International Journal of Hypertension

The Emerging Role of Heme Oxygenase and Its Metabolites in the Regulation of Cardiovascular Function

Guest Editors: David E. Stec, Kazunobu Ishikawa, David Sacerdoti, and Nader G. Abraham

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Editorial

The Emerging Role of Heme Oxygenase and Its Metabolites in the Regulation of Cardiovascular Function

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Heme oxygenase (HO) is an essential enzyme for normal homeostasis of the cardiovascular system. It is the enzyme responsible for the breakdown of heme to iron, biliverdin, and carbon monoxide (CO) gas. Biliverdin is then subsequently reduced to bilirubin by the ubiquitous enzyme biliverdin reductase. HO enzymes are essential for the turnover of red blood cells as well as of heme containing proteins within the cell. However, in recent years numerous studies have demonstrated an important role for HO and its metabolites, CO, and bilirubin in regulating various physiological processes throughout the cardiovascular system. More importantly, alterations in HO have been identified in numerous pathological conditions, and therapies which induce HO have been found to provide beneficial outcomes in numerous cardiovascular disease processes. This special issue consists of 9 original research articles, 4 review articles, and 1 clinical study; all of which highlight the important role that HO enzymes have in the regulation of the cardiovascular system.

The important role of HO in the regulation of kidney function and blood pressure is highlighted in studies by F. Botros et al., S. Quadri et al., and E. Csongradi et al. HO-1 induction with hemin results in marked increases in renal blood flow and glomerular filtration rate (GFR) which are associated with increases in urine flow and sodium excretion. HO-1 induction with hemin also attenuates the decrease in renal blood flow to acute angiotensin II (Ang II) treatment. The important role of HO-1 to counteract the effects of Ang II is further demonstrated in studies in by E. Csongradi et al. in which blockade of intrarenal HO-1 increases blood pressure in Ang II-dependent hypertension through increases in intrarenal superoxide production. The important antioxidant actions of HO and its metabolites are also made evident in studies by E. George et al. who demonstrate that both HO-1 induction and treatment with the HO metabolites CO and bilirubin attenuate the increase in oxidant production in placental explants in response to hypoxia. HO induction as well as CO and bilirubin also attenuate hypoxia-induced sFlt production which has important implications for pregnancy-induced hypertension or preeclampsia. Studies by T. Kawakami et al. also highlight the important antioxidant role of HO and its metabolites. In these studies, HO-1 induction lowers oxidative stress levels which in turn improves vascular function, lowers blood pressure, and prevents renal injury in extracellular superoxide dismutase (EC-SOD, SOD3) knockout mice. The important role of HO in the modulation of oxidative stress in the differentiation of bone-marrow-derived mesenchymal stem cells (MSCs) and its potential role in diseases such as diabetes, inflammation, osteoporosis, and hypertension are reviewed by L. Vanella et al. HO-1 regulates MSC differentiation process by shifting the balance of MSC

differentiation in favor of the osteoblast lineage by decreasing oxidative stress and increasing osteogenic markers such as alkaline phosphatase and BMP-2.

The important role of HO in target organ injury is also highlighted in this special issue. K. Chandrashekar et al. demonstrate the important role of HO-1 in preserving renal function and protecting the kidney against renal injury in a model of subpressor Ang II-induced kidney injury. M. Constantin et al. detail the therapeutic potential of HO and its metabolite CO in lung inflammation, acute lung injury, lung transplantation, and pulmonary hypertension.

HO enzymes play a critical role in the regulation of vascular function and the protection of the vasculature from injury. The important role of HO enzymes in this capacity as well as the potential targeting of vascular HO for therapeutics is reviewed by E. Marcantoni et al. The importance of vascular HO-1 in the suppression of vascular inflammation is demonstrated in studies by K. Ishikawa et al. who show that HO-1 knockout mice exhibit enhanced vascular inflammation, atherosclerotic lesions, and increased oxidation of HDL.

HO enzymes have an important interaction with nitric oxide (NO) in the regulation of vasculature tone. HO in the vasculature has been previously reported to both induce as well as inhibit the formation of NO depending on the degree of HO-1 induction. A. Daiber et al. examine the complex relationship between HO and NO and its implications in organic nitrate therapy and argue for a beneficial role of HO-1 induction to protect against tolerance to effective organic nitrate-based therapies for cardiovascular disease. The relationship between HO and NO is further investigated in a clinical study by D. Sacerdoti et al. who demonstrate the important role for HO in the maintenance of blood flow in the face of NO inhibition as well as the important role for HO in protecting the bioavailability of vascular-derived NO in humans.

The role of HO and its metabolites in metabolic diseases such as diabetes and obesity is also highlighted in this special issue. A. Elmarakby et al. report that induction of HO-1 is able to lower renal oxidative stress, inflammation, and injury in diabetic spontaneously hypertensive rats (SHRs) suggesting that induction of HO-1 may be a potential therapeutic approach for diabetic nephropathy. J. Cao et al. elegantly demonstrate that HO-1 induction with the Apo AI mimetic, L-4F, decreases blood pressure, insulin resistance, blood glucose, and adiposity in HO-2 knockout mice. These results suggest that HO-1 induction could be a viable therapeutic approach for the correction of both metabolic and cardiovascular disorders in obesity.

Taken together, the series of articles in this special issue highlight the important regulatory role of HO and its metabolites in the cardiovascular system. The articles also demonstrate the important role that HO enzymes play in protecting the cardiovascular system from diseases such as hypertension, atherosclerosis, kidney disease, diabetes, and obesity. They also shed light on the potential therapeutic role for HO-1 induction to combat these diseases. It is clear from these articles that the HO system is indeed emerging as both an important regulator of cardiovascular function as well as an attractive target for the development of effective therapeutics.

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The Guest Editors would like to express their sincerest gratitude to the contributors of the articles for this special issue of the *International Journal of Hypertension*. They would also like to express their deepest appreciation for the external reviewers who assisted in the review process as well as the Editorial and Production teams at Hindawi Publishing Corporation for making this *Special Issue* possible.

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Research Article

Apo A1 Mimetic Rescues the Diabetic Phenotype of HO-2 Knockout Mice via an Increase in HO-1 Adiponectin and LKBI Signaling Pathway

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Insulin resistance, with adipose tissue dysfunction, is one of the hallmarks of metabolic syndrome. We have reported a metabolic syndrome-like phenotype in heme oxygenase (HO)-2 knockout mice, which presented with concurrent HO-1 deficiency and were amenable to rescue by an EET analog. Apo A-I mimetic peptides, such as L-4F, have been shown to induce HO-1 expression and decrease oxidative stress and adiposity. In this study we aimed to characterize alleviatory effects of HO-1 induction (if any) on metabolic imbalance observed in HO-2 KO mice. In this regard, HO-$2^{(-/-)}$ mice were injected with 2 mg/kg/day L-4F, or vehicle, i.p., for 6 weeks. As before, compared to WT animals, the HO-2 null mice were obese, displayed insulin resistance, and had elevated blood pressure. These changes were accompanied by enhanced tissue (hepatic) oxidative stress along with attenuation of HO-1 expression and activity and reduced adiponectin, pAMPK, and LKB1 expression. Treatment with L-4F restored HO-1 expression and activity and increased adiponectin, LKB1, and pAMPK in the HO-$2^{(-/-)}$ mice. These alterations resulted in a decrease in blood pressure, insulin resistance, blood glucose, and adiposity. Taken together, our results show that a deficient HO-1 response, in a state with reduced HO-2 basal levels, is accompanied by disruption of metabolic homeostasis which is successfully restored by an HO-1 inducer.

