The pancreatic cancer model also demonstrated that in the absence of Nrf2, pancreatic intraepithelial neoplasia was significantly reduced [77]. Thus, it appears that Nrf2 can be regulated by specific oncoproteins (K-Ras, B-Raf) to increase tumorigenesis by reduction of ROS through detoxification and antioxidant responses that creates a more favorable cellular microenvironment. The findings described here support the other primary mouse study which suggests that the initiated cells are reduced in mice lacking Nrf2 and therefore, the cellular environment in those mice lacks the favorable protections of mice with sufficient Nrf2 [37].

Lastly, in a Lewis lung carcinoma (3LL) mouse metastasis model, Nrf2-deficient mice on a C57/Bl6/J background developed a significantly higher number of lung metastatic nodules than wild-type mice [78]. The total cancer incidence in the Nrf2-deficient mice was 100% compared to 28.6% in the WT mice. Pulmonary and bone marrow inflammation, including myeloid-derived suppressor cells (MDSCs), was also elevated in the Nrf2-deficient mice bearing metastatic nodules. MDSCs can suppress CD8+ T-cell populations through reactive oxygen species (ROS) and thus may be suppressing the immune response in these animals [79]. Keapl mutant mice with increased levels of Nrf2 protein were resistant to metastasis [78] and demonstrated reduced levels of ROS. In these studies, Nrf2 appears to play a role in the prevention of metastasis, which is the reverse of findings observed in other mouse lung cancer studies. However, the mechanisms regulating metastasis differ from the earlier stages of carcinogenesis, which were modeled in the other mouse studies. Additionally, the strain background used in the metastatic studies (B6) differs from those used in the previously described mouse studies (BALB and B6/129/SJL). These strains are known to differ in many phenotypes, including the polarity of their immune systems, which could influence responsiveness [80].

As of yet, no studies in mice have examined the effects of altered Nrf2 in SCLC or SCC.

4. Chemotherapeutic Strategies Using the NRF2 Pathway

By the nature of its cellular functions of alleviating oxidative stress, Nrf2 is important in the prevention of disease onset and progression and has established a definitive role in cancer prevention [10, 12, 13, 18]. As mentioned above, using Nrf2-knockout mice in a LLC model demonstrated that the absence
of Nrf2 led to accelerated colonization and proliferation of metastatic cancer cells in the lungs [78]. However, recent genetic evidence demonstrates an upregulation of NRF2 in various human cancers including lung cancer, thereby suggesting possible protumorigenic involvement in several stages of cancer, such as promotion and progression. Along with increased NRF2 signaling, mutations in KEAP1 and NRF2 and constitutive expression of NRF2, are known to enable tumor cells to hijack the NRF2 pathway as a mechanism to resist chemotherapeutic agents [10, 43, 56, 58, 81, 82]. Cancer chemoprevention/chemotherapy is the use of plant-based (phytochemicals) or synthetic chemical compounds to prevent, suppress, delay, or reverse the development of invasive cancer. This concept has been expanded to target all stages of cancer development [83, 84]. One strategy to use drugs to stimulate or to inhibit NRF2 in lung. We refer the readers to the following excellent reviews for NRF2 and NRF2 pathway to determine when it would be advantageous to use drugs to stimulate or to inhibit NRF2 in lung. We refer the readers to the following excellent reviews for NRF2 and general chemoprevention [12, 82], while we focus on lung herein.

4.1. Studies Demonstrating Beneficial Effects of Stimulating NRF2 . Dietary and medicinal plants are major sources of phytochemicals, which have played an important role in cancer treatment [83]. Dietary components that increase reduced glutathione (GSH) levels and induce phase II enzymes are known to activate transcription through the NRF2-ARE pathway. While various research studies have shown that human consumption of cruciferous vegetables, such as broccoli and brussel sprouts, both induces NRF2 and decreases risk of lung cancer [85–89], it should be noted that further chronic studies need to be implemented to confirm this linkage. Because polymeric black tea polyphenols (PBP) protect against B(a)P-induced DNA adduct formation in vitro, Patel et al. investigated the role of Nrf2 in phase II enzyme induction by PBP extract in murine pulmonary tissues in vivo [84]. Pretreatment with PBP followed by sacrifice day post-B(a)P induced total cellular levels of Nrf2 protein and increased nuclear accumulation of Nrf2 in the lungs. PBP extract also induced ARE-mediated Nqo1 and Gst gene expressions, while the Keap1 levels in the lungs remain unaltered. These results suggest that homeostatic maintenance of KEAP1 levels and the presence of phytochemicals at the beginning of initiation may be linked to a possible role of NRF2 acting as a tumor suppressor. Some phytochemicals utilize other pathways in conjunction with the NRF2 pathway to affect their anticancer activities. Curcumin, the principal curcuminoid of the Indian spice turmeric, has exhibited anti-initiating effects via the transcriptional regulators of phase I and II enzymes in mice [90]. Previous to inoculation with B(a)P as the carcinogen, mice were treated with dietary curcumin and the mechanisms of curcumin-mediated anti-initiation were investigated. Curcumin inhibited B(a)P-induced phase I enzyme activities by significantly decreasing AhR-DNA binding and thereby decreasing the subsequent activation of phase I enzymes. Curcumin also enhanced nuclear translocation of Nrf2 and Nrf2-ARE binding in vivo, leading to increases in phase II enzymes in the lungs [90]. Recent studies (described earlier) using B(a)P and dioxin demonstrated coinduction of AhR and Nrf2 leading to a reduction in DNA-adduct formation and also supporting the curcumin findings [60]. However, due to the anti-inflammatory effects of curcumin [91] and the known importance of inflammation in lung cancer [92–94], involvement of other pathways cannot be ignored.

While the previously mentioned studies involve pretreatment with chemopreventive compounds, there is also evidence that Nrf2 has a postinitiation role in experimentally induced lung carcinogenesis. Sulforaphane (SFN), an isothiocyanate isolated from cruciferous vegetables such as broccoli, is the most potent naturally occurring inducer of phase II enzymes, and although most pathways induced are Nrf2-dependent, some Nrf2 independent mechanisms have been shown, such as direct regulation of glutathione levels [95]. One in vivo investigation determined if Nrf2 contributes to pulmonary protection based on the timing of treatment with the phytochemical [96]. Whether SFN was introduced prior to the first dose of the carcinogen or after cellular initiation, increased phase II enzymes, decreased phase I enzymes and significant reduction of oxidative damage were observed [96]. Collectively, the NRF2 pathway appears important in facilitating chemopreventive measures in the lungs, as long as the pathway itself has not been altered/mutated.

There have also been some recent breakthroughs for new therapies, most notably in regards to suicide gene therapy and pro-drug activation of tumor-selective compounds [97, 98]. A lentiviral (LV) vector expressing herpes simplex virus thymidine kinase (HSV-TK/GCV) under the regulation of ARE (LV-ARE-TK/GCV), was constructed and its constitutive ARE hyperactivity was used to selectively target lung cancer cells for suicide gene therapy [97]. The vector was tested in human lung AC cells and a mouse xenograft model of lung cancer. In both settings, the vector was effective in decreasing cell viability and tumor size [97]. A drug in phase II clinical trials designed to promote tumor hypoxia was recently found to be bioactivated by a novel nitroreductase (AKR1C3) that was directly controlled by NRF2 levels. Subsequent microarray analysis of 2490 cancer patients demonstrated normative upregulation of AKR1C3 in tumor tissues, suggesting that NRF2 elevation and its sequelae could be used to enhance the specificity and efficacy of novel chemotherapeutics [98].

4.2. Studies on the Effects of Inhibition of the NRF2 Pathway . Yamamoto and colleagues found a high incidence/frequency occurrence of loss of KEAP1 function in patients with lung cancer [9, 56, 99]. The low KEAP1 activity resulted in multiple effects and ultimately resistance to chemotherapeutic agents,
constitutive activation of the NRF2 pathway [13]. In addition, for the use in cancers in which genetic mutations cause rational for the development of new NRF2 inhibitory agents for example, cisplatin [9, 56, 58]. Thus, there is a strong leading to elevated Based on the studies reviewed, it appears that both mutations 5. Conclusions 6. Oxidative Medicine and Cellular Longevity and progression, whereas during metastasis, Nrf2 is observed to be anti-tumorigenic. Overall, the mechanism by which these pro-tumorigenic effects occur likely involves increased cytoreduction, including decreased cell death (by apoptosis and necrosis) and increased proliferation, increased detoxification and upregulation of antioxidant pathways regulated by oncogenes, such as Kras, commonly mutated during initiation events in humans and mice [37, 74]. This ultimately produces a more favorable pulmonary microenvironment for the tumors to develop [77]. In the case of the LLc metastasis model, ROS suppression of CD8+ T cells leading to suppression of the entire immune system is likely the culpable mechanism [78]. The findings in mice may also explain the differences observed between activating the NRF2 pathway and chemotherapeutic resistance, since not all studies examined every stage of carcinogenesis. Some of the therapies discussed are also not necessarily NRF2-specific, such as sulforaphane [95], and thus, other mechanistic pathways cannot be excluded. Thus, there are more studies that need to be done to fully understand the role of NRF2 in lung cancer, including assessment of the different types and stages of lung cancer. It may be that only certain stages and subtypes will be sensitive to the effects of Nrf2 mutations. The discovery that the NRF2 pathway has a dual role in cancer should not be seen as a death knell for the chemotherapeutic/chemopreventive drugs that utilize this pathway. What is of importance is that the biologic context in which these drugs are administered must be considered to maximize the efficacy of any cancer treatment regimen. As shown from the research already conducted, the chemotherapeutic options currently available appear to be highly dependent on the homeostatic state of the NRF2 pathway within the actual lung tumor.

Authors’ Contribution
A. K. Bauer, T. Hill, and C. M. Alexander contributed equally to the paper.

Conflict of Interests
The authors declare that there are no conflicts of interests.

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Preventive and Therapeutic Effects of MG132 by Activating Nrf2-ARE Signaling Pathway on Oxidative Stress-Induced Cardiovascular and Renal Injury

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So far, cardiovascular and renal diseases have brought us not only huge economic burden but also serious society problems. Since effective therapeutic strategies are still limited, to find new methods for the prevention or therapy of these diseases is important. Oxidative stress has been found to play a critical role in the initiation and progression of cardiovascular and renal diseases. In addition, activation of nuclear-factor-E2-related-factor-2- (Nrf2-) antioxidant-responsive element (ARE) signaling pathway protects cells and tissues from oxidative damage. As a proteasomal inhibitor, MG132 was reported to activate Nrf2 expression and function, which was accompanied with significant preventive and/or therapeutic effect on cardiovascular and renal diseases under most conditions; therefore, MG132 seems to be a potentially effective drug to be used in the prevention of oxidative damage. In this paper, we will summarize the information available regarding the effect of MG132 on oxidative stress-induced cardiovascular and renal damage, especially through Nrf2-ARE signaling pathway.

1. Introduction

The World Health Organization reports that chronic diseases as the leading cause of mortality in the world cause approximately 17 million people to die prematurely each year and keep steadily growing [1, 2]. What is more, this largely invisible epidemic is the worst in low- and middle-income countries, which could forego billions of dollars in national income as a result of these diseases. For example, the estimated losses in China from 2005 to 2015 are 558 billion dollars [1]. Cardiovascular diseases (CVD), a group of common chronic diseases, are the largest causes of morbidity and mortality worldwide. Chronic kidney disease (CKDs), also known as a microvascular disease, is an increasing public health concern too. CKD not only increases the risk of CVD and disease expenditure but also has a major impact on patients, health services, and society burden [3–5]. Thus, it is a priority to find effective drugs to treat CVD and CKD.

Epidemiological studies have shown several risk factors for patients with CVD and CKD, such as heredity [6, 7], diabetes [8, 9], anemia [10], and hyperlipidemia [11, 12], but nontraditional risk factors such as oxidative stress may also contribute to these diseases [13, 14]. Our understanding of how oxidative stress contributes to cardiovascular and renal diseases has undergone considerable evolution over the past two decades. In recent years, reactive oxygen species (ROS) have come to be recognized as taking part not only in normal intracellular signaling for survival, but also in contributing to cytotoxicity [15]. Therefore, antioxidant therapy seems a preventive or therapeutic solution for the oxidative damage. Reportedly antioxidants such as vitamin E have been used in the treatment of human cardiovascular and renal disease; however, despite that there is one study supporting the therapeutic effect of vitamin E on these diseases [16], most of the clinical studies have failed to materially impact the course of the diseases [17, 18]. The possible reasons might...
include inefficiency of monoantioxidant used such as vitamin E only. Therefore, supplemental or upregulating endogenous multiple antioxidant levels may be a more efficient approach than mono-antioxidant therapy.

There are highly regulated cellular defense systems, including the redox-sensitive nuclear-factor-E2-related-factor-2-(Nrf2-) antioxidant-responsive element (ARE) pathway. Nrf2 is a transcription factor to regulate the expression of a battery of antioxidant genes and other cytoprotective phase II detoxifying enzymes through binding ARE [19, 20]. Therefore, Nrf2-ARE pathway promises to be a valuable therapeutic target for the prevention of oxidative stress and damage. Accumulating investigation has demonstrated that proteasome inhibitor MG132 could protect cells and tissues against oxidative damage because it could activate the Nrf2-ARE signaling pathway, leading to an upregulation of detoxifying and antioxidant genes [21–24]. In this paper, we thus focus on the antioxidant effect of MG132 on oxidative stress-induced cardiovascular and renal diseases.