1. Introduction

Obesity, metabolic syndrome, and associated insulin resistance are major contributors to cardiovascular disease, the leading cause of mortality in the United States [1]. Insulin resistance is characterized by hyperglycemia and increase in lipolysis and free fatty acid levels and increased hepatic triglyceride secretion and sterol-regulatory element-binding protein-1 (SREBP-1) [2, 3]. SREBPs are transcription factors known to regulate genes involved in fatty acid and cholesterol synthesis and are regulated by pAMPK [3, 4]. AMPK is phosphorylated and activated by the major kinase, LKB1, and acts as a metabolic checkpoint that is suppressed in hyperglycemic conditions [4–8]. Hyperglycemia and associated increase in reactive oxygen species (ROS) are known to decrease HO levels [1, 9, 10]. There are two forms of HO, the inducible HO-1 and the constitutively expressed HO-2 [4, 11]. HO-1 and -2 catabolize heme into equimolar concentrations of carbon monoxide, bilirubin, and free iron, generating an antioxidant effect and increasing nitric oxide (NO) bioavailability and providing cardiovascular protection [4, 11]. HO-1 is the major cytoprotective moiety of the HO system because of its rapid inducibility by a broad spectrum of compounds and conditions including stress. However, recent studies using HO-$2^{(-/-)}$ mice suggest that HO-2 is also critical for cellular homeostasis and for upregulation of HO-1 [4, 11]. When HO-1 increases, levels of antioxidant and anti-inflammatory molecules increase and the level of reactive oxygen species (ROS) decreases [9]. The benefits of increased levels of HO-1 protein include the prevention of

high blood pressure, decreased vasoconstrictors, increased vasodilators, and the inhibition of oxidative stress [1, 10, 12].

The effects of HO are also associated with an increase in adiponectin, a protein hormone that modulates many metabolic processes and can improve cardiovascular function while downregulating proinflammatory factors [1, 9, 13, 14]. Adiponectin exists in three different forms trimer, hexamer, and high molecular weight (HMW) with HMW adiponectin being the form that attenuates cardio-vascular disease [1, 14]. In both, obese subjects and animals, the plasma levels of adiponectin are inversely related to insulin sensitivity [4, 13, 15, 16]. The upregulation of HO-1 is associated with an increase in adiponectin levels and correlates with decreased inflammatory cytokines, IL-1, IL-6, and TNFα [1, 9].

Recently developed HO-2 null mice have displayed characteristics of a metabolic syndrome-like phenotype with enhanced systemic inflammatory and oxidative stress response. Curiously, these mice also demonstrate a failure to induce stress-dependent HO-1 upregulation along with suppression of adiponectin levels. That attenuated HO-1 upregulation in an HO-2 null mouse is accompanied by metabolic imbalance led us to examine the effects of an HO-1 inducer in such a setting. The apo-A1 mimetic peptide, L-4f, was administered to HO-2 null mice so as to rescue HO-1 expression. This apo-A1-mimetic peptide was synthesized from amino acids that improved the ability of HDL to protect LDL against oxidation in animals with atherosclerosis [2]. L-4F treatment resulted in reduced adiposity, evident by decreased visceral fat content, in conjunction with improved energy balance and metabolic homeostasis in HO-2 null mice. These changes were further characterized by increases in HO-1 and adiponectin levels along with enhanced cellular expression of LKBI-pAMPK in the liver tissues.

2. Materials and Methods

2.1. Animal Protocol. All animal experiments followed an institutionally approved protocol in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. The HO-2 null mice are direct descendents of the HO-2 mutants produced [17]. These well-characterized HO-2 null mice have a C57BL/6 $\times$ 129/Sv genetic background that was used on age- and gender-matched controls. Homozygote HO-$2^{(-/-)}$ null and B6/129SF2/J (WT) mice were used for the studies. Mice were divided into three groups (10 mice/group): WT, HO-$2^{(-/-)}$ + vehicle, and HO-$2^{(-/-)}$ + L-4F. Beginning at 20 weeks of age when the mice had established diabetes, L-4F (i.e., Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH2) synthesized from L-amino acids as previously described (Stephen P 2008) at a dose of 200 μg/100 g daily in 2 mL vehicle, or vehicles (ABCT: ammonium bicarbonate buffer at pH 7.4 containing 0.01% Tween 20) were administered intraperitoneally (i.p.) for 6 weeks. Mice were fed a normal chow diet and had access to water *ad libitum*. Glucose monitoring was performed using an automated analyzer (Life scan Inc., Milpitas, CA, USA). Blood pressure was measured by the tail cuff method before and every 7 days after L-4F administration. Body weights of HO-$2^{(-/-)}$ and WT mice at the beginning of the experiment were 28 $\pm$ 2 g and 20 $\pm$ 2 g, respectively. Glucose levels were 160 $\pm$ 20 and 121 $\pm$ 20 mg/dL for HO-$2^{(-/-)}$ and WT mice, respectively. At the time of sacrifice the body weight of all mice was measured. The subcutaneous and visceral fat in the abdomen, mesenteric fat, and fat around the liver, kidney, spleen, and heart were dissected free, pooled for each mouse and weighed. Blood samples were collected in K_3EDTA tubes at sacrifice, and the plasma was separated. Liver samples were flash frozen in liquid nitrogen for further studies.

2.2. Western Blot Analysis. Frozen hepatic samples were pulverized in T-PER (ThermoFisher Scientific, Rockford, IL, USA) homogenization buffer, rotated for 1 hour at 4°C, and then centrifuged at 12,000 rpm for 25 minutes at 4°C. The supernatant was collected and protein was quantified using the BCA protein assay (Pierce Biotechnology, Inc., Woburn, MA). Protein expression analysis was preformed through immunoblotting with antibodies against HO-1 (Stressgen Biotechnologies Corp., Victoria, BC, Canada), adiponectin, pAKT, AKT, pAMPK, and AMPK (Cell Signaling Technology, Inc. Beverly, MA, USA) were used. Imaging and quantification were done using the Odyssey imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

2.3. Real-Time Quantitative PCR. Total RNA was recovered from liver following the Perfect Pure Tissue Kit (5Prime, IN Gaithersburg, MD, USA) RNA extraction protocol with DNase treatment. cDNA was made using the Improm Reverse Transcriptase kit (Promega, Madison, WI, USA). Primer sequences for mouse HO-1 were 5′-CAGCCCCAC-CAAGTTCAAAC-3′ and 5′-TCAGGTGTCATCTCCAGA-GTGTTC-3′, adiponectin 5′-AGCCGCTTATATGTATCG-CTCA-3′ and 5′-TGCCGTCATAATGATTCTGTTGG-3′, AMPK 5′-CGCAGACAGCCCCAAAG-3′ and 5′-AGAGAC-TTGGGCTTCGTTGTGT-3′, LKB1 5′-TGCTGGACTCCG-AGACCTTA-3′ and 5′-CCTGCGCAGCTTTTTCTTC-3′, AKT 5′-GAACCGTGTCCTGCAGAACTCTAG-3′ and 5′-GTGGGTCTGGAATGAGTACTTGAG-3′, and GAPDH 5′-CCAGGTTGTCTCCTGCGACT-3′ and 5′-ATACCAGGA-AATGAGCTTGACAAAGT-3′. The thermal cycling conditions were 95°C for 20 seconds followed by 40 cycles of 95°C for 3 minutes, 60°C for 30 seconds, and finally 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds.

2.4. O_2^- Production. liver samples were placed in scintillation vials (2 per vial) containing 1 mL of Krebs-HEPES buffer, pH 7.4, and lucigenin (5 μmol/L) for 30 min at 37°C. Lucigenin chemiluminescence was measured in a liquid scintillation counter (LS6000TA, Beckman Instruments) and superoxide production quantified as previously described [2].

2.5. Glucose and Insulin Tolerance Tests. After 6 h fast, mice were injected intraperitoneally with glucose (2.0 g/kg body weight). Blood samples were taken at various time points (0–120 min), and blood glucose levels and serum insulin levels were measured. For determination of insulin tolerance, mice were injected intraperitoneally with insulin (2.0 U/kg). Blood

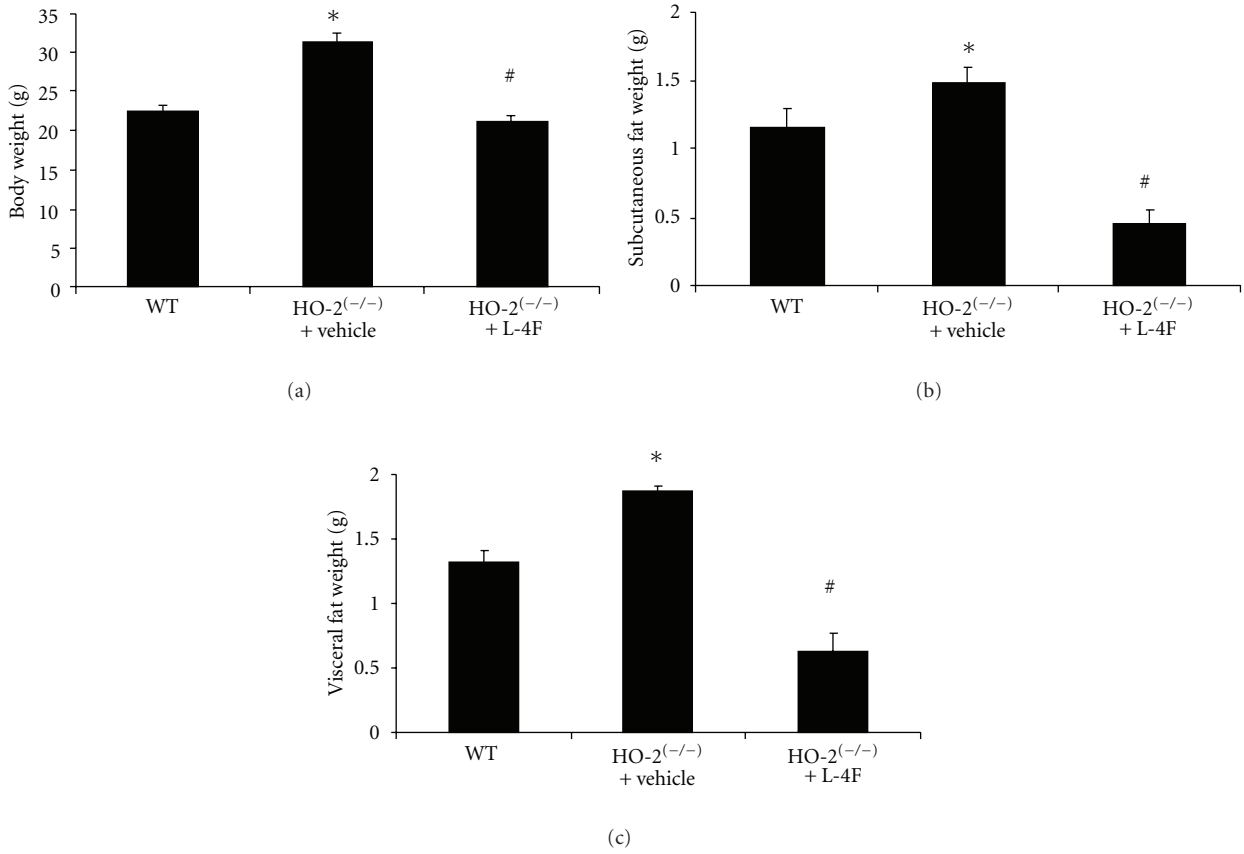

Figure 1: Effect of HO-2 deletion on body weight and adiposity. Figures showing wild-type (WT), HO-2 null (HO-$2^{(-/-)}$) and L-4F treated HO-$2^{(-/-)}$ mice (HO-$2^{(-/-)}$ + L-4F) after 6 weeks of treatment. (a) Effect of L-4F on body weight on HO-$2^{(-/-)}$ mice. (b) Effect of L-4F on subcutaneous body weight on HO-$2^{(-/-)}$ mice. (c) Effect of L-4F on visceral body weight on HO-$2^{(-/-)}$ mice. Results are means ± SE, $n = 6$, $^{*}P < 0.05$ versus WT; $^{\#}P < 0.05$ versus HO-$2^{(-/-)}$.

samples were taken at various time points (0–90 min), and blood glucose levels were measured.

2.6. Measurement of HO Activity. Tissue HO activity, in liver samples from WT, HO-$2^{(-/-)}$ treated and untreated mice, was assayed as described previously [18, 19] using a technique in which bilirubin, the end product of heme degradation, was extracted with chloroform, and its concentration was determined spectrophotometrically (dual UV/VIS beam spectrophotometer lambda 25; PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA) using the difference in absorbance at a wavelength from $\lambda 460$ to $\lambda 530$ nm with an absorption coefficient of 40 $mM^{-1}\,cm^{-1}$. Under these conditions, HO activity was linear with protein concentration, time-dependent, and substrate-dependent [18, 19].

2.7. Statistical Analyses. Statistical significance between experimental groups was determined by the Fisher method of analysis of multiple comparisons ($P < 0.05$). For comparison between treatment groups, the null hypothesis was tested by a single-factor ANOVA for multiple groups or unpaired t-test for two groups.

3. Results

3.1. Effect of HO-2 Deletion on Body Weight, Fat Content, Blood Pressure, and Metabolic Parameters. In Figure 1(a) we show that HO-2 deletion significantly increased body weight when compared to WT mice and was reversed after six weeks of L4-F treatment. A similar pattern was observed in weight reduction of subcutaneous and visceral fat by administration of L4-F in HO-$2^{(-/-)}$ mice as shown in Figures 1(b) and 1(c). Furthermore, we determined that the random blood glucose levels in the HO-2 null mice were significantly increased compared to WT and were reversed back to normal baseline with L4-F treatment (Figure 2(a)). Both systolic and diastolic blood pressures were significantly elevated in HO-2 null mice as compared to WTs ($P < 0.05$) (Figure 2(b)). This increased body weight, adiposity, and elevated blood pressure suggests metabolic syndrome like phenotype in HO-2 KO mice, which was successfully reversed by HO-1 induction.