2. Oxidative Stress and Nrf2-ARE Signaling Pathway

2.1. Oxidative Stress. ROS, a necessary evil of aerobic life, are routinely produced as a byproduct of aerobic metabolism, oxidative phosphorylation, environmental stressors, disease, or even natural aging process [25]. ROS generation is an important signaling mechanism in cells [26]. Our body is under constant oxidative attack from ROS so that a complex antioxidant system that generally defends this attack in balance has been evolved [15]. Oxidative stress is defined by the imbalance between the production of ROS and the endogenous antioxidant mechanisms that counteract the effects of ROS or repair the resulting damages [27]. Under physiological conditions, several tightly controlled oxidative pathways contribute towards ROS productions, while several endogenous antioxidant enzymatic mechanisms account for ROS depletion [28]. Either caused by reduced detoxification or increased generation, ROS can lead to widespread and indiscriminate cellular damage. As the central cause of oxidative stress, ROS at homeostatic levels have diverse actions on cell function. For instance, ROS can activate protein kinases (such as mitogen-activated protein kinases (MAPK)) [29] and upregulate redox-sensitive factors (such as NF κB and AP-1) [30, 31]. On the other hand, it can be detrimental to cellular homeostasis by leading to opening ion channels [32] and major cellular macromolecules damage, including lipid peroxidation [33], DNA oxidation [34], and protein modification [35]. These damages, if left unrepaired, can lead to mutations that cause diseases.

2.2. Mechanism of the Nrf2-ARE Signaling Pathway in Oxidative Stress-Associated Injury. There is an upsurge of interest in Nrf2-ARE system because it plays a key role in the cell’s response to oxidative stress [36–38]. Nrf2, a cap-n-collar family of nuclear basic leucine zipper transcription factors, is at the central of this system and regulates cellular defenses against ROS. Nrf2-ARE signaling pathway is regulated by complex and poorly understood mechanisms. Kelch-like ECH-associated protein 1 (Keap1), known as an actin cytoskeleton-associated protein, binds very tightly to Nrf2 and anchors this transcription factor in the cytoplasm [39]. Keap1 also serves as a substrate adaptor for Cullin-3 (Cul3) that binds to ring-box 1 to form the E3 ubiquitin-ligase complex. The latter ultimately leads to ubiquitination and proteasomal degradation of Nrf2; thereby the ability of Nrf2 to induce phase II detoxification enzyme genes is repressed, as shown in Figure 1 [40–43].

When exposed to various stimuli such as oxidative stress, certain antioxidants, and chemopreventive agents, the Nrf2/Keap1 complex will be disrupted by modifying two (Cys273 and Cys288) of the 25 cysteine residues of Keap1 [44], allowing the cytoplasmic-to-nuclear translocation of Nrf2. In the nucleus, Nrf2 increases gene expression of phase II detoxifying enzymes such as glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), NAD (P) H:quinine

![Figure 1](image-url)
oxidoreductase 1 (NQO1), and heme oxygenase 1 (HO-1) [45, 46]. As shown in Figure 1(b), the transcriptional activation of these antioxidant enzymes is thought to be mediated by ARE or electrophile response element, which is found at the 5-flanking region of the phase II detoxification enzyme genes [47].

Modification of the Nrf2/Keap1 complex and Nrf2 nuclear translocation is important to Nrf2-ARE-pathway-dependent gene expression, and several signaling pathways are associated with these processes. For example, one component of these pathways is MAPKs. Both extracellular signal-regulated kinase (ERK) and p38MAPK have been found to induce Nrf2 translocation and HO-1 expression through diallyl sulfide in HepG2 cells [48]. In addition, protein kinase C (PKC) is also associated with Nrf2-dependent antioxidant enzyme expression. Huang et al. reported that PKC promotes Nrf2 phosphorylation at Ser-40, which yields the dissociation of Nrf2 from Keap1 in HepG2 cells. Data revealed that PKC-induced Nrf2 phosphorylation is critical to ARE-dependent antioxidant enzyme expression [49, 50]. Taken together, regulation of the upstream kinases involved, such as phosphatidylinositol 3-kinase (PI3 K), ERK, and PKC, provides a valuable tool for the investigation of Nrf2/Keap1 complex-controlled gene transcription [51].

3. Effects of Ubiquitin-Proteasome System (UPS) and MG132 on Nrf2-ARE Signaling Pathway

3.1. UPS. Proteins in eukaryotic cells are continually being synthesized and degraded. Two proteolytic systems, the lysosomal systems and UPS, are mainly responsible for this homeostasis. The lysosomal system is the principal mechanism for degrading proteins with long half-life and is the only system in cells for degrading organelles and large protein aggregates or inclusions [52]. The UPS pathway, as a highly specific extralysosomal system, plays a pivotal role in the degradation of misfolded and damaged proteins within the eukaryotic cells. Moreover, the UPS is also essential for selective degradation of short-lived and regulatory proteins involved in a wide variety of fundamental cellular processes, including cell cycle control [53], apoptosis [54], transcriptional regulation [55], proliferation [56], cell surface receptors expression [57], ion channels modulation [58], and Nrf2 degradation [59].

The UPS consists of three parts: the 76-amino acid protein ubiquitin, the multisubunit complex 26S proteasome, and three enzymes, including ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligase (E3) which are involved in a 3-step enzymatic cascade process [53, 60]. In an energy-dependent stepwise process catalyzed by three enzymes (E1, E2, and E3), target proteins for the proteasomal degradation are conjugated to multiple units of ubiquitin yielding a polyubiquitinated proteins. In the next step, unfolding ubiquitinated proteins are recognized, hydrolyzed, and then degraded by the 26S proteasome [61], which was illustrated in Figure 2(a). Proteasome, a highly conserved catalytic enzyme complex, is a large multisubunit protease and the most common form is known as 26S proteasome. It is composed of one catalytic 20S core particle (CP or 20S proteasome) and one or two 19S regulatory particles (RP or 19S proteasome) (Figure 2(b)). The 26S proteasome is a 2.5 MD protein complex which presents in the nucleus and cytoplasm of all eukaryotic cells [62, 63]. Known as 20S proteasome, the large core unit with a molecular mass of approximately 700 kDa is made up of two outer α rings and two inner β rings, which consists of 7 structurally similar α and β subunits, respectively [62]. The 20S proteasome contains proteolytic active sites that are sequestered within an interior space and performs several peptidolytic functions to maintain cellular homeostasis [64]. On the other hand, the 19S proteasome is able to recognize polyubiquitylated target proteins and take part in their deubiquitylating, unfolding,
and translocation into the interior space of the 20S proteasome for destruction [62].

3.2. Proteasome Inhibitor MG132 and Nrf2-ARE Signaling Pathway. MG132 (Z-Leu-Leu-Leu-CHO), a peptide aldehyde proteasome inhibitor, was constructed by Roca et al. in 1994 and has been widely used in proteasome biology, allowing for the identification of new therapeutic targets and the development of novel therapeutic strategies. MG132 is a substrate analogue and potent transition-state inhibitor and mainly exhibits the chymotrypsin-like activity of the proteasome [65, 66]. When cells are exposed to this cell-permeable, potent, highly specific, and reversible proteasome inhibitor, MG132 will reduce degradation of ubiquitin-conjugated Nrf2 by inhibiting activity of the β subunits of the core particle of 26S proteasome without affecting its ATPase or isopeptidase activities. Subsequently, undegraded Nrf2 will be released from the Nrf2/Keap1 complex and translocate into the nucleus. Then Nrf2 binds to ARE and upregulates transcription of antioxidant genes (Figure 2(b)).

The stabilization of Nrf2 by proteasome inhibition and subsequent transcriptional activation of its downstream genes have been shown in different cell types in earlier studies [24, 42, 67–69]. Recently, several studies have demonstrated that MG132 has the capacity of activating Nrf2-ARE signaling pathway in a variety of disease conditions [22, 70, 71]. This antioxidant response is known to be dose dependent. Low-dose MG132 exposure improves cellular fitness accompanied by the up-regulation of heat-shock proteins, GST, and Nrf2 [22, 68, 72] while high-dose MG132 yields an opposing effect that leads to apoptosis and even severe oxidative stress [73, 74]. Although the precise mechanism by which MG132 exerts antioxidant effects has not been fully understood, one well-accepted hypothesis is that the antioxidative effect of MG132 is related to the prevention of Nrf2 degradation through its suppression of UPS and subsequent translocation of Nrf2 from cytoplasm into the nucleus [41]. In Huang et al.’s study, the phosphorylation of Nrf2 at serine 40 appears to be a critical event in the release of Nrf2 from Keap1 and the translocation of Nrf2 from cytosol into the nucleus [49]. However, whether MG132 can provoke Nrf2 phosphorylation remains unknown; therefore, further investigations are needed to make this mechanism clear.

Despite that MG132 inhibition of proteasome results in an elevation of Nrf2 expression, the compensatory induction of proteasome activity was also noticed. For instance, elevated proteasome subunit synthesis upon proteasome inhibition by MG132 is well conserved in human squamous cells [75, 76]. Interestingly, Nrf2, as a degradation target of proteasome, was also thought to mediate the proteasome recovery by increasing the 20S proteasome and the Pa28αβ (11S) proteasome regulator protein levels through a transcriptional feedback loop [77]. However, other studies demonstrated that the compensatory increase in proteasome subunit gene expression was Nrf1 dependent, instead of Nrf2 [75, 76]. Therefore, the exact mechanisms by which proteasomal activity is compensatively increased remain systemic studies.

4. Effect of MG132 on Oxidative Stress-Induced Cardiovascular and Renal Injury: Nrf2-Dependent Pathway

4.1. Preventive Effect of MG132

4.1.1. Cardiovascular Injury. With regard to CVD, many of the pathogenic components of the disease are associated with oxidative stress, such as inflammation, LDL oxidation, and endothelial dysfunction. Overproduction and accumulation of ROS severely damage DNA, proteins, and lipids, resulting in further tissue damage and organ dysfunction. Compelling evidence supports the idea that supraphysiological levels of ROS (or called oxidative stress) play an important role in the pathophysiology of various CVDs, including endothelial dysfunction [78, 79], atherosclerosis [80, 81], and ischemia-reperfusion injury [82].

Our previous study indicated that high glucose could lead to ROS generation in both primary neonatal and adult cardiomyocytes from wild-type mouse heart. Whereas, in Nrf2 knockout cells from Nrf2 knockout mice, ROS were significantly higher under basal conditions and high glucose markedly further increased ROS production in concentration- and time-dependent manners [83]. Nrf2 was shown to mediate the basal expression and induction of ARE-controlled NQO1 and HO-1, at both mRNA and protein levels in cardiomyocytes [83]. Persuasive evidence has suggested that activation of antioxidant genes through Nrf2-ARE-dependent mechanism might yield protection against oxidative stress-associated injury in CVD [19, 84]. This antioxidant effect of proteasome inhibitor MG132 was confirmed by a Germany group [23]. Exposure to 0.5µM MG132 for 48 h proved to be nontoxic and protected neonatal rat cardiac myocytes against H2O2-mediated oxidative stress [23]. Another study from China investigated the effects of long-term MG132 treatment on cardiac hypertrophy in vivo. This study showed that treatment with MG132 (0.1 mg/kg/day) for 8 weeks attenuated pressure-overload-induced cardiac hypertrophy and improved cardiac function in abdominal aortic banding rats [85]. Recently a study from our group showed that therapeutic effect of MG132 on diabetic cardiomyopathy is associated with its suppression of proteasomal activities [86]. Mechanistically MG132 may upregulate Nrf2-mediated anti-oxidative function and downregulate NF-κB-mediated inflammation.

In a similar study, we treated STZ-diabetic mice with sulforaphane at 0.5 mg/kg daily in five days of each week for 3 months. Sulforaphane treatment completely prevented diabetes-induced aortic pathogenic changes by attenuating oxidative stress, inflammation, and fibrosis in the aorta [87]. The aortic protection by sulforaphane treatment from diabetes was also accompanied with a significant upregulation of Nrf2 expression and function (reflected by its downstream genes: HO-1, NQO1, and SOD1 expression) [87]. MG132 was also used in several vascular diseases. For instance, nontoxic inhibition of the proteasome using MG132 was found to protect against oxidative stress-induced endothelial dysfunction through increasing depressed SOD1 expression [71]. This finding is in line with a previous report...
that MG132 could liberate Nrf2 from Keap1 and translocate to nucleus to bind DNA with up-regulation of its downstream antioxidant genes [24]. Hemin is released from hemoglobin after central neuronal system hemorrhage and may cause ROS accumulation which contributes to cell loss in surrounding tissue. Pretreatment with 1 \(\mu M\) MG132 for 2 h prevented approximately half of heme-mediated oxidative injury by up-regulation of Nrf2 and HO-1 [88].