To investigate if the metabolic syndrome, observed in HO-2 null mice, is associated with insulin resistance, we performed insulin sensitivity and glucose tolerance tests. Insulin administration to WT, HO-2 null, and L4-F treated HO-2 null mice produced a rapid decrease in glucose levels in the WT and L-4F treated HO-2 KO mice compared to HO-2

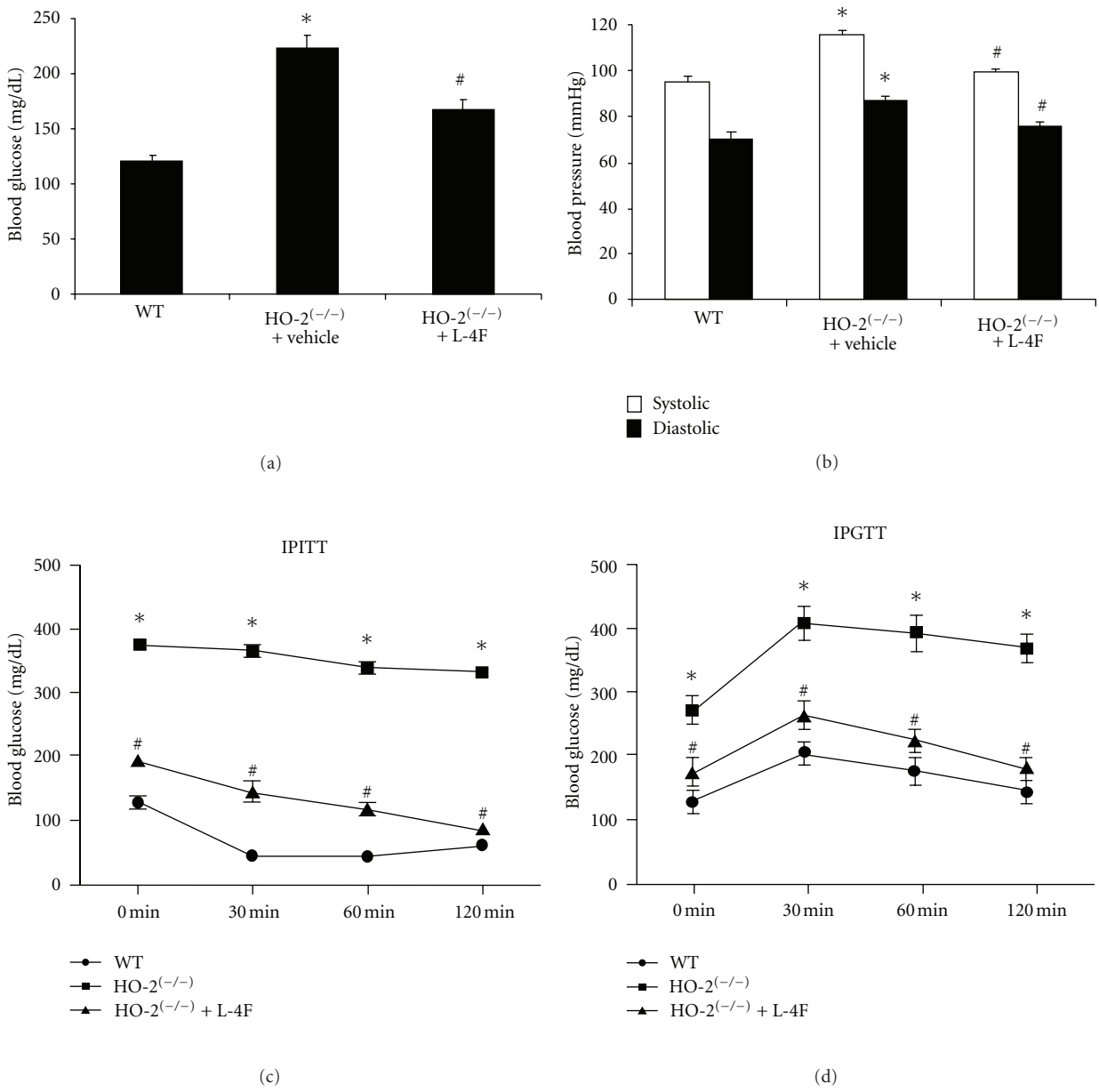

Figure 2: Effect of L-4F on blood glucose, blood pressure, insulin sensitivity, and glucose tolerance in HO-2 null mice. (a) Blood glucose. Values are means ± SE, $n = 6$, $^{*}P < 0.05$ versus WT; $^{\#}P < 0.05$ versus HO-2$^{(-/-)}$. (b) Blood pressure values are means ± SE, $n = 6$, $^{*}P < 0.05$ versus respective WT, $^{\#}P < 0.05$ versus respective HO-2$^{(-/-)}$. HO-2$^{(-/-)}$. (c) Intraperitoneal insulin sensitivity (IPITT) and glucose tolerance (IPGTT) (d) tests were performed as described in research designs and methods. Results are means ± SE, $n = 6$. (IPITT) $^{*}P < 0.05$ versus WT, $^{\#}P < 0.05$ versus HO-2$^{(-/-)}$, (IPGTT). $^{*}P < 0.05$ versus WT, $^{\#}P < 0.05$ versus HO-2$^{(-/-)}$.

null ($P < 0.05$), suggesting improved sensitivity of HO-2 null mice with L-4F treatment, decreasing from 333.7 ± 6.0 mg/dL for HO-2 null mice to 84.7 ± 4.9 mg/dL in the L-4F treated HO-2 null mice (Figure 2(c)). Plasma glucose levels at all times were significantly elevated in the HO-2 null mice compared to the L-4F treated HO-2 null mice. Glucose administration to all mice rapidly increased the glucose level after 30 min and remained elevated in the HO-2 null mice, compared to the L4-F treated HO-2 null and WT mice which returned to initial levels at 120 min (Figure 2(d)).

3.2. Effect of HO-2 KO on Tissue Redox, HO-1 Expression, and Activity. Analysis of lucigenin-detectable chemiluminescence demonstrated enhanced oxidative stress in hepatic samples from HO-2 KO versus WT mice ($P < 0.05$). This increase in O_2^- generation was attenuated ($P < 0.05$) in HO-2 null mice treated with L-4f for 6 wks (Figure 3(a)). In Figure 3(b), we show that the HO-2 null mice express significantly ($P < 0.05$) lower levels of HO-1 as compared to WT mice; however levels are restored with L-4F treatment. A daily injection of L-4F for 6 weeks also resulted in a

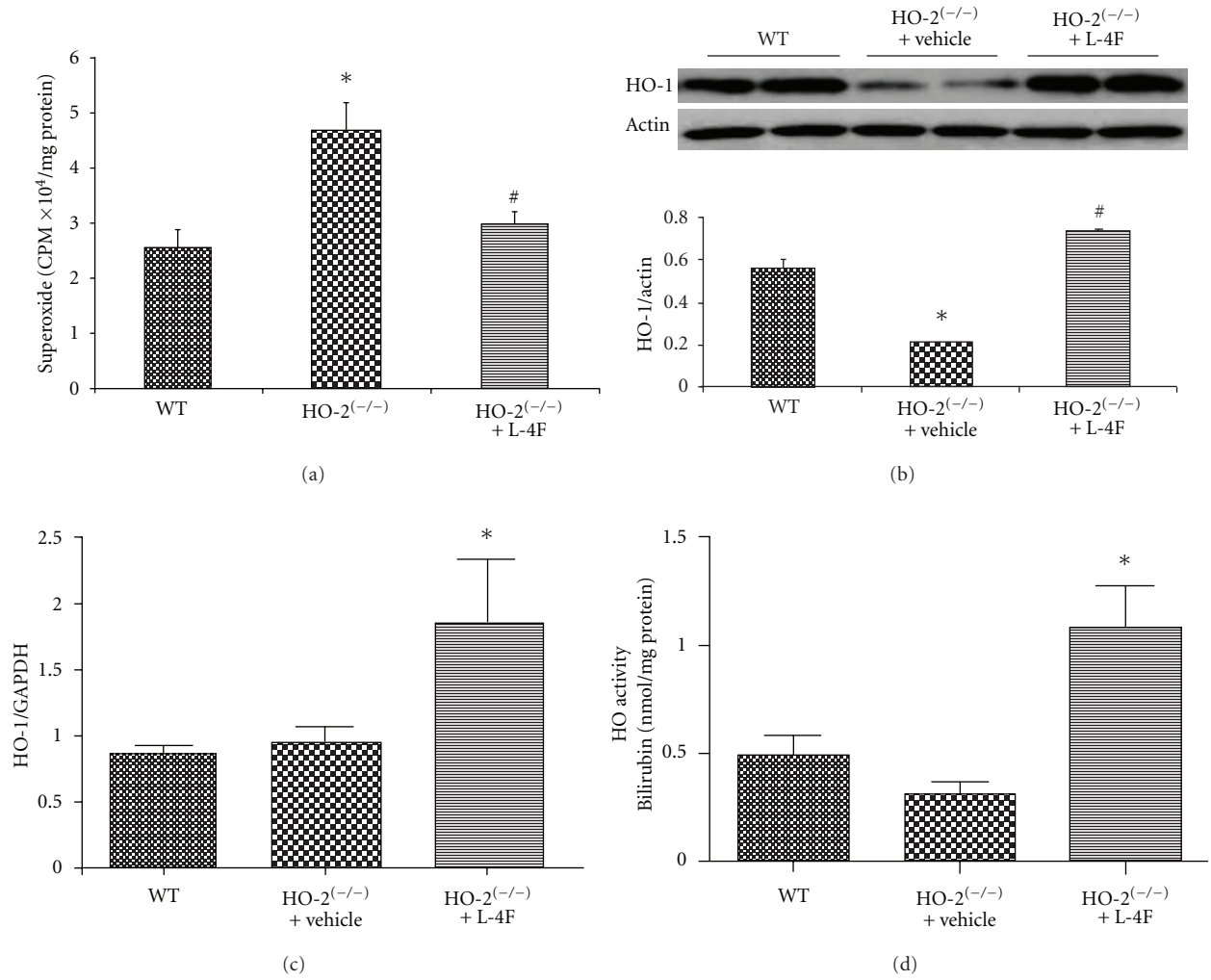

Figure 3: Effect of L-4F on redox status, HO-1 levels, and activity in HO-2 null mice. (a) Levels of superoxide produced in WT, HO-2$^{(-/-)}$ and L-4F treated HO-2$^{(-/-)}$ mouse liver. Values are means ± SE, $n = 6$, $^{*}P < 0.05$ versus WT, and $^{\#}P < 0.05$ versus HO-2$^{(-/-)}$. (b) Western blot, densitometry analysis and (c) mRNA expression of hepatic tissues for HO-1 expression. Values are means ± SE, $n = 6$, $^{*}P < 0.05$ versus WT, $^{\#}P < 0.05$ versus HO-2$^{(-/-)}$ (d). Effects, L-4F on HO-1 activity measured as described in materials and methods. Results are means ± SE, $n = 6$, $^{*}P < 0.05$ versus HO-2$^{(-/-)}$.

significant increase in HO-1 mRNA levels compared to the HO-2 null mice (Figure 3(c)). HO-2 deletion impairs HO-1 inducibility leading to a decrease in HO activity. In Figure 3(d) we show that treatment with L-4F in the HO-2 null mice significantly increases HO-1 activity.

3.3. Effect of HO-2 KO on Hepatic Adiponectin and pLKB1/pAMPK Signaling. Western blot analysis demonstrated that HO-2 deletion is associated with significant decrease in the expression of adiponectin when compared to age-matched WT (Figure 4(a)). Treatment with L-4F increased these levels by 2-fold to levels significantly higher than those measured in HO-2 null mice (Figure 4(a)). Consistent with the changes in protein shown in Figure 4(a), we show with real-time PCR that adiponectin levels significantly increase with treatment with L-4F compared to untreated in the HO-2 null mice (Figure 4(b)). To elucidate the mechanism involved in the changes observed with L-4F treatment, we determined expression and activity of signaling pathways that may be involved in the process. Interestingly, the expression of activated AMPK was regulated by HO-2 deletion since the HO-2 null mice expressed significantly lower levels of pAMPK albeit normal levels of AMPK. L-4F restored the levels of pAMPK in the HO-2 null mice without affecting total AMPK levels (Figure 4(c)). Furthermore, there was a significant increase in LKB1 expression in the L-4F treated HO-2 null mice (Figure 4(d)), which suggests that the AMPK/LKB1 pathway could play a role in the L-4F mediated resource of HO-2$^{(-/-)}$ phenotype. In addition, to elucidate modulation of AKT-dependent pathways by HO-1 induction via L-4F, immunoblot assessment of pAKT/AKT was performed which exhibited enhanced ($P < 0.05$) pAKT/AKT levels in HO-2 KO (1.39 ± 0.12) versus WT (1.02 ± 0.09) mice. This effect of HO-2 deletion on pAKT expression was unaffected by L-4F administration in HO-2 null mice (1.46 ± 0.14).