4.1.2. Renal Injury. Similar to CVD, oxidative stress is also the major player in the process of many kidney diseases, including acute kidney injury (AKI) [89, 90], ischemia reperfusion-induced renal injury [91], primary glomerulonephritis [92–96], diabetic nephropathy [97–101], lupus nephritis [102–104], and antineutrophil cytoplasmic antibodies-associated vasculitis [105, 106].

Previous work has indicated that impaired renal function in hypercholesterolemic pigs is improved by chronic proteasome inhibition with MLN-273 [107]. In a recent study, enhanced renal proteasome activity was found during lipopolysaccharide-induced AKI in human kidney cells. Suppression of proteasome activity using 10 \(\mu M\) MG132 for 18 h can attenuate lipopolysaccharide-induced AKI [108]. In another AKI model, cisplatin-induced nephrotoxicity was markedly ameliorated by MG132 treatment both in vivo and in vitro [109].

Antifibrotic effect of MG132 at low doses has been observed in rat renal fibroblasts and mesangial cells [110, 111]. As we know, oxidative stress plays an important role in pathogenesis of diabetic nephropathy. Zheng et al. provided experimental evidence indicating that Nrf2-ARE signaling pathway activation by sulforaphane or cinnamic aldehyde can be used therapeutically to relieve renal damage induced by type 1 diabetes. This idea was confirmed by our recent study [112]. We treated type 1 diabetic mice with sulforaphane at 0.5 mg/kg daily for five days for each for 3 months. At the end of 3-month treatment with sulforaphane one set of mice was sacrificed to perform the experimental measurements (3-month time point). The second set of mice was aged for 3 additional months without further sulforaphane treatment (6-month time point). Our results revealed that sulforaphane significantly prevented diabetes-induced renal inflammation, oxidative damage, and fibrosis by activation of Nrf2-ARE signaling pathway in the kidney at 3-month time point, but not at 6-month time point, suggesting the requirement of continual use of sulforaphane for its sustained effect [112]. In another STZ-induced diabetes rat model, MG132 was administered at a dose of 10 \(\mu g/kg/day\) via intraperitoneal injection once daily for 3 months. After MG132 treatment, renal Nrf2 and its downstream antioxidants (SOD1, CAT, and GPx) were upregulated and diabetic renal damage was also improved [22].

4.2. Therapeutic Effect of MG132

4.2.1. Cardiovascular Injury. A recent study from our group suggested that therapeutic effect of MG132 on diabetic cardiomyopathy is associated with its suppression of proteasomal activities [86]. Diabetic mice showed significant cardiac dysfunction, heart structural derangement, and remodeling (fibrosis and hypertrophy), as well as increased systemic and cardiac oxidative damage and inflammation. All of these pathogenic changes were reversed by MG132 treatment. In addition, MG132 treatment significantly increased cardiac expression of Nrf2 and its downstream antioxidant genes and also significantly decreased the expression of Ik-\(\beta\) and the nuclear accumulation and DNA binding activity of NF-\(\kappa\)B in the heart. Therefore, the possible mechanisms might include both up-regulating Nrf2-mediated anti-oxidative function and downregulating NF-\(\kappa\)B-mediated inflammation induced by MG132.

4.2.2. Renal Injury. The therapeutic effect of MG132 on diabetic nephropathy was also reported by our group [113]. Three-month old transgenic type 1 diabetic (OVE26) mice displayed renal dysfunction with albuminuria and then were treated with MG132 (10 \(\mu g/kg/day\)). After 3-month treatment with MG132, diabetes-induced renal oxidative damage, inflammation, fibrosis, and eventual dysfunction were significantly attenuated accompanied with a significant decrease in 20S proteasome activity decrease and activation of Nrf2-ARE signaling pathway. In vitro study using human renal tubular HKII cells confirmed the role of Nrf2 in the prevention of diabetes-induced renal damage. HKII cells were treated with high glucose (27.5 mM) for 48 h. During that time, MG132 (2 \(\mu M\)) and palmitate (300 \(\mu M\)) were added in the last 9 h and 6 h, respectively. Immunofluorescent staining for Nrf2 showed that Nrf2 expression and nuclear accumulation were decreased in high glucose plus palmitate group but increased in MG132 treatment group. MG132 treatment also significantly prevented the increase of connective tissue growth factor overexpression in the cells treated with high glucose plus palmitate. What's more, silencing the Nrf2 gene with its specific siRNA abolished MG132 decrease of high glucose and palmitate-induced connective tissue growth factor overexpression. These results suggested that MG132 upregulates Nrf2 function via inhibition of diabetes-induced proteasomal activity, leading to the therapeutic effect on diabetic nephropathy.

4.3. Dose-Dependent Effects of MG132 on Cardiovascular and Renal Injury. It should be mentioned that whether cells have beneficial response to MG132 also depend on several factors, including the type of cells, the dose of MG132, and the exposure time. Contrast to the studies discussed above, several studies in cardiac myocytes showed an opposite conclusion. Exposure of myocytes to high doses of MG132 (10 \(\mu M\)) in short term enhanced the cellular damage [114, 115]. Available evidence suggests that toxic inhibition of proteasome function induces programmed cell death in proliferating endothelial cells [116]. Similarity, proteasome inhibitor MG132 has been shown to affect cell growth and death through formation of ROS and depletion of GSH in As4.1 juxtaglomerular cells [117–119]. In order to explain this interesting phenomenon, Meiners et al. have systemically analyzed dose-dependent effects of proteasome inhibition with MG132 using human umbilical cord vein cells [120].
They found that nontoxic doses of MG132 (70 nM) induced a defined, dose-dependent transcriptional response by up-regulating anti-oxidative enzymes (e.g., SOD1, GPx) that were accompanied by protection against H2O2-induced oxidative stress, whereas high doses of MG132 (200 nM) induced apoptosis in endothelial cells [120]. In general, nontoxic proteasome inhibition might offer a new therapeutic approach for the treatment of oxidative stress-associated cardiovascular and renal diseases.

5. Other Mechanisms by Which MG132 Protects Cells against Oxidative Damage

Although MG132 protects cardiovascular and renal damage from oxidative stress predominantly via Nrf2-ARE signaling pathway, other possible mechanisms should not be ignored. Among these mechanisms, the relatively well-studied one is IκB-NF-κB pathway. Recent studies suggested that hyperglycemia enhances 26S proteasome activity through peroxynitrite/superoxide-mediated PA700-dependent proteasomal activation, which elevates NF-κB-mediated renal and aortic inflammatory response in early diabetes. Importantly, these alterations were abolished by MG132 administration [121]. Another in vivo study demonstrated that MG132 attenuated oxidative stress-induced damage by suppressing NF-κB in coronary arterioles in type 2 diabetic mice, because increased NAD(P)H oxidase and NF-κB activity in diabetes was attenuated by MG132 administration [122]. Similar situation was also found in H2O2-treated microvascular endothelial cells in vitro [123] and heart of rats with pressure overload in vivo [124]. Besides IκB-NF-κB pathway, MG132 can play a key role in cellular defense system by suppressing MAPK signaling pathway [125, 126] and blocking the degradation of vascular protective molecules [127].

6. Conclusions

Accumulating observation has illustrated that a great range of cardiovascular and renal diseases have been associated with oxidative stress. Given that Nrf2-ARE signaling pathway plays critical roles in preventing oxidative stress-associated injury, Nrf2 activators are supposed to be used clinically as a new strategy. In a phase 2, double-blind, randomized, placebo-controlled clinic trial, Dinkova-Kostova et al. used bardoxolone methyl, which has the ability to activate Nrf2 [128], to treat 227 patients with CKD for 52 weeks [129]. Results suggested that patients receiving bardoxolone methyl had significant increases in estimated glomerular filter rate compared with those given placebo, accompanied by only mild adverse effects, such as muscle spasms, hypomagnesemia, and gastrointestinal effects. Similar outcomes were obtained in a subgroup study for diabetic nephropathy [129]. With the recent US Food and Drug Administration approval of bortezomib (Velcade) for the treatment of relapsed multiple myeloma, the proteasome inhibition has been established as a powerful and promising therapeutic strategy for oxidative stress damage [130, 131]. Although, to our knowledge, no evidence has been proved that MG132 can be used in patients with oxidative stress-induced cardiovascular and kidney diseases, it is increasingly apparent that MG132 has the antioxidant effect by up-regulation of Nrf2-ARE signaling pathway both in vitro and in vivo. Thus, MG132 may become another candidate for clinical application for the patients with cardiovascular and renal diseases. However, what is the dose window of MG132 in treatment of oxidative damage in human disease? What is the mechanism of MG132 to promote Nrf2 to release from Keap1? All these questions remain unanswered yet. Therefore, further research focusing on the effect of MG132 on Nrf2-ARE signaling pathway and the underlying mechanisms is urgently needed.

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Research Article

Nrf2 Is Crucial to Graft Survival in a Rodent Model of Heart Transplantation

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Currently, the sole treatment option for patients with heart failure is transplantation. The battle of prolonging graft survival and modulating innate and adaptive immune responses is still being waged in the clinic and in research labs. The transcription factor Nrf2 controls major cell survival pathways and is central to moderating inflammation and immune responses. In this study the effect of Nrf2 levels in host recipient C57BL/6 mice on Balb/c allogeneic graft survival was examined. Importantly, Nrf2−/− recipient mice could not support the graft for longer than 7.5 days on average, whereas activation of Nrf2 by sulforaphane in Nrf2+/+ hosts prolonged graft survival to 13 days. Several immune cells in the spleen of recipient mice were unchanged; however, CD11b+ macrophages were significantly increased in Nrf2−/− mice. In addition, IL-17 mRNA levels were elevated in grafts transplanted into Nrf2−/− mice. Although Nrf2 appears to play a crucial role in graft survival, the exact mechanism is yet to be fully understood.

1. Introduction

Heart transplantation is the only restorative technique for end-stage heart failure, and allograft rejection remains a significant barrier to successful transplantation. The immune response to an allograft is an ongoing interchange between the innate and adaptive immune systems, which if left unchecked will ultimately lead to rejection of the transplanted organ. In addition, implanted grafts are recognized as “non-self” by the recipient immune system due to discordant major histocompatibility complex (MHC); what is more, other immunogenic signals such as tissue damage resulting from organ procurement and ischemia/reperfusion damage can also promote host immune responses and graft rejection.

The transcription factor nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) or “Nrf2” is a master regulator of the cellular antioxidant response. Nrf2 promotes cell survival by binding the antioxidant response element (ARE) in the promoter of downstream target genes that promote detoxification of xenobiotics and scavenging of reactive oxygen species (ROS). The benefit of Nrf2 activation has been demonstrated in various pathological states such as diabetes, chemoprevention, and cardiovascular and neurological diseases [1–5].

The role of Nrf2 in mediating cellular inflammation and immunity is rapidly becoming established. Activation of Nrf2 appears to antagonize inflammatory pathways such as TGFβ1 and NF-κB [6, 7]. Nrf2’s influence on survival of transplants (i.e., liver or cardiac stem cells) has been investigated from the side of the donated tissue [8–10]. However, Nrf2 genotype and/or modulation on the side of the recipient host have not yet been investigated. The purpose of the present study was to determine how Nrf2 in recipient hosts affects graft survival in an allogeneic mouse model of heart transplantation.

2. Methods

2.1. Animals and Surgery. Nrf2+/+ (WT) and Nrf2−/− (KO) C57BL/6 mice (described previously [11]) aged 8–12 weeks
were used as recipient mice for heart transplant studies. Six–eight-week-old Balb/c mice were purchased from Jackson Labs and used as donors for heart transplant studies. All animals received water and food *ad libitum*. Nrf2 WT mice were left untreated or given sulforaphane (SF) (WT + SF) by i.p. injection (12.5 mg/kg body weight) one day prior to transplant and every 48 h after transplant until animals were sacrificed. KO mice were left untreated, or a smaller subset were left untreated or given sulforaphane (SF) (WT + SF). Animals were injected 1 day prior to transplant and then every 48 h until tissues were harvested.

### Table 1: Primers used in qPCR experiments.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward 5'-3'</th>
<th>Reverse 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Odc</em></td>
<td>GCCAGTAACGGAGGTCCAGAT</td>
<td>ATCATCAGTGCAATCCGTA</td>
</tr>
<tr>
<td><em>IL-17</em></td>
<td>TGTTAGGTCACCTCAAGATC</td>
<td>GAGGATATCTATCAGGGTCTTCA</td>
</tr>
<tr>
<td><em>CD11b</em></td>
<td>AGCCCCACACTAGCATCAA</td>
<td>TCCATGTCACAGGCAAGAG</td>
</tr>
<tr>
<td><em>F4/80</em></td>
<td>GGAGGACATCCACTCTGG</td>
<td>TGATGACTTTGCTTGCATGC</td>
</tr>
</tbody>
</table>

### Table 2: Overall survival of Balb/c donor hearts.

<table>
<thead>
<tr>
<th>Recipient genotype/treatment</th>
<th>WT</th>
<th>KO</th>
<th>WT + SF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival time (days)</td>
<td>9.63</td>
<td>7.25</td>
<td>16</td>
</tr>
<tr>
<td>Time in days (Ave ± SD)</td>
<td>9.07 ± 0.54</td>
<td>7.03 ± 0.46</td>
<td>12.76 ± 1.63^{*}</td>
</tr>
</tbody>
</table>

### Figure 1: Study design diagram. A schematic of the study design, time points, and N-size for each group is provided. Briefly, donor hearts and recipient spleens were harvested at 2 days and 5 days following transplant, and donor hearts were harvested at the final day of graft survival for analysis. For the Nrf2^{+/−} group treated with sulforaphane (SF), animals were injected 1 day prior to transplant and then every 48 h until tissues were harvested.