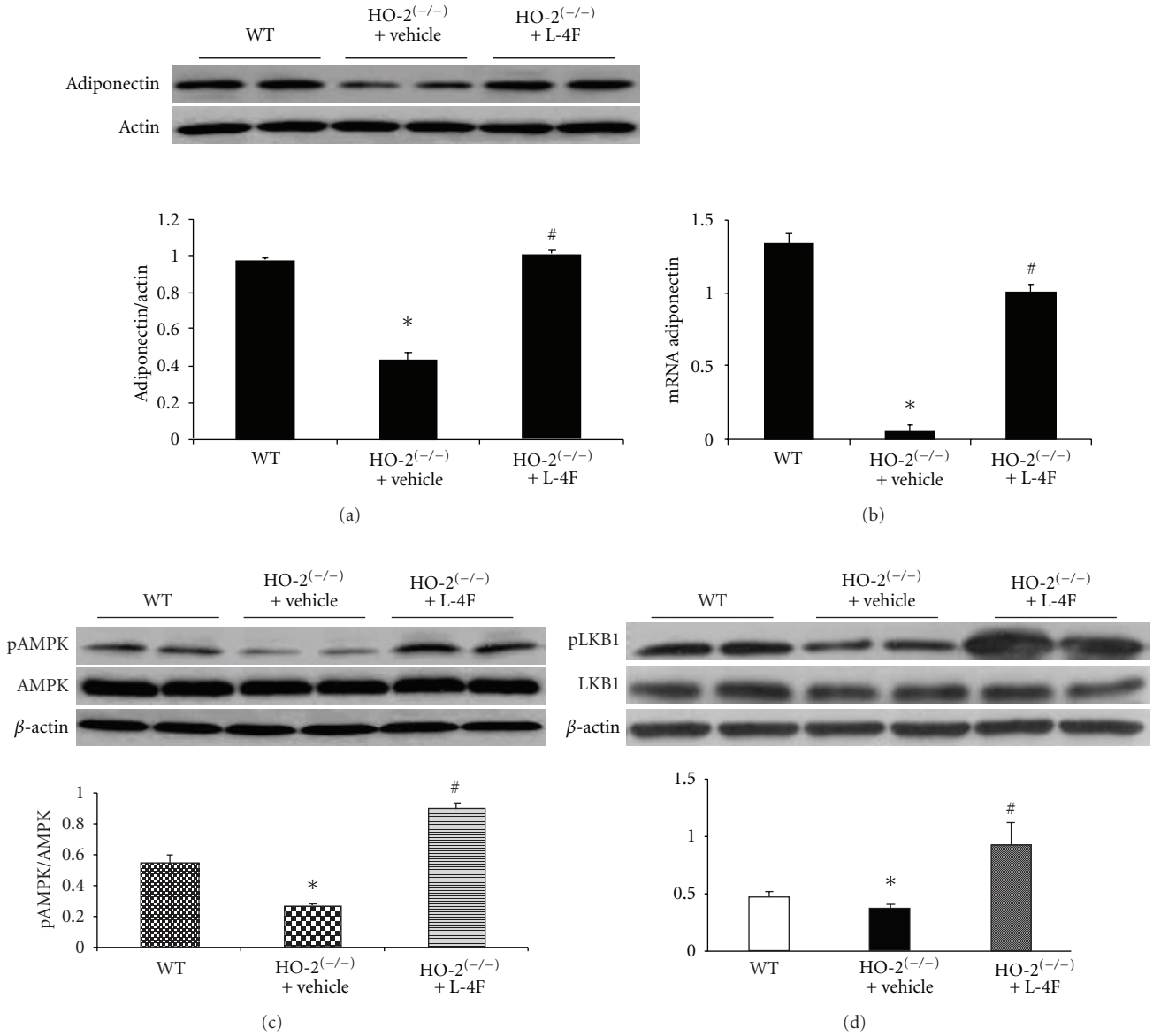

Figure 4: Effect of L-4F on adiponectin pAMPK and LKB1 expression in HO-2 null. L-4F was administered daily for 6 weeks and liver tissues analyzed for adiponectin protein (a) and mRNA (b) expression. Protein bands were analyzed by densitometry and plotted against a relative expression to actin. Results are means ± SE, $n = 6$, $^{*}P < 0.05$ versus WT, $^{\#}P < 0.05$ versus HO-$2^{(-/-)}$. (c) Western blot and densitometry analysis of hepatic tissues for pAMPK and AMPK. Values are means ± SE, $n = 5$, $^{*}P < 0.05$ versus WT, $^{\#}P < 0.05$ versus HO-$2^{(-/-)}$. (d) Expression for LKB1 in WT, HO-2 KO with and without L-4F. Values are means ± SE, $n = 6$, $^{*}P < 0.05$ versus WT, $^{\#}P < 0.05$ versus HO-$2^{(-/-)}$.

4. Discussion

The data presented here shows that treatment of HO-2 null mice with L-4F rescues the key markers of metabolic syndrome via an increase in HO-1 and adiponectin through a signaling mechanism involving the LKB1/AMPK signaling pathway (Figure 5). Interestingly, L-4F had no effect on activated AKT (pAKT), suggesting selectivity of L-4F to pAMPK.

First key finding presented here is in line with earlier reports [4, 11] suggesting a role of HO-2 in mediating HO-1 upregulation. HO-2 null mice were characterized by disruption of metabolic homeostasis and displayed increased body weight, adiposity, insulin resistance with elevated blood pressure, and oxidative stress. Pathophysiological conditions such as these have historically been shown to be associated with increase in cellular defense mechanisms including HO-1 [20]. HO-2, a constitutively expressed isoform, supports sustenance of basal redox status in the cells, and its knockdown is not surprisingly met with oxidative stress. HO-1 induction, however, in this HO-2 knockdown state fails to occur even in the presence of added pathophysiological insult such as metabolic syndrome. These observations delineate the essential role of HO-2 in stress-induced HO-1 induction. This failure of HO-1 upregulation could further dampen

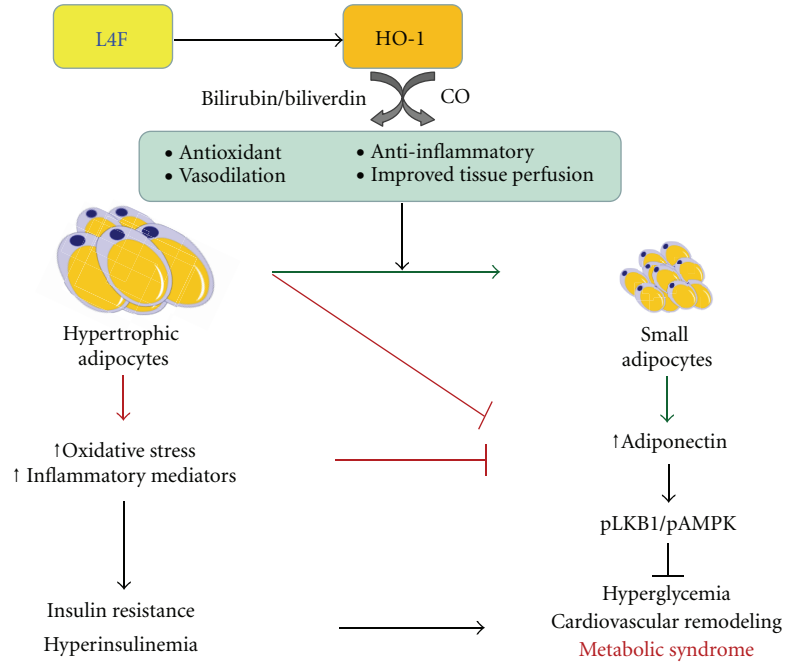

Figure 5: Schematic, the upregulation of HO-1 and adiponectin levels by L-4F coincides with increased pAMPK and LKBI levels providing a signaling mechanism by which L-4F rescues the metabolic phenotype and improves vascular function.

cellular defenses and contribute towards the phenotypic alteration observed in these animals. Restoration of HO-1 expression and activity accompanied by phenotype reversal further supports the role of deficient HO-1 in mediating, at least partly, clinicopathological alterations observed in HO-2 null state. Previous reports have documented physical interactions of the two HO isoforms [21], which could contribute towards HO-2-dependent induction of HO-1.

Second key observation of this study is the modulatory effect of heme-HO system on adiponectin and associated metabolic signaling pathways and their role in alleviating metabolic pathologies observed in an HO-2 KO state. One major marker of obesity is inflammation, which produces an excess of reactive oxygen species, specifically superoxide [11]. When there is chronic exposure to an excess of superoxide, adiponectin and HO-1 levels decrease significantly and contribute to the pathogenesis of insulin resistance [2, 4, 9, 12]. An increase in HO-1 levels increases adiponectin levels, which is known to possess a vascular protective role, preserve endothelial function, and improve insulin sensitivity through glucose uptake [1, 9]. Treatment with L-4F is shown to increase both HO-1 and adiponectin levels in vitro and in vivo [2, 15] while decreasing superoxide (Figure 3(a)), further supporting the idea that L-4F improves the phenotype in the metabolic syndrome mouse model through an increase in insulin sensitivity and glucose tolerance.

L-4F treatment significantly increased pAMPK and LKB1 levels, all associated with improved insulin sensitivity [15]. P-AMPK is known to act in the regulation of cell survival, protect against oxidative stress [15, 22–24], and, when activated, contribute to glucose transport, fatty acid oxidation, and increased mitochondrial function [2, 25]. It is known that crosstalk between AMPK and AKT can regulate nitric oxide bioavailability and vascular function [22, 23, 26]. However L-4F did not affect the protein expression or activation of AKT, suggesting a pathway more specific to AMPK. LKB1 is a serine-threonine kinase that directly phosphorylates AMPK and decreases lipogenesis [4, 5, 8]. We show that L-4F induces LKB1 in HO-2 null mice, indicating that HO-1 mediates the transcriptional regulation of LKB1 by L-4F to activate AMPK.

In conclusion, as depicted in the schematic (Figure 5), the upregulation of HO-1 and adiponectin levels by L-4F coincides with increased pAMPK and LKBI levels, providing a signaling mechanism by which L-4F rescues the metabolic syndrome phenotype and improves energy balance. Thus, L-4F could provide as a beneficial drug treatment to complement conventional therapeutic of disease associated with disruption of metabolic homeostasis.