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2.2. Histology. Transplanted donor hearts were formalin fixed and paraffin embedded, and sections were cut at ~4 µm thickness for hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) analysis. Hearts stained with H&E were evaluated for general histology and rejection according to the International Society for Heart and Lung Transplantation [13]. Briefly, heart sections from day 2 and day 5 following transplantation were evaluated by a trained pathologist and assessed for the grade of rejection defined as follows: 1R = mild, focal perivascular and/or interstitial infiltrate without myocyte damage; 2R = medium, multifocal infiltrate with myocyte damage; 3R = severe, diffuse polymorph infiltrate with extensive myocyte damage and/or edema and/or hemorrhage and/or vasculitis; or “Quilty” = inflammation from the outside-in, that is, starting from the epicardium and encroaching on the myocardium, possibly due to infection rather than rejection.

2.3. Flow Cytometry Analysis. Single-cell suspensions of spleen were obtained by mincing the organs through a cell strainer dish and stained according to standard procedures. If not mentioned, all the antibodies were purchased from BioLegend (San Diego, USA). Flow cytometry was performed on a BD FACSCanto or Accuri C6 flow cytometer (BD Biosciences, San Jose, USA). Cell surface staining of CD3, CD4, CD8, B220, NK, NKT, macrophages, and DCs was
performed following standard protocols; total splenocytes were stimulated with PMA (50 ng/mL)/Ionomycin (1 μg/mL) (in vitro with monensin) for 4 h; then intracellular staining of FoxP3, IFN-γ, IL17, and IL4 was done using the eBioscience staining buffer. All the data were analyzed using FlowJo software (FlowJo, Ashland, USA) excluding cell doublets.

2.4. RNA Isolation and qPCR. Total RNA was isolated from donor heart tissues using Trizol. RNA quality was assessed using a NanoDrop (Wilmington, DE) where the 260/280 ratios were obtained. Samples with a ratio of 1.7–2.1 were utilized for downstream gene analysis. Approximately 1 μg total RNA from each sample was reverse-transcribed using M-MLV (Promega, Madison, WI), oligo-DT, random primers, and dNTPs from Roche (Indianapolis, IN). cDNA was then diluted ~1:25 in nuclease free water to be used for qPCR. All primers used were intron spanning, and gene expression levels were analyzed using TaqMan chemistry with primers and probes designed in Roche’s Universal Probe Library Design Center. All experiments were conducted on a LightCycler 480 (Roche). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [14], and studies were designed according to the MIQE guidelines [15]. For all housekeeping and target genes assessed, standard curves were evaluated on 10-fold serial dilutions of control cDNA to determine reaction efficiency. Where efficiencies between housekeeping and target genes differed >5%, efficiencies could be accounted for in the LightCycler 480 software during analysis of relative gene expression. A complete list of target genes and primers is provided in Table 1. Specificity of primer pairs was evaluated through the use of both no-RT and water template control samples. Only primers that did not amplify more than one product or any product in the no-RT and water controls were used for final experiments.

2.5. Statistical Analysis. One-way ANOVA using the Tukey-Kramer post hoc analysis for differences between means assessed statistical differences between animal groups and time points for survival times and flow cytometry. Data from qPCR was analyzed using relative comparison from the Roche LightCycler software, which is based on the $2^{-\Delta\Delta CT}$ method. Efficiencies of each primer set were determined by standard curve analysis and considered by the software algorithm for determining relative expression over a housekeeping gene, ornithine decarboxylase (Odc).

3. Results

3.1. Activation of Nrf2 Prolongs Graft Survival of Heart Transplants in Mice. To examine the role of Nrf2 in heart transplant graft survival, hearts from Balb/c mice were transplanted into C57BL/6 Nrf2$^{+/+}$ (WT) with or without sulforaphane (WT + SF) treatment or Nrf2$^{-/-}$ (KO) mice. Table 2 illustrates that loss of Nrf2 function in mice results in a significantly shorter survival time of the Balb/c graft when compared to their wild-type counterparts, implying that Nrf2 has a protective role in graft survival. Notably, treatment of recipient Nrf2$^{+/+}$ mice with the Nrf2 activator SF significantly extended the survival of the graft to nearly 13 days. Figure 2 provides a representative illustration of Nrf2 levels in grafts 2 days after transplantation. Note the minimal Nrf2 expression in a few nuclei of the Balb/c donor hearts transplanted into Nrf2 WT (Figure 2(a)) and KO (Figure 2(b)) mice, whereas Nrf2 expression is increased in grafts where Nrf2 WT recipient mice received SF treatment (Figure 2(c)). It is important to note that treatment with SF in the Nrf2$^{-/-}$ mice showed no effects on overall survival time of the graft (data not shown), indicating that SF treatment protects the heart graft from rejection largely through Nrf2 activation. Therefore, activation of Nrf2 has a great therapeutic potential to prolong graft survival in transplantation.

3.2. Treatment of Recipient Mice with Sulforaphane Delays Transplant Rejection. Donated Balb/c hearts were analyzed at 2 and 5 days following transplantation for histology and rejection classification. A summary of the analysis and complete data presentation is provided in Table 3, and representative
**Table 3: Sulforaphane pretreatment delays graft rejection.**

<table>
<thead>
<tr>
<th></th>
<th>WT day 2 (grade N/total N)</th>
<th>KO day 2 (grade N/total N)</th>
<th>WT + SF day 2 (grade N/total N)</th>
<th>WT day 5 (grade N/total N)</th>
<th>KO day 5 (grade N/total N)</th>
<th>WT + SF day 5 (grade N/total N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No rejection</td>
<td>1/5</td>
<td>2/3</td>
<td>5/5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1R</td>
<td>1/5</td>
<td>1/3</td>
<td></td>
<td>1/4</td>
<td>1/4</td>
<td></td>
</tr>
<tr>
<td>Grade 2R</td>
<td>3/5</td>
<td></td>
<td>3/4</td>
<td>3/4</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>Quilty</td>
<td></td>
<td></td>
<td>4/4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*N = 3–5 animals for each group. Data is displayed as “N”—meeting histological criteria/total analyzed “N.” Heart sections were formalin fixed and paraffin embedded prior to being stained with H&E for histology analysis. Definition of terms: 1R = mild, focal perivascular and/or interstitial infiltrate without myocyte damage, 2R = medium, multifocal infiltrate with myocyte damage, “Quilty”: inflammation from the outside-in, that is, starting from the epicardium, possibly due to infection rather than rejection.

**Figure 3: Representative histology from donor Balb/c hearts.** Hearts isolated at 2 and 5 days following transplant were analyzed by histology for rejection grading. Representative images display no rejection (a), Grade 1 rejection (b), and Grade 2 rejection (c) or “Quilty” phenomenon (d). Images were taken at 20x.

images of the pathological histology used to categorize each animal are displayed in Figure 3. In summary, Nrf2+/- and -/- animals showed some low-grade rejection (see Figure 3(b) for representative image of Grade 1R) at 2 days after transplant as similar levels of lymphocyte infiltration were observed. Remarkably, Nrf2+/- animals pretreated with SF showed no histological signs of rejection 2 days after transplant (see Figure 3(a) for representative image), suggesting that activation of Nrf2 can delay transplant rejection. However, all animals showed significant signs of graft rejection 5 days following transplant (see Figure 3(c) for representative image of Grade 2R), with Nrf2+/- animals all displaying signs of “Quilty,” a phenomenon thought to be independent of rejection, showing infiltrate originating from the epicardium and infiltrating inward (see Figure 3(d) for representative image of “Quilty”). These data suggest that activation of Nrf2 with SF is able to delay graft rejection and infiltrates of immune cells for at least the first 2 days after transplant, possibly contributing to the significant increase in overall graft survival observed in these animals.

3.3. Nrf2 Influences IL-17 Production in the Spleen of Recipients. T cells are predominating players for acute graft rejection (for review, see [16]). To investigate a potential mechanism mediating rejection in the Nrf2 KO mice, we analyzed the T-cell immune responses in the spleens of
Figure 4: T-cell populations are generally unchanged in spleens from transplant recipients. Flow cytometry analysis on spleens isolated from recipient mice (Nrf2 WT, KO, or WT + SF) at either day 2 (2 d) or day 5 (5 d) following transplantation. No significant differences were found in T-cell populations including total (CD3⁺) (a), Tregs (b), CD8⁺ (c), CD4⁺ (d), Th1⁺ (e), Th2⁺ (f), Tc1⁺ (g), or Tc2⁺ (h). N-size ranged from 4 to 7 animals per group, per time point.
recipient mice. There were no remarkable differences in the numbers of total T cells (CD3+, CD4+, CD8+, Th1, Th2, Tc1, or Tc2) in the spleens of recipients (Figure 4). However, mRNA levels of the Th17 cytokine and IL-17 were markedly higher in the donated hearts transplanted to KO recipients at 2 days following transplant (Figure 5). Together with the notion that loss of Nrf2 function facilitates heart graft rejection (Table 2), the elevated IL-17 production in the graft likely contributes to the rejection. Consistent with the histological analysis, at 5 days after transplant IL-17 mRNA levels in the donated hearts are elevated in all recipient groups regardless of genotype or treatment, implying that other factors exist in the graft-specific immune response due to the altered Nrf2 function.

3.4. Nrf2 KO Mice Generate a Significantly Greater Macrophage Population in Response to Transplant. Evaluation of spleens from recipient C57BL/6 mice for non-T-cell immune cells revealed a significant elevation in CD11b+ F4/80+ macrophages in KO mice (Figure 6(a)), whereas other non-T-cell populations such as NK, DC, and B cells are unchanged regardless of genotype, treatment, or time point after transplantation (Figure 7). These data imply a possible link of elevated macrophage population in the spleens of the KO recipient mice to heart graft rejection. To assess whether this additional macrophage population in the KO animals correlated with increased macrophage infiltration of the graft, we evaluated CD11b expression by IHC, as well as CD11b and F4/80 mRNA expression in the grafts from each animal group. Analysis by IHC for CD11b in formalin-fixed, paraffin-embedded grafts yielded inconclusive results due to minimal positive staining within the graft (data not shown). Additionally, evaluation of mRNA levels of CD11b and F4/80 did not show any notable changes in expression (less than 2-fold) between groups when data were normalized to the Nrf2 WT day 2 group (Figure 6(b)).

4. Discussion

Currently heart transplantation is the only option for end-stage heart failure disease, and prolonging graft survival is still the subject of intense research. In this study the effects of Nrf2 on host defense and allogeneic graft survival were examined. Our results support that Nrf2 is required to maintain graft survival and that activation of the Nrf2 pathway could prove to be beneficial in extending the life of the transplanted heart.

The role of Nrf2 in the innate and adaptive immune systems continues to be uncovered. Recently, Nrf2 has been implicated in altering maturation of dendritic cells (DCs) [17, 18]. In addition, activation of Nrf2 in older mice upregulates Th1 immunity by maintaining DC redox equilibrium [19]. Although we did not directly assess DC maturity or costimulatory responses in the current study, no alterations in total DC population were observed in the spleens of Nrf2 WT, KO, or WT + SF treated mice. What is more, activation of Nrf2 by the common food preservative, tert-butylhydroquinone (tBHQ), appears to skew CD4+ T cells toward Th2 differentiation; however, there were no observable differences in CD4+ or Th2+ T cells in any of our treatment groups. In the current study we utilized a well-known Nrf2 activator, the isothiocyanate sulforaphane. One possibility is that the mechanism of action of either or both of these activators could have off-target effects that influence T-cell differentiation through a non-Nrf2-mediated pathway.

Nrf2 KO mice have been shown to suffer from splenomegaly and spleen cell death and inflammation in older age, also resulting in hemolytic anemia caused by increased sequestration of IgG-bound erythrocytes in
the spleen [20]. Although loss of Nrf2 has been implicated in other autoimmune disorders [21], the anemia in the study by Lee et al. was instead damage induced from increased oxidative stress. It is hard to determine whether an underlying immune-mediated anemia in the KO mice in this study could have affected the systemic immune response to graft rejection; however, given the young age of the mice in the present study, it is unlikely.

In the case of bacterial infection and sepsis, Nrf2 has been shown to contribute to mobilizing the immune response and counteract the oxidative stress in monocytes [22, 23]. In fact Nrf2 is critical at modulating an “appropriate” level of inflammatory response so as not to be damaging to the host organs and cells. Loss of Nrf2 dramatically impacts survival during experimental sepsis due to deregulated inflammation and activation of Nrf2 can improve survival [24]. Some evidence of this was seen in the present study where CD11b⁺ macrophages were significantly elevated in the spleen of Nrf2 KO at 2 days after heart transplantation, indicating a disproportionate innate response in the early days after transplant. However, follow-up analysis by IHC and qPCR could not conclusively provide evidence of increased macrophage infiltration in the Nrf2 KO recipient animals. Nrf2 WT mice when given SF did not display a significantly lower CD11b⁺ count. One possibility is that Nrf2 activation by SF was able to minimize the innate response to early graft rejection thereby extending the overall graft survival by ∼5 and ∼3 days over KO and WT mice, respectively. Taken together, the increased macrophage number in the KO spleens may not result in increased infiltration into the graft; however, increased macrophages in the peripheral lymphoid organs could result in production of additional factors such as cytokines, ROS activity, and stress responses, which lead to the earlier rejection.