Abbreviations:

HO-1/HO-2:	Heme oxygenase 1, 2
ROS:	Reactive oxygen species
EC-SOD:	Extracellular superoxide dismutase
NO:	Nitric oxide
AKT:	Protein kinase B
pAKT:	Phosphorylated protein kinase B
AMPK:	AMP-activated protein kinase
pAMPK:	Phosphorylated AMP-activated protein kinase
O_2^-:	Superoxide
LKB1:	Serine/threonine kinase 11.

Acknowledgments

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References

[1] A. Nicolai, M. Li, D. H. Kim et al., "Heme oxygenase-1 induction remodels adipose tissue and improves insulin sensitivity in obesity-induced diabetic rats," *Hypertension*, vol. 53, no. 3, pp. 508–515, 2009.

[2] S. J. Peterson, D. H. Kim, M. Li et al., "The L-4F mimetic peptide prevents insulin resistance through increased levels of HO-1, pAMPK, and pAKT in obese mice," *Journal of Lipid Research*, vol. 50, no. 7, pp. 1293–1304, 2009.

[3] T. Porstmann, C. R. Santos, B. Griffiths et al., "SREBP Activity Is Regulated by mTORC1 and Contributes to Akt-Dependent Cell Growth," *Cell Metabolism*, vol. 8, no. 3, pp. 224–236, 2008.

[4] K. Sodhi, K. Inoue, K. H. Gotlinger et al., "Epoxyeicosatrienoic acid agonist rescues the metabolic syndrome phenotype of HO-2-null mice," *Journal of Pharmacology and Experimental Therapeutics*, vol. 331, no. 3, pp. 906–916, 2009.

[5] R. J. Shaw, M. Kosmatka, N. Bardeesy et al., "The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 10, pp. 3329–3335, 2004.

[6] S. P. Hong, F. C. Leiper, A. Woods, D. Carling, and M. Carlson, "Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 15, pp. 8839–8843, 2003.

[7] S. A. Hawley, J. Boudeau, J. L. Reid et al., "Complexes between the LKB1 tumor suppressor, STRADα/β and MO25α/β are upstream kinases in the AMP-activated protein kinase cascade," *Journal of Biology*, vol. 2, no. 4, article 28, 2003.

[8] J. M. Lizcano, O. Göransson, R. Toth et al., "LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1," *EMBO Journal*, vol. 23, no. 4, pp. 833–843, 2004.

[9] M. Li, D. H. Kim, P. L. Tsenovoy et al., "Treatment of obese diabetic mice with a heme oxygenase inducer reduces visceral and subcutaneous adiposity, increases adiponectin levels, and improves insulin sensitivity and glucose tolerance," *Diabetes*, vol. 57, no. 6, pp. 1526–1535, 2008.

[10] N. G. Abraham, M. Li, L. Vanella, S. J. Peterson, S. Ikehara, and D. Asprinio, "Bone marrow stem cell transplant into intrabone cavity prevents type 2 diabetes: role of heme oxygenase-adiponectin," *Journal of Autoimmunity*, vol. 30, no. 3, pp. 128–135, 2008.

[11] F. Seta, L. Bellner, R. Rezzani et al., "Heme oxygenase-2 is a critical determinant for execution of an acute inflammatory and reparative response," *American Journal of Pathology*, vol. 169, no. 5, pp. 1612–1623, 2006.

[12] S. J. Peterson and W. H. Frishman, "Targeting heme oxygenase: therapeutic implications for diseases of the cardiovascular system," *Cardiology in Review*, vol. 17, no. 3, pp. 99–111, 2009.

[13] T. Bobbert, J. Weicht, K. Mai, M. Möhlig, A. F. H. Pfeiffer, and J. Spranger, "Acute hyperinsulinaemia and hyperlipidaemia modify circulating adiponectin and its oligomers," *Clinical Endocrinology*, vol. 71, no. 4, pp. 507–511, 2009.

[14] F. Haugen and C. A. Drevon, "Activation of nuclear factor-κB by high molecular weight and globular adiponectin," *Endocrinology*, vol. 148, no. 11, pp. 5478–5486, 2007.

[15] S. J. Peterson, G. Drummond, D. H. Kim et al., "L-4F treatment reduces adiposity, increases adiponectin levels, and improves insulin sensitivity in obese mice," *Journal of Lipid Research*, vol. 49, no. 8, pp. 1658–1669, 2008.

[16] A. R. Nawrocki, M. W. Rajala, E. Tomas et al., "Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to peroxisome proliferator-activated receptor γ agonists," *Journal of Biological Chemistry*, vol. 281, no. 5, pp. 2654–2660, 2006.

[17] K. D. Poss, M. J. Thomas, A. K. Ebralidze, T. J. O'Dell, and S. Tonegawa, "Hippocampal long-term potentiation is normal in heme oxygenase-2 mutant mice," *Neuron*, vol. 15, no. 4, pp. 867–873, 1995.

[18] N. G. Abraham, T. Kushida, J. McClung et al., "Heme oxygenase-1 attenuates glucose-mediated cell growth arrest and apoptosis in human microvessel endothelial cells," *Circulation Research*, vol. 93, no. 6, pp. 507–514, 2003.

[19] N. G. Abraham, G. Scapagnini, and A. Kappas, "Human heme oxygenase: cell cycle-dependent expression and DNA microarray identification of multiple gene responses after transduction of endothelial cells," *Journal of Cellular Biochemistry*, vol. 90, no. 6, pp. 1098–1111, 2003.

[20] N. G. Abraham and A. Kappas, "Pharmacological and clinical aspects of heme oxygenase," *Pharmacological Reviews*, vol. 60, pp. 79–127, 2008.

[21] Y. H. Weng, G. Yang, S. Weiss, and P. A. Dennery, "Interaction between heme oxygenase-1 and -2 proteins," *Journal of Biological Chemistry*, vol. 278, no. 51, pp. 50999–51005, 2003.

[22] R. Shibata, K. Sato, D. R. Pimentel et al., "Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms," *Nature Medicine*, vol. 11, no. 10, pp. 1096–1103, 2005.

[23] S. Kovacic, C. L. Soltys, A. J. Barr, I. Shiojima, K. Walsh, and J. R. B. Dyck, "Akt activity negatively regulates phosphorylation of AMP-activated protein kinase in the heart," *Journal of Biological Chemistry*, vol. 278, no. 41, pp. 39422–39427, 2003.

[24] A. L. Kruger, S. Peterson, S. Turkseven et al., "D-4F induces heme oxygenase-1 and extracellular superoxide dismutase, decreases endothelial cell sloughing, and improves vascular reactivity in rat model of diabetes," *Circulation*, vol. 111, no. 23, pp. 3126–3134, 2005.

[25] D. B. Shackelford and R. J. Shaw, "The LKB1-AMPK pathway: metabolism and growth control in tumour suppression," *Nature Reviews Cancer*, vol. 9, no. 8, pp. 563–575, 2009.

[26] A. L. Kruger, S. J. Peterson, M. L. Schwartzman et al., "Upregulation of heme oxygenase provides vascular protection in an animal model of diabetes through its antioxidant and antiapoptotic effects," *Journal of Pharmacology and Experimental Therapeutics*, vol. 319, no. 3, pp. 1144–1152, 2006.