An additional avenue worth pursuing in the future of this work is the specific scavenging of ROS that is mediated by the Nrf2 pathway. Ischemia-reperfusion injury is a common issue with organ transplantation and has been shown previously to dictate the rejection response over that of recipient immunity [25]. One explanation of poor transplant tolerance in Nrf2 KO mice could be due to their lack of ability to scavenge ROS. Ischemia-reperfusion injury inherently generates high levels of ROS that establish an environment of inflammation and infiltrating leukocytes. Treatment of recipient Nrf2 WT mice with SF could decrease ROS production and assist in minimizing inflammation as well as altering the chemokine environment in the graft to delay intrusion of recipient immune effectors.

Interestingly, there were only subtle differences between T-cell and non-T-cell populations in the recipient spleens of mice in this study. Although CD11b⁺ macrophages were the only cell population that reached statistical significance, Th17 cells did trend to being higher in Nrf2 KO mice, which was supported by the overwhelming presence of IL-17 mRNA in grafts taken 2 days after transplant from KO mice. Conversely, IL-17 mRNA was notably reduced in grafts harvested 2 days after transplant from WT mice treated with SF. These data support that though there may not be differences in T-cell differentiation or total population, perhaps trafficking of immune cells is somehow different between Nrf2 WT and KO genotypes.

5. Conclusions

The work presented here displays the importance of Nrf2 in graft survival in an allogeneic mouse model of heart transplant. These data provide an initial insight into the therapeutic benefit of Nrf2 activation during transplant. Yet more work needs to be done to prove the hypotheses laid forth in this study, as well as to fully interrogate how Nrf2
Figure 7: No alterations in non-T-cell immune populations regardless of Nrf2 genotype. Flow cytometry analysis on spleens isolated from recipient mice (Nrf2 WT, KO, or WT + SF) at either day 2 (2 d) or day 5 (5 d) following transplantation did not reveal other differences in non-T-cell immune cells such as NK (a), NKT (b), dendritic cells (c), or B cells (d).

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(and its activation) is able to prolong graft survival upon heart transplant. Though the mechanism is critically important, the implications of using Nrf2 activators in a clinical setting to improve allogeneic graft survival are established by our current work.

Conflict of Interests

The authors have no conflict of interests to disclose, such as financial holdings, professional affiliations, advisory positions, board memberships, patent holdings, and the like, or involvement with any commercial secondary interests.

Authors’ Contribution

W. Wu and Q. Qiu contributed equally to this work.

References


Research Article

Low-Dose Radiation Activates Akt and Nrf2 in the Kidney of Diabetic Mice: A Potential Mechanism to Prevent Diabetic Nephropathy

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Repetitive exposure of diabetic mice to low-dose radiation (LDR) at 25 mGy could significantly attenuate diabetes-induced renal inflammation, oxidative damage, remodeling, and dysfunction, for which, however, the underlying mechanism remained unknown. The present study explored the effects of LDR on the expression and function of Akt and Nrf2 in the kidney of diabetic mice. C57BL/6J mice were used to induce type 1 diabetes with multiple low-dose streptozotocin. Diabetic and age-matched control mice were irradiated with whole body X-rays at either single 25 mGy and 75 mGy or accumulated 75 mGy (25 mGy daily for 3 days) and then sacrificed at 1–12 h for examining renal Akt phosphorylation and Nrf2 expression and function. We found that 75 mGy of X-rays can stimulate Akt signaling pathway and upregulate Nrf2 expression and function in diabetic kidneys; single exposure of 25 mGy did not, but three exposures to 25 mGy of X-rays could offer a similar effect as single exposure to 75 mGy on the stimulation of Akt phosphorylation and the upregulation of Nrf2 expression and transcription function. These results suggest that single 75 mGy or multiple 25 mGy of X-rays can stimulate Akt phosphorylation and upregulate Nrf2 expression and function, which may explain the prevention of LDR against the diabetic nephropathy mentioned above.

1. Introduction

Radiation at high doses is known to cause cytotoxic effects in vitro and in vivo; however, radiation at low doses induces an adaptive effect or hormesis, showing a protective effect on subsequent challenges-induced damage in vitro and in vivo [1, 2]. One of the major mechanisms for low-dose radiation (LDR) to induce adaptive response is the induction of cellular protective components that offer the protection against the damage induced by subsequent challenges including radiation, chemicals, and even diseases [3].

Diabetes mellitus (DM) is a global health problem due to its serious complications. Among diabetic complications, nephropathy probably is one of the major complications to increase the mortality of diabetic patients or impact their life quality. Although mechanisms by which diabetes induces the development of nephropathy are multiple, excessive production of reactive oxygen species (ROS) diabetic condition seems the primary factor [4–6]. We have phenomenally
observed the protective effect of LDR on various diabetic complications, including the testicular, renal, and cardiac damages in diabetic rats and mice [7–10]. In fact, an early study from other groups also implicated the protective effect of LDR on brain damage in the diabetic rat model [11]. In order to explore the protective mechanisms by which LDR prevents diabetic complications, we propose LDR may stimulate cell survival and growth signaling pathways and up-regulate antioxidant system.

Among cell survival pathways, the protein kinase B (PKB)/Akt, as a family of serine/threonine protein kinases, plays important roles in a diverse numbers of processes including cell survival, cell growth, gene expression, apoptosis, protein synthesis, energy metabolism, and oncogenesis. Several publications have demonstrated the protective role of Akt in preventing the pathogenesis of diabetic nephropathy [12].

Nuclear factor E2–related factor-2 (Nrf2) is a key transcription factor in regulating intracellular redox balance and a sensor of oxidative and electrophilic stress. Nrf2 regulates intracellular antioxidants, phase II detoxifying enzymes, and many other proteins that detoxify xenobiotics and neutralize ROS and/or RNS to maintain cellular redox homeostasis. NAD(P)H quinone oxidoreductase (NQO1), heme oxygenase-1 (HO-1), and glutathione S-transferase are among the well-studied Nrf2 target genes that are upregulated through the antioxidant response element regulatory element in response to oxidative stress [13–15]. The important role of Nrf2 in combating oxidative stress induced by diabetes has been demonstrated by the increased cardiac and renal sensitivity of Nrf2−/− mice to diabetes [13, 16, 17].

The present study, therefore, was to test our hypotheses that LDR protection from diabetes-induced renal damage may include the stimulation of cell survival pathways and the upregulation of antioxidant system. To this end, we have used a type 1 diabetic model that was induced by streptozotocin (STZ) as used before [7–10]. We irradiated these diabetic mice and age-matched controls to different forms of LDR, and then immediately investigate whether LDR can stimulate Akt as a key cell survival pathway and Nrf2 expression and transcription function as the most important antioxidative mechanism. We found that single 25 mGy of X-rays did not, but single 75 mGy or accumulated 75 mGy (25 mGy daily × 3) of X-rays could significantly stimulate Akt function. These two forms of LDRs also significantly upregulate Nrf2 expression and transcription. The latter was reflected by stimulation of Nrf2 downstream antioxidants including NQO1 and HO-1. Therefore, stimulation of these cell survival pathway and antioxidative mechanisms may play the critical role in the prevention of diabetic renal damage and dysfunction by LDR.

2. Materials and Methods

2.1. Animals. Eight weeks old male C57BL/6j mice were purchased from and also housed in Jilin University Animal Center at 22°C with a 12:12-light-dark cycle and free access to rodent chow and tap water for ≥2 weeks before being used for experiments. All animal procedures were approved by the University Animal Care and Use Committee, which is certified by the Chinese Association of Accreditation of Laboratory Animal Care. Body weights of mice were measured every 3 days.

2.2. Induction of Type 1 Diabetes. Mice were randomly divided into two groups, a STZ-treated diabetic group and age-matched control group. STZ (Sigma Chemical, St. Louis, MO) was freshly dissolved in 0.05 M sodium citrate buffer (pH 4.5). Both groups of mice were fasted overnight and then were given by intraperitoneal injection of STZ at 60 mg/kg body weight daily for 6 consecutive days or equivalent volume of the citrate buffer. Blood glucose level was determined using a Freestyle glucometer 1 week after the last injection of STZ. We considered mice to be diabetic when blood glucose was ≥12 mmol/L.

2.3. Whole-Body LDR. A Phillips therapeutic X-ray machine (XSS 205FZ) was operated at 200 kV and 10 mA in the presence of 1.0 mm Al and 0.5 mm Cu filters. LDR was given to mice in whole body at dose of 25 mGy or 75 mGy with a dose rate of 12.5 mGy/min.

Mice from both diabetic and age-matched control groups were randomly divided into two groups with and without LDR that is, total four groups: control, LDR, DM, and DM/LDR. For LDR groups, mice were further divided into 3 groups: a single dose of 25 mGy, a single dose of 75 mGy, and accumulated 75 mGy (25 mGy daily for 3 days). Eight mice from each group were euthanized at 1, 3, 6, 9, and 12 h after LDR exposure. The protocol was approved by the Committee on the Ethics of Animal Experiments of Jilin University, Jilin, China (Permit Number: 2007-0011). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.4. Western Blotting. Tissue lysates were prepared in lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 50 mmol/L Tris, pH 8.0, 1 mmol/L EDTA, 10 mg/L phenylmethylsulfonyl fluoride) using a homogenizer on ice. Then supernatants were collected after centrifugation at 12,000 rpm at 4°C for 10 min. Protein concentration was determined by the Bradford assay. Equal amount (50 μg protein/lane) of proteins and prestained molecular weight marker (Gibco-BRL, Gaithersburg, MD) were loaded onto 12% SDS-polyacrylamide gels in a minigel apparatus (Mini-Protean II; Bio-Rad). After being separated on an SDS-PAGE gel, proteins were transferred to PVDF membranes (0.45 μm pore size; Millipore). Nonspecific binding to the membrane was blocked for 1 h at room temperature with 5% nonfat milk in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20). Membranes were then incubated at 4°C with primary antibody in blocking buffer containing 5% nonfat milk shaking overnight. Primary antibodies used in the present study include anti-mouse Nrf2 antibody (1:500, Abcam, Cambridge, MA), anti-mouse NQO1 antibody (1:500, Abcam), anti-mouse Akt antibody, anti-mouse Akt2 antibody
(1:1000, Cell Signaling Technology, Danvers, MA), and HO-1 antibody (1:100, Santa Cruz, CA). After washing with TBST three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h (CST 1:2000). The same membrane was probed with β-actin (1:1000) as loading control. Blots were developed with enhanced chemiluminescent reagent and target band density was scanned using a Molecular Dynamics 300A Laser Densitometer.

2.5. Immunohistochemical Staining. Sections were blocked with Superblock buffer (Pierce, Rockford, IL) for 30 min. Sections then were incubated with primary antibodies of anti-Akt, anti-phospho-Akt, anti-Akt2, and anti-Nrf2, as used for Western blotting assays in 1:50 dilution overnight at 4°C. Following washing with PBS for 3 times, these sections were incubated with biotin-labeled secondary antibody at room temperature for 1 h, followed by color development with diaminobenzidine for 2 min.

2.6. Statistical Analysis. Data were collected from repeated experiments and were presented as means ± SD (n = 8). Comparisons were performed by one-way ANOVA for the different groups, followed by a post hoc Tukey’s test for the difference between groups using statistical software SPSS 14.0. Differences were considered to be significant at P ≤ 0.05.

3. Results

3.1. Diabetic Animal Model and LDR Effects. Whole-blood glucose levels were similar among experimental groups before induction of diabetes by STZ. One week after the last dose of STZ, whole-blood glucose levels were examined. Once hyperglycemia was diagnosed, diabetic mice and age-matched control mice were exposed or shamed to LDR either at single 25 mGy, single 75 mGy, or accumulated 75 mGy (e.g.: 25 mGy daily for 3 days). Body weights among groups were same before study. STZ-induced diabetes prevented the body-weight gain that occurred in age-matched control group (Figure 1(a)). No effect of LDR on the body-weight gain no matter exposure levels (Figure 1(a)). Figure 1(b) shows that whole-blood glucose levels in the DM group significantly increased and LDR did not affect whole-blood glucose levels either in control or diabetic mice.

3.2. LDR Increased Renal Akt Function under Diabetic Conditions. Immunohistochemical staining revealed that the phosphorylated Akt, predominantly localized in the glomerulus of the kidney, was significantly increased at 1, 3 and 6 h after diabetic mice were exposed to 75 mGy of X-rays (Figure 2(a)). Western blot showed that there was no significant difference for the expression level of renal Akt phosphorylation at Ser 473 (p-Akt) between Control and DM groups. Exposure of control mice to 75 mGy did not significantly increase renal p-Akt level at the times 1–12 h after irradiation compared to control mice (Figures 2(b), 2(c)).

3.3. LDR Increased Renal Akt2 Expression under Diabetic Conditions. Immunohistochemical staining revealed that the expression of Akt2, predominantly localized in the glomerulus of the kidney, was significantly increased in DM/LDR group at 3 and 6 h after irradiation with 75 mGy of X-rays (Figure 3(a)). Compared to control, exposure of control mice to 75 mGy did not significantly change renal Akt2 expression at any postirradiation time-point. Renal Akt2 expression was significantly decreased in DM group compared with Control, detected by immunohistochemical staining and western blot (Figures 3(a)–3(d)). Compared to diabetic mice, exposure of diabetic mice to a single 25 mGy LDR also did not affect renal Akt2 expression level (Figure 3(b)); however, exposure to either a single 75 mGy (Figures 3(b), 3(d) or an accumulated 75 mGy (25 mGy × 3, Figures 3(c), 3(d) significantly increased renal Akt2 levels from 3–9 h with a peak at 6–9 h for the single 75 mGy and from 3–6 h with a peak at 6 h for the accumulated 75 mGy, respectively (Figure 3(d)).

3.4. LDR Partially Prevented Diabetic Downregulation of Renal Nrf2 Expression. Immunohistochemical staining revealed that Nrf2 expression, predominantly localized in the glomerulus (Figure 4(a)). The intensity of Nrf2 staining was significantly lower in the kidney of diabetic mice than control or diabetic mice treated with LDR at post 6 h (Figure 4(a)). Western blotting showed that the accumulation of Nrf2 was significantly lower in the kidney of diabetic mice than that of corresponding control mice (Figures 4(b), 4(c)). Exposures of diabetic mice to LDR at single 25 mGy did not have any impact on diabetic inhibition of Nrf2 expression; however, exposure to the single 75 mGy significantly prevented diabetic inhibition of the expression of renal Nrf2 in a time-dependent manner from 1 to 6 h after irradiation (Figures 4(b), 4(c)). Exposure of diabetic mice to the accumulated 75 mGy (25 mGy × 3) also significantly prevented the inhibition of renal Nrf2 expression in diabetic kidney at 3 and 6 h after LDR (Figures 4(b), 4(c)).

3.5. LDR Partially Prevented Diabetic Downregulation of Renal Nrf2 Function. Since Nrf2 is a transcription factor to positively regulate the expression of several downstream genes to play important role in the prevention of oxidative stress and damage, we have examined several of its downstream genes to functionally evaluate Nrf2 in the kidney of diabetic mice with and without LDR. Renal NQO1 expression was found to significantly decrease in diabetic group compared with control, detected by western
Figure 1: Effects of LDR on the body weight and blood glucose in diabetic mice. Diabetic and age-matched control mice were irradiated in whole body with LDR (25 mGy, $3 \times 25$ mGy, or 75 mGy). Body weights (a) and fasting blood glucose levels (b) were measured before and after diabetes as well as before and after LDR at indicated time points ($n = 8$). Data was presented as mean ± SD. *$P < 0.05$ versus Control. Pre-STZ: before diabetes induction by STZ; Pre-LDR: before diabetic and age-matched control mice were irradiated with LDR.
Figure 2: Effects of LDR on renal Akt function in diabetic mice. Renal tissues from mice exposed to sham, single 25 mGy, 75 mGy or $3 \times 25$ mGy of X-rays were collected at 1, 3, 6, 9, and 12 h after LDR to localize the phosphorylated Akt (p-Akt) at Ser473 with immunohistochemical staining ((a), the representative images of the staining were from the group of 75 mGy of LDR). The expression levels of total Akt and p-Akt were also examined by western blotting (b), followed by quantitative analysis (c). Data was presented as mean ± SD. *$P < 0.05$ versus DM. Definition of the abbreviations is provided in Figure 1 legend.
Figure 3: Effects of LDR on renal Akt2 expression in diabetic mice. Animals were treated and renal tissues were collected as described in Figure 2. The localization of Akt2 expression in the kidney was examined by immunochemical staining (a) and the expression of Akt2 and total Akt were detected by western blotting (b) followed by quantitative analysis (c). Data was presented as mean ± SD. *P < 0.05 versus Control, #P < 0.05 versus DM.
Figure 4: Effects of LDR on renal Nrf2 expression in diabetic mice. Animals were treated and renal tissues were collected as described in Figure 2. The localization of Nrf2 expression in the kidney was examined by immunochemical staining (a) and the expression of Nrf2 were detected by western blotting (b) followed with quantitative analysis (c). Data was presented as mean ± SD. *P < 0.05 versus Control; #P < 0.05 versus DM.
Effects of LDR on renal NQO1 expression in diabetic mice. Animals were treated and renal tissues were collected as described in Figure 2, respectively. The expression of NQO1 was detected by western blotting (b) followed with quantitative analysis (c). *P < 0.05 versus Control, #P < 0.05 versus DM.

Renal HO-1 expression, examined by western blot, significantly decreased in DM groups compared to corresponding groups (Figure 6). Exposure of diabetic mice to single 25 mGy X-rays did not have any impact on its expression. In contrast, single 75 mGy or accumulated 75 mGy X-rays significantly prevented diabetic inhibition of HO-1 expression at 3–9 h after irradiation (Figure 6).

4. Discussion

In the present study, we have explored for the first time the effect of LDR on renal expression of Akt as cell survival pathway and Nrf2 as antioxidant transcription regulator in normal and diabetic conditions. We provided the following new findings: 75 mGy of X-rays can stimulate Akt signaling pathway and upregulate Nrf2 expression and function in diabetic kidneys; single exposure of 25 mGy did not, but
three repeated 25 mGy of X-rays could offer similar effects as single 75 mGy exposure on the stimulation of Akt phosphorylation and the upregulation of Nrf2 expression and function.

In our recent study we have demonstrated that multiple exposures of STZ-induced diabetic mice to 25 mGy of X-rays significantly prevented diabetes-induced renal oxidative damage, inflammation, and fibrosis as well as renal dysfunction [9]. This prompted us to know whether there is a stimulation of either cell survival signaling or antioxidant pathway under diabetic condition by exposure to LDR. In the literature, several studies have used in vitro models to show that LDR was able to stimulate cell survival signaling such as Akt phosphorylation [18, 19]. However, whether LDR can stimulate Akt phosphorylation in the tissue of LDR-irradiated animals remain elusive, particularly under diabetic conditions. We found here that single or accumulated 75 mGy of X-rays can significantly stimulate the increase
of renal Akt phosphorylation at 1–12 h with a peak at 6 h. We have reported that in the kidney of type 1 diabetic mice induced by STZ as used here, Akt plays an important role in preventing diabetes-induced renal cell death [20]. Similar antiapoptotic effect of Akt on the tissue or in vitro cultured cells in response to various pathogenic stimuli were also extensively reported [21–24]. Therefore, we assumed that the stimulation of Akt phosphorylation by LDR in the kidney of diabetic mice may be one of the possible mechanisms by which LDR prevents the development of diabetic nephropathy [9].

In our previous study, we have demonstrated that diabetes induced a significant increase of renal oxidative damage that was accompanied with renal inflammation and fibrosis, leading to renal dysfunction [9]. We also reported that LDR protection of rat testes from diabetes-induced cell death and oxidative damage was along with an upregulation of testicular superoxide dismutase levels [10]. These two pieces of important information stimulates us to ask whether LDR protect the kidney also through upregulation of antioxidants. Since Nrf2 plays a critical regulation of cellular antioxidant function and LDR was able to upregulate Nrf2 expression in cultured cell line [25], we investigated whether diabetes affects renal Nrf2 expression and whether LDR upregulates Nrf2 expression and function to increase the expression of antioxidants under diabetic conditions. Here we found that diabetes significantly down-regulate renal Nrf2 expression (Figure 4) and function (Figures 5 and 6), which is consistent with other studies indicating the downregulation of Nrf2 under different pathogenic conditions, including diabetes [26].

We further found that exposure of diabetic mice to either single or accumulated 75 mGy of X-rays can significantly upregulate Nrf2 expression at 3–6 h after irradiation. The upregulated Nrf2 function by single or accumulated 75 mGy of X-rays was also reflected by the upregulation of its down-stream antioxidants, including NQO1 at 3–6 h and HO-1 at 3–9 h (Figures 5 and 6). The difference of peak expression times and durations of these down-stream antioxidants suggests the slight difference of Nrf2 in regulating these antioxidant components at transcriptional level. Activation of Nrf2 by sulforaphane in vitro and by sulforaphane or MG132 in vivo has been reported to suppress high-level glucose-induced oxidative stress and metabolic dysfunction in human microvascular endothelial cells [27] and reduced diabetic proteinuria in STZ-induced diabetic rats [28, 29]. In addition, a recent study demonstrated that Nrf2 can bind to Bcl-2 gene antioxidant response element to upregulate antiapoptotic protein Bcl-2, leading to a prevention of apoptotic cell death [30]. Therefore, upregulation of Nrf2 expression and transcription function may not only turn on antioxidant mechanisms by increasing the expression of antioxidants, but also upregulate antiapoptotic pathways to reduce diabetes-induced renal cell death.

In terms of the hormesis, the optimal and effective doses were extensively documented as 75 mGy [7, 31–34]. Here we also showed a significantly stimulating effect of 75 mGy X-rays on the stimulation of Akt phosphorylation and the upregulation of Nrf2 expression and function. However, accumulated 75 mGy that was given by 3 daily exposures at 25 mGy was similarly effective as single 75 mGy on the stimulation of Akt phosphorylation, but single 25 mGy of X-rays did not have such effect. Our finding is consistent with a recent study that showed that repeated 20 mGy computed tomography scans did not induce genomic instability in reticulocytes, but conferred a significant resistance to larger doses of radiation-induced genomic instability in the bone marrow cells of irradiated mice while exposures to single computed tomography scans exhibit transient genotoxicity, enhanced apoptosis, and enhanced radiation sensitization to subsequent large dose of radiation [35]. This explains why in our previous studies repeated exposure to 25 mGy for 8–12 weeks could significantly prevent diabetes-induced renal and cardiac damage [9]. This is also consistent with the finding by Normura et al., that is, continuous low-dose-rate gamma irradiation ameliorates diabetic nephropathy and increases life span in db/db mice through the activation of renal antioxidants [36].

In summary, we have demonstrated that single or accumulated 75 mGy of X-rays can stimulate Akt signaling pathway and upregulate Nrf2 expression and function in diabetic kidneys. Considering that multiple exposure of diabetic mice to 25 mGy X-rays significantly prevented the development of diabetic nephropathy, we assumed here that the stimulated Akt phosphorylation and upregulated Nrf2 expression and function by LDR in the kidney of diabetic mice may be the mechanisms, in part at least, for the prevention by LDR of diabetic nephropathy.

Conflict of Interests

The authors declare that they have no conflict of interests with SPSS 14.0 software, financial foundation, or any other third party.

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References


Research Article

Prevention of Diabetic Nephropathy by Sulforaphane: Possible Role of Nrf2 Upregulation and Activation

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The present study was to investigate whether sulforaphane (SFN) can prevent diabetic nephropathy in type 1 diabetic mouse model induced by multiple low-dose streptozotocin. Diabetic and age-matched control mice were given SFN at 0.5 mg/kg body weight daily for 3 months. At the end of 3-month SFN treatment, the diabetic nephropathy, shown by renal inflammation, oxidative damage, fibrosis, and dysfunction, was significantly prevented along with an elevation of renal Nrf2 expression and transcription in diabetes/SFN group compared with diabetic group. However, this renal prevention by SFN was not seen when the 3-month SFN-treated diabetic mice were aged for additional 3 months without further SFN treatment. Nrf2-mediated renal protective effects in diabetes were evaluated in human renal tubular HK11 cells transfected with control and Nrf2 siRNA and treated with 27.5 mM mannitol or high glucose plus palmitate (300 μM). Blockade of Nrf2 expression completely abolished SFN prevention of the profibrotic effect induced by high glucose plus palmitate. These results support that renal Nrf2 expression and its transcription play important roles in SFN prevention of diabetes-induced renal damage. However, the SFN preventive effect on diabetes-induced renal pathogeneses is not sustained, suggesting the requirement of continual use of SFN for its sustained effect.

1. Introduction

Diabetic nephropathy is characterized by initial oxidative stress, inflammatory response, thickening of basement membranes, expansion of mesangial matrix and interstitial fibrosis, podocytes and renal cell death, increased albuminuria, and renal dysfunction [1–3]. Reactive oxygen or nitrogen species (ROS or RNS) production in response to hyperglycemia, advanced glycosylation end products, hyperlipidemia, inflammatory cytokines including transforming growth factor (TGF)-β1, and hypertension contributes to these renal pathogenic changes [1]. However, supplying exogenous antioxidants failed to demonstrate a significant therapeutic effect on human diabetic complications [1–4]. Therefore, a strategy to upregulate endogenous multiple antioxidants, instead of exogenously supplying a single or limited combination of antioxidants, would be more efficient approach to prevent or treat diabetic complications [4].

The transcription factor NFE2-related factor 2 (Nrf2) as one member of the cap “n” collar family is a master regulator of cellular detoxification responses and redox status [5]. Under physiological conditions kelch-like ECH-associated protein 1 (KEAP1) binds to Nrf2 and sequesters it in the cytoplasm [6]. Under basal conditions, KEAP1 mediates rapid ubiquitination and subsequent degradation of Nrf2 by the proteasome [6]. Upon exposure of cells to
oxidative stress or electrophilic compounds, Nrf2 dissociates from KEAP1 and translocates into the nucleus to bind to antioxidant-responsive elements in the genes encoding antioxidant enzymes, namely, NADPH quinone oxidoreductase (NQO1), heme oxygenase-1 (HO-1), glutathione S-transferase, superoxide dismutase (SOD), catalase (CAT), and γ-glutamylcyclsteine synthetase. Upregulation of these Nrf2-dependent antioxidants promotes detoxification, and anti-inflammatory function [6, 7]. A growing body of evidence has indicated a critical role for activator-induced Nrf2 upregulation in the prevention of diabetic complications, including nephropathy [7, 8].

Sulforaphane (SFN) is an organosulfur compound that exhibits anticancer and antidiabetic properties in experimental models and obtained from cruciferous vegetables such as broccoli, brussels sprouts, or cabbages [9]. Studies showed that SFN metabolites were detected in all tissues at 2 and 6 h after gavage, with the highest concentrations in the small intestine, prostate, lung, and kidney, suggesting that SFN is bioavailable and will be an effective chemoprevention agent for these tissues [10]. SFN has garnered particular interest as an indirect antioxidant due to its extraordinary ability to induce expression of several enzymes via the KEAP1/Nrf2 pathway [9, 10]. Several studies have shown the preventive effect of SFN via induction of Nrf2 on chemical or ischemia-induced renal damage [11, 12]. A recent study has shown that after chronic treatment with SFN for 4 months diabetic mice exhibited significant renal prevention from diabetes-induced damage most likely via induction of Nrf2-mediated antioxidant pathway [13]. However, it was unclear whether a sustained renal protection would be seen in diabetic mice beyond the time-point of SFN treatment.

The present study investigated whether treatment of diabetic mice for 3 months with SFN could prevent the development of diabetic nephropathy three months after terminating SFN treatment. These would determine whether SFN has sustained protective effects in controlling diabetes-induced renal damage. We used a type 1 diabetic mouse model induced with multiple low-dose streptozotocin (MLD-STZ) as previously demonstrated by our laboratory [14]. In addition to animal experiments, we utilized in vitro cultures of human renal tubular HK 11 cells to modulate Nrf2 expression and determine a mechanistic role of Nrf2 in diabetic nephropathy.

2. Results

2.1. Effects of SFN on the Blood Glucose and Renal Function in Diabetic Mice. After the onset of hyperglycemia, diabetic and age-matched control mice were subcutaneously given SFN at 0.5 mg/kg daily for 3-months. At the end of 3 month treatment of SFN one set of mice were sacrificed to perform the experimental measurements (3-month time-point). The second set of both diabetic and control mice were aged for additional 3 months without further SFN treatment (6 month time-point). We demonstrate that after MLD-STZ induction of diabetes, blood glucose levels in diabetes (DM) groups with or without SFN treatment were significantly increased without difference between DM and DM plus SFN (DM/SFN) group (Table 1).

It was also shown that diabetes significantly increased albumin-to-creatinine ratio (ACR) at 3 months of diabetes and further increased it at 6-months of diabetes. Treatment with SFN for 3-month partially attenuated diabetes-increased ACR at 3-month time-point, but this partial reduction of ACR seen at 3 months time-point was diminished when it was examined at 6 month time-point. Similar to ACR, the ratio of kidney weight (KW) to tibia length (TL) was significantly increased in diabetes group compared to control animals, and SFN treatment of diabetic mice decreased this ratio at 3-month time-point, but not at 6-month time-point (Table 1).

2.2. SFN Prevented Diabetes-Induced Renal Fibrosis, Inflammation, and Oxidative Stress. Histological examination with hematoxylin and eosin (H&E) staining showed the significantly progressive changes of the renal structure, including glomerular basement membrane thickening, mesangial cell proliferation, increased mesangial matrix, and Kimmelstiel Wilson (K-W) nodules along with renal tubular epithelium damage and a large number of protein casts in DM group (Figure 1(a)). SFN treatment significantly prevented these changes in the DM/SFN group, examined at 3-month time-point, but not at 6-month time-point (Figure 1(a)).

We examined the glycogen accumulation in the kidney by periodic acid-Schiff (PAS) staining, which showed that diabetes induced a significant glycogen accumulation in a time-dependent manner from 3 to 6 months. This effect was significantly prevented by SFN treatment, examined at 3 month time-point, but not at 6 months (Figure 1(b)).

Diabetes-induced renal fibrosis was further confirmed by the increased renal protein expression of transforming growth factor (TGF)-β1 and connective tissue growth factor (CTGF) as two critical profibrotic mediators (Figure 1(c)). SFN prevention of diabetes-induced TGF-β1 and CTGF expression was only observed at the 3-month time-point (Figure 1(c)).

Since inflammation and oxidative stress have been suggested to play an important role in diabetes-induced renal pathogenesis [15–18], we performed Western blot assay for the renal expression of inflammatory cytokines, plasminogen activator inhibitor (PAI)-1 (Figure 2(a)), and tumor necrosis factor (TNF)-α (Figure 2(b)), which were progressively increased in DM group and partially prevented by SFN treatment only at 3-month time-point.

The next study using Western blotting assay showed the significant increase in renal oxidative damage, by detection of 3-nitrotyrosine (3-NT) accumulation as an index of nitrosative damage (Figure 2(c)) and 4-hydroxy-2-nonenal (4-HNE) accumulation as an index of lipid peroxidation (Figure 2(d)). The oxidative and nitrative damage-induced diabetes was prevented almost completely by SFN treatment only at 3-month time-point.

2.3. SFN Upregulated the Expression of Nrf2 and Its Downstream Genes In Vivo. Above results showed that SFN can protect diabetic induction of renal dysfunction, fibrosis,
Figure 1: SFN prevented diabetes-induced renal structural changes and fibrosis. Diabetic and age-matched mice were treated with SFN at 0.5 mg/kg daily for five days in each week for 3 months, and then some were used to perform the experimental measurements at the end of 3-month SFN treatment (3-month time-point). Some of these diabetic and age-matched control mice with and without 3-month SFN treatment were kept for additional 3 months without further SFN treatment and then were sacrificed for study (6-month time-point). To examine renal morphology sections were stained with hematoxylin and eosin (a). Periodic acid-Schiff staining was used to examine glycogen accumulation (b). Bar = 100 μM. Western blotting assay was performed for measuring fibrosis by TGF-β1 and CTGF protein expression (c). Data were presented as means ± SD (n = 6 at least) * P < 0.05 versus control; ( # P < 0.05 versus DM group).
Figure 2: SFN prevented diabetes-induced renal inflammation and oxidative stress. Western blotting assay was performed for measuring the expression of inflammatory cytokines PAI-1 (a) and TNF-α (b), and oxidative damage accumulation of 3-NT (c) and 4-HNE (d). Data were presented as means ± SD (n = 6 at least). (*P < 0.05 versus control; #P < 0.05 versus DM group).
inflammation, and oxidative damage. One of SFN effects is induction of Nrf2; therefore, whether SFN protects the kidney from diabetes by activating Nrf2 was examined first by measuring renal Nrf2 expression and its transcription function. Nrf2 expression at mRNA (Figure 3(a)) and protein (Figure 4(a)) levels, measured by real-time PCR and Western blot, respectively, was significantly increased in the kidney of SFN-treated control mice and diabetic mice at 3-month time-point, but not at 6 months. There was a significant additive increase in the renal Nrf2 expression in DM/SFN group, compared to DM group at the end of 3-month SFN treatment.

Next, we explored Nrf2 transcriptional function by examining the expression of its downstream target antioxidant genes at mRNA and protein levels. (1) At 3-month time-point, diabetes significantly increased expression of renal NQO1 and HO-1, but significantly decreased the expression of renal cytosolic SOD (SOD1), mitochondrial SOD (SOD2), and CAT at both mRNA (Figure 3) and protein levels (Figure 4). Renal expression of NQO1, HO-1, SOD1, SOD2, and CAT at mRNA and protein levels in the group of DM/SFN was significantly higher than that in DM group (Figures 3 and 4) (2) At 6-month time-point, there was no significant difference for the renal expression of NQO1 and HO-1 among groups, but there were significant decreases in renal expression of SOD1, SOD2, and CAT at both mRNA and protein levels in DM and DM/SFN groups compared to control and SFN groups (Figures 3 and 4). No significant differences were observed between DM and DM/SFN samples.

2.4. *SFN Protected High-Glucose (HG)-Induced Inflammatory and Fibrotic Responses via Upregulating Nrf2 Expression and Function In Vitro.* The *in vivo* experiments discussed above suggest a role for Nrf2 in SFN-mediated inhibition of renal damage seen in 3-month diabetic mice. In the following studies, the direct role of Nrf2 in the reduction of diabetic renal damage was further defined using human kidney proximal tubular (HK 11) cells. First we demonstrated that SFN induced Nrf2 and HO-1 protein expression in a dose-dependent manner in HK 11 cells (Figure 5(a)). HK11 cells were treated with and without HG (27.5 mM) for 48 h with palmitate (Pal, 300 μM) for the last 6 h with the presence or absence of SFN (3 μM for 12–48 h). Western blotting assay showed that HG/Pal treatment significantly increased CTGF expression, which was time-dependently prevented by pretreatment with SFN (Figure 5(b)).

Subsequently HK 11 cells were treated with HG in the presence of SFN (3 μM) for 48 h, and, at the last 6 h, Pal (300 μM) was added into the culture medium. Expression of CTGF, Nrf2, and HO-1 was analyzed by Western blotting assay (Figure 5(c)). Both SFN and HG/Pal can significantly increase Nrf2 and HO-1 expression, but only HG/Pal increased CTGF expression. Figure 5 also showed that SFN/HG/Pal can synergistically increase Nrf2 and HO-1 expression, along with complete inhibition of CTGF expression induced by HG/Pal.

2.5. **Silencing Nrf2 by Small Interfering RNA (siRNA) Abolished the Prevention of HG/Pal-Induced Profibrotic Effect by SFN.** HO-1 and Nrf2 expression was upregulated in SFN, HG/Pal and SFN/HG/Pal groups (Figure 5(c)), suggesting upregulation of Nrf2 transcriptional function. Thus, we determined a direct role of Nrf2 in SFN-mediated prevention of HG/Pal-induced profibrotic effect by silencing Nrf2 expression. HK 11 cells were transfected with human specific Nrf2 siRNA (NFE2L2HSS181505) or control siRNA for 48 h, and then incubated with new medium containing either 5.5 mM or 27.5 mM glucose for additional 48 h. At the last 9 h and 6 h during the second 48 h incubation, SFN (3 μM) and Pal (300 μM) were added, respectively (Figure 6). Western blotting analysis showed that with the control siRNA, SFN treatment upregulated Nrf2 and HO-1 expression with a significant prevention of HG/Pal-induced CTGF; however, with the Nrf2 specific siRNA, SFN treatment could not upregulate the expression of either Nrf2 or HO-1 and also did not prevent HG/Pal-induced CTGF expression (Figure 6). This result demonstrates the requirement of Nrf2 expression and function for SFN prevention of HG/Pal-induced renal fibrotic response.

3. **Discussion**

Using MLD-STZ-induced type 1 diabetic mouse model, we demonstrated the importance of SFN-induced Nrf2 expression in the renal protection against diabetes-induced damage. This was reflected by the significant reduction of diabetes-increased ACR and the ratio of kidney weight to tibia length as the index of renal dysfunction, and also reduction of diabetes-induced renal oxidative damage, inflammation, fibrosis, and structural abnormalities, observed at the 3-month time-point, but not at 6-month time-point.
Figure 3: SFN upregulated renal expression of Nrf2 and its downstream genes at mRNA level. Nrf2 (a) and its downstream genes, NQO1 (b), HO-1 (c), SOD1 (d), SOD2 (e), and CAT (f) expression at mRNA level were detected by RT-PCR. Data were presented as means ± SD (n = 6 at least). (∗P < 0.05 versus control; ∗∗P < 0.05 versus DM group).
Figure 4: SFN upregulated renal expression of Nrf2 and its downstream genes at protein level. Nrf2 (a) and its downstream genes, NQO1 (b), HO-1 (c), SOD1 (d), SOD2 (e), and CAT (f) expression in protein level was detected by Western blotting assay. Data were presented as means ± SD (n = 6 at least). (∗P < 0.05 versus control; #P < 0.05 versus DM group).
Figure 5: SFN protected hyperglycemia/hyperlipidemia-induced fibrotic response along with activation of Nrf2 in vitro. HK11 cells were treated with different doses of SFN (0.5 μM–10 μM) for 24 h, and then expression of Nrf2 and HO-1 at protein levels was measured by Western blotting assay (a). HK11 cells were treated with high glucose (HG, 27.5 mM) for 48 h, and, at the last 6 h, palmitate (Pal, 300 μM) was added into the culture medium. Cells were treated with SFN (3 μM) for different times (from 12 h to 48 h), and then expression of CTGF was analyzed by Western blotting assay (b). HK11 cells were treated with HG and SFN for 48 h, and, at the last 6 h, Pal was added into the culture medium as described in panel B. The expression of CTGF, Nrf2, and HO-1 at protein levels was analyzed by Western blotting assay (c). Data are presented as mean ± SD from at least three separate experiments. (∗P < 0.05 versus control group; #P < 0.05 versus HG/Pal group).
presented as mean ± SD from at least three separate experiments. (*P < 0.05 versus control group; †P < 0.05 versus HG/Pal group).

Emerging evidence indicates that Nrf2 was upregulated in the cells in vitro and tissues in vivo when they are challenged by oxidative stress. Several studies have indicated the induction of ROS and/or RNS by HG in the cultured renal cells [19–21]. Using human mesangial cells, Jiang et al. demonstrated HG-induced elevation of nuclear protein level of Nrf2 along with an upregulation of the mRNA level of NQO1, HO-1, and glutathione S-transferase [20]. Using human renal tubular cells, we provided evidence here that exposure to HG plus palmitate also significantly increased the expression of Nrf2 and its downstream gene HO-1 (Figures 5(a) and 5(c)).

Upregulation of Nrf2 and its downstream antioxidant genes in response to hyperglycemia were found not only in cultured cells, but also in the kidney of diabetic mice. Jiang et al. have examined activation of Nrf2 in the kidneys of STZ-induced diabetic mice using MLD-STZ-induced type 1 diabetes in C57BL/6 mice. They found that, at 4 months of diabetes, Nrf2 expression in the glomeruli of diabetic mice was increased and the nuclear accumulation of Nrf2 was also increased, along with an upregulation of NQO1 [20]. Consistent with this study, we also demonstrated the increased expression of Nrf2 and its downstream genes NQO1 and HO-1 at both mRNA and protein levels, which, however, was observed only at 3-month timepoint, but not at 6-month timepoint (Figures 3 and 4). For the discrepancy of Nrf2 expression at different timepoints of diabetes, observed here, we assumed that as an adaptive mechanism, Nrf2 is quickly upregulated in cells and tissues in response to oxidative stress at early stage, but downregulated in cells or tissues exposed to overwhelming or long-lasting oxidative stress. In fact, this assumption was in an agreement with the finding from previous studies in other disease models. For instance, acute cigarette smoke exposure led to Nrf2 activation in human macrophages, but Nrf2 expression was significantly decreased in pulmonary macrophages from smokers with chronic exposure to cigarettes [22]. Rats with chronic renal failure caused by 5/6 nephrectomy exhibited significant increases in oxidative stress and inflammation in the remaining kidney. In these rats, Nrf2 expression and function was reduced mildly at six weeks but reduced markedly at 12 weeks after nephrectomy [23].

The role of Nrf2 in the prevention of diabetic nephropathy was first explored by Yoh et al. using Nrf2-KO mice [24]. They used STZ to induce diabetes in both Nrf2-KO and the wild-type (WT, C57BL/6) mice, showing that compared to WT diabetic mice, Nrf2-KO diabetic mice exhibited an increased susceptibility to the development of diabetic nephropathy, which was confirmed by Jiang et al. [20]. In addition, upregulation of Nrf2 expression with its activators was found to also provide the preventive effect on diabetes-induced renal damage. For instance, treatment of diabetic mice with resveratrol [25], 1% tBHQ [26], or MG132 [27] in STZ-induced diabetic mice, renal expression of Nrf2, and downstream antioxidants was increased along with significant decreases in renal damage and fibrosis. In addition, Zheng et al. have used SFN to treat STZ-induced diabetic model in C57 BL/6 mice for 4 months, to demonstrate the upregulation of renal Nrf2 and its downstream targets NQO1 and γ-GCS with significant alleviation of renal damage [13]. Using FVB mice, the present study also demonstrated that treatment of STZ-induced diabetic mice for 3-month provided a similar preventive effect on diabetic nephropathy to those in Zheng’s study.

However, our present study here provided the new evidence to indicate that the preventive effect of SFN on diabetic nephropathy observed at the end of SFN treatment was not sustained as this protective effect was significantly diminished when examined at 3 months after terminating SFN treatment (i.e., at 6-month time-point). The lack of sustained preventive effect of SFN on renal damage is probably due to the lack of persistent upregulation of Nrf2 and its downstream target genes as shown by our results. Since in our study we used SFN at 0.5 mg/kg/day in 5 days/week for 3-months while Zheng et al. used SFN at...
12.5 mg/kg/day in 3 times/week for 4 months [13], whether the dosage of SFN was too low to induce a persistent upregulation of renal Nrf2 needs to be further investigated.

In summary, we have investigated whether SFN as one of Nrf2 activators can protect diabetic nephropathy using a type 1 diabetes model. We treated diabetic and age-matched control mice with SFN at 0.5 mg/kg for five days of each week for 3 months, resulting in a significant prevention of diabetes-induced progression of renal damage and dysfunction. The renal protection was observed in the diabetic mice only at the end of 3-month SFN treatment, but not sustained for the additional 3 months without further SFN treatment. The renal prevention from diabetes was accompanied with a significant upregulation of Nrf2 expression and function in the kidney. In cultured renal tubular cell study, we define the direct role of Nrf2 in SFN protection from HG/Pal-induced fibrotic effect because silencing Nrf2 gene could completely abolish the prevention of HG/Pal effects by SFN. These results suggest that diabetic nephropathy can be prevented by SFN most likely via upregulation of Nrf2 expression and function, but, to reach this goal, SFN treatment should be continually supplemented.

4. Materials and Methods

4.1. Animals. FVB male mice, 8–10 weeks age, were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in the University of Louisville Research Resources Center at 22°C with a 12 h light/dark cycle with free access to standard rodent chow and tap water. All experimental procedures for these animals were approved by the Institutional Animal Care and Use Committee of the University of Louisville, which is compliant with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication number 85-23, revised 1996).

For induction of type 1 diabetic mouse model, mice were injected intraperitoneally with MLD-STZ (Sigma-Aldich, St. Louis, MO, USA), dissolved in 0.1 M sodium citrate buffer (pH4.5) at 50 mg/kg body weight daily for 5 consecutive days while age-matched control mice were received multiple injections of the same sodium citrate buffer. Five days after the last injection, mice with hyperglycemia (blood glucose levels ≥ 250 mg/dl) were defined as diabetic, as before [28]. SFN (Sigma-Aldich) was subcutaneously injection at 0.5 mg/kg for five days of each week for 3 months. Dose of SFN was used based on published information [29]. Mice were randomly allocated into four groups (n = 6 at least per group): normal control, SFN, DM, and DM/SFN. Since SFN was dissolved in dimethyl sulfoxide (DMSO) and diluted in PBS, mice serving as vehicle controls were given the same volume of PBS (1% DMSO).

4.2. Mouse Urinary ACR Detection. Mouse urine was collected at 3 months and 6 months of diabetes, respectively. Urinary albumin (Bethyl Laboratories Inc., Montgomery, TX, USA), and urinary creatinine (BioAssay Systems) were measured according to manufacturers’ procedures provided with these kits. Mouse urinary ACR was calculated as ACR = urinary albumin/urinary creatinine (μg/mg).

4.3. Renal Histopathological Examination. After anesthesia, kidneys were isolated to take a piece of it for fixation in 10%-buffered formalin. The fixed tissue was dehydrated in graded alcohol series, cleared with xylene, embedded in paraffin, and sectioned at 5 μm thickness. To examine the basic structure change of renal tissue, sections were stained with H and E. PAS staining was also used for examination of glycogen and collagen contents, as described before [30, 31]. Sections stained for H and E and PAS then were assessed using a Nikon Eclipse E600 microscopy system.

4.4. Real-Time PCR. Collected kidney was snap frozen in liquid nitrogen and kept at −80°C. Total RNA was extracted using the TRizol Reagent (Invitrogen, Carlsbad, CA, USA). RNA concentrations and purities were quantified using a NanoDrop ND-1000 spectrophotometer. First-strand complementary DNA (cDNA) was synthesized from total RNA according to manufacturer’s protocol from the RNA PCR kit (Promega, Madison, WI, USA). Reverse transcription was performed using 1 μg of total RNA in 12.5 μL of the solution containing 4 μL 25 mM MgCl₂, 4 μL AMV reverse transcriptase 5x buffer, 2 μL dNTP, 0.5 μL RNase inhibitor, 1 μL of AMV reverse transcriptase, and 1 μL of oligo dT primer, which were added with nuclease-free water to make a final volume of 20 μL. Reaction system was run at 42°C for 50 min and 95°C for 5 min. Primers of HO-1, NQO1, SOD1, SOD2, and CAT were purchased from Applied Biosystems (Carlsbad, CA, USA). Real-time PCR was carried out in a 20 μL reaction buffer that included 10 μL of TaqMan Universal PCR Master Mix, 1 μL of primer, and 9 μL of cDNA with the ABI 7300 Real-Time PCR system. Fluorescence intensity of each sample was measured at each temperature to monitor amplification of the target gene. Comparative cycle time (CT) was used to determine fold differences between samples [32].

4.5. Western Blotting Assay. Renal tissues were homogenized and HK 11 cells were sonicated in RIPA buffer (Santa Cruz). Total protein was extracted and separated on 10% SDS-PAGE gels and transferred to a nitrocellulose membranes (Bio-rad, Hercules, CA, USA). Membranes were blocked with a 5% nonfat dried milk for 1 h at room temperature and then incubated overnight at 4°C with the following antibodies: 3-NT (1 : 1000 dilution), 4-HNE (1 : 1000 dilution), TNF-α (1 : 500 dilution), PAI-1 (1 : 2000 dilution), CTGF (1 : 500 dilution), TGF-β1 (1 : 500 dilution), Nrf2 (1 : 500 dilution), HO-1 (1 : 500 dilution), NQO1 (1 : 500 dilution), SOD1 and SOD2 (1 : 2000 and 1 : 5000 dilution, resp.), CAT (1 : 5000 dilution), and β-actin (1 : 2000 dilution), all which were purchased from Santa Cruz Biotech. Inc. except for PAI-1 from BD Bioscience, TNF-α from Abcam, 3-NT from Millipore, and 4-HNE from Alpha Diagnostic International. After three washes with tris-buffered saline (pH 7.2) containing 0.05% Tween 20, membranes were incubated with the appropriate secondary antibodies for 1 h at room temperature. Antigen-antibody complexes were then visualized using an enhanced
chemiluminescence kit (Thermo scientific, Rockford, IL, USA) [33].

4.6. Cell Culture, Treatments, and Measurements. HK11 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12 supplemented with 5% fetal bovine serum (FBS, Invitrogen). To recapture the in vivo diabetic model in an in vitro condition, HK11 cells were exposed to D-glucose in a final concentration of 27.5 mM (HG) with 2% BSA and 1% FBS for 48 h and at the last 6 h, palmitate (300 μM) was added into the culture medium. To exclude a hyperosmotic effect, 5.5 mM D-glucose plus 22 mM D-mannitol (Sigma) was added as hyperosmotic control. To mimic the in vivo study, SFN dissolved in DMSO was added (3 μM final concentration) for 48 h. The effect of Nrf2 and its downstream gene expression on glucose-induced CTGF expression was defined with corresponding siRNA. CTGF, Nrf2, and HO-1 expression was measured by Western blotting assay.

Nrf2 expression and translocation into nuclear were monitored by fluorescent microscope. HK11 cells were cultured on chamber slides for 12 h to allow the cells to attach to the flask and then subjected to above experimental conditions. The chamber slides were incubated with rabbit anti-Nrf2 antibody (1 : 50 dilution, Abcam, Cambridge, MA) overnight at 4°C, with Cy3-conjugated goat anti-rabbit IgG antibody, and costained with Dapi for the nuclei. The fluorescent staining was analyzed under fluorescent microscope.

4.7. siRNA Transfection. HK 11 cells were transfected with either the negative control siRNA (Invitrogen) or siRNA specific to human Nrf2 (NFE2L2HSS181505, Invitrogen) by Lipofectamine TM 2000 transfection reagent (Invitrogen) according to manufacturer’s instructions for 48 h followed by SFN (3 μM) and HG (27.5 mM) treatment for additional 48 h as described above. The sense and antisense sequences of the primers were 5′-CAACUGACAGAAGUGACAAAUU-3′ and 5′-AUAUUGUCACUGUCUGUUG-3′; transfection efficiency was assessed by Western blot analysis for Nrf2 and HO-1 protein expression. Effects of siRNA knockdown of Nrf2 on the expression of CTGF were assessed by Western blot analysis at indicated time points.

4.8. Statistical Analysis. In vivo and in vitro data were collected from at least 6 animals each group and repeated cell experiments, respectively, and were presented as means ± SD. We used Image Pro Plus 6.0 software to identify the positive staining, Image Quant 5.2 to analysis western blotting. Comparisons were performed by one or two-way ANOVA for the different groups, followed by post hoc pairwise repetitive comparisons using Tukey’s test with Origin 7.5 Lab data analysis and graphing software. Statistical significance was considered as P < 0.05.

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