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Research Article

The Modulatory Role of Heme Oxygenase on Subpressor Angiotensin II-Induced Hypertension and Renal Injury

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Angiotensin II (AngII) causes hypertension (HTN) and promotes renal injury while simultaneously inducing reno-protective enzymes like heme oxygenase-1 (HO-1). We examined the modulatory role of HO on sub-pressor angiotensin II (SP-AngII) induced renal inflammation and injury. We first tested whether the SP-AngII-induced renal dysfunction, inflammation and injury are exacerbated by either preventing (chronic HO-1 inhibition) or reversing (late HO-1 inhibition) SP-AngII-induced HO (using tin protoporphyrin; SnPP). We next examined whether additional chronic or late induction of SP-AngII-induced HO (using cobalt protoporphyrin; CoPP), prevents or ameliorates renal damage. We found that neither chronic nor late SnPP altered blood pressure. Chronic SnPP worsened SP-AngII-induced renal dysfunction, inflammation, injury and fibrosis, whereas late SnPP worsened renal dysfunction but not inflammation. Chronic CoPP prevented HTN, renal dysfunction, inflammation and fibrosis, but surprisingly, not the NGAL levels (renal injury marker). Late CoPP did not significantly alter SP-AngII-induced HTN, renal inflammation or injury, but improved renal function. Thus, we conclude (a) endogenous HO may be an essential determining factor in SP-AngII induced renal inflammation, injury and fibrosis, (b) part of HO's renoprotection may be independent of blood pressure changes; and (c) further induction of HO-1 protects against renal injury, suggesting a possible therapeutic target.

1. Introduction

Angiotensin II (AngII) is one of the major factors playing a role in the development of chronic kidney disease. It does so by virtue of its hemodynamic, prooxidant, proinflammatory, and profibrotic effects. However, along with its detrimental effects, AngII has been found to induce adaptive, protective pathways. One such cytoprotective system is via heme oxygenase (HO). HO is the rate-limiting step in the metabolism of heme, breaking it down into biliverdin with the resultant release of ferric iron and carbon monoxide [1–3]. It exists as two isoforms, HO-1 and HO-2. HO-1 is an inducible form that is upregulated by various stimuli including lipopolysaccharide, nitric oxide, and AngII [1, 2], whereas HO-2 is constitutively expressed [3]. The cytoprotective properties of HO are attributed to the antioxidant, anti-inflammatory, and vasorelaxant actions of the end products of heme metabolism; biliverdin with the resultant bilirubin and carbon monoxide [4]. Previous studies have demonstrated the importance of HO in dampening the hypertensive and renal vasoconstrictor effects of AngII [5–7]. However, these studies employed pressor doses of AngII. Such high doses of AngII may not accurately reflect the balance between the injurious and adaptive factors that are triggered by physiological doses of AngII. A model that is more analogous to clinical situations is one in which AngII levels are elevated, but within a pathophysiologic range. This is achieved by chronically infusing subpressor doses of AngII (SP-AngII). This model is characterized by the development of salt-sensitive hypertension (HTN), increased expression of proinflammatory factors, oxidative stress, and progressive renal injury [4, 5, 8–10]. We recently examined the renal vascular effects of acute

inhibition of HO after 2 weeks of SP-AngII [11]; however, the modulatory effects of HO on SP-AngII-induced renal inflammation and injury are incompletely understood. In the present study, we evaluated the role of SP-AngII-induced HO-1 in modulating the injurious effects of SP-AngII. We tested whether chronically inhibiting HO exacerbates SP-AngII-induced renal inflammation and injury and also whether inhibiting HO late in the course of SP-AngII alters the renal dysfunction. In addition, because inducing overexpression of HO-1 may represent a potential therapeutic target for preventing AngII-induced damage, we tested whether chronically inducing HO-1 prevented renal injury and whether inducing HO-1 late in the course of SP-AngII-induced HTN ameliorates renal inflammation and damage.

2. Methods

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health.

2.1. Experimental Groups and Design. Male Sprague Dawley rats (Harlan Teklad, Indianapolis, IN USA) (263 ± 20 g) maintained on standard rat chow with water ad libitum were used in all experiments. All animals were inserted with osmotic minipumps (2ML2, Alzet minipumps, DURECT Corp., Cupertino, CA, USA), which infused either a vehicle (saline) or AngII (200 ng/kg/min) subcutaneously for 14 days as previously described [6, 12, 13]. We used this dose of SP-AngII because it increases plasma AngII to levels that do not cause immediate vasoconstriction, yet reliably cause HTN and lead to renal inflammation and injury within the time frame of the experiments. We then tested the involvement and effects of HO in these animals. For this we modulated HO activity in two ways: the first by preemptively modulating the activity of HO (treating throughout the SP-AngII infusion) and second by reversing or enhancing its activity after SP-AngII has been present for 12 days. Initially, we tested whether chronically inhibiting HO (tin protoporphyrin (SnPP) 30 μmol/kg, i.p., every 3 days) worsens renal inflammation and injury. We then tested whether blocking the effect of HO near the end of the 2 -week infusion (late SnPP; 50 μmol/kg, i.p. on day 12), worsens the renal parameters. Because further induction of HO-1 may protect against AngII-induced HTN, we tested whether chronic induction of HO-1 (cobalt protoporphyrin (CoPP) 30 μmol/kg, i.p., every 3 days) blunts renal inflammation and injury. Finally, we tested whether inducing HO-1 near the end of the SP-AngII infusion (late CoPP; 50 μmol/kg, i.p, on day 12) can ameliorate the adverse affects of SP-AngII.

Systolic blood pressure (SBP) was measured during the 14-day course of SP-AngII/vehicle infusion by tail cuff plethysmography (TCP) (Harvard Apparatus, Holliston MA, USA) in conscious, trained animals. On day 14 of the protocol, blood was collected and the animals were housed in metabolic cages, and urine was collected for 24 hours. The animals were then sacrificed, and the kidneys were harvested. All organs were weighed and flash-frozen using liquid nitrogen.

2.2. Measurements

2.2.1. Renal Function, Oxidative Stress, Inflammation, and Injury. Renal function was assessed by measuring plasma creatinine using a Quantichrom Creatinine Assay kit (BioAssay Systems). The kidney cortices were homogenized using a tissue homogenizer (IKA Works) in radioimmunoprecipitation assay (RIPA) buffer (10 μL of phenylmethylsulfonyl fluoride (PMSF) +10 μL sodium orthovanadate +10–20 μL protease inhibitor cocktail per mL of 1X RIPA lysis buffer). A bicinchoninic acid (BCA; Pierce, Rockford, IL, USA) protein assay kit was used for the calorimetric detection and quantification of the total protein in the kidney homogenates. Renal inflammation was estimated by analyzing the renal interleukin-6 levels (IL-6). Kidney cortex homogenates from all groups were analyzed simultaneously for IL-6 levels (Quantikine Rat IL-6 Immunoassay kit) (R&D Systems, Minneapolis MN, USA). Finally renal injury was determined by measuring the following: urinary levels of neutrophil gelatinase-associated lipocalin levels (NGAL, by ELISA; Assay Designs, Ann Arbor, MI, USA); renal tissue levels of kidney injury marker (KIM-1, by ELISA; R&D Systems, Minneapolis, MN, USA); apoptosis, as measured by cytochrome-C levels (by ELISA; R&D Systems, Minneapolis, MN, USA), and the profibrotic signal, transforming growth factor- beta 1 (TGF-β1; by ELISA; R&D Systems, Minneapolis, MN, USA).

2.3. HO Activity. HO activity was determined by measuring the amount of bilirubin produced using a well established protocol [14]. Briefly, the kidney cortices were processed using a homogenization buffer (sucrose, monobasic and dibasic potassium phosphate, EDTA and PMSF at ph 7.7) The protein content of this homogenate was measured and then mixed with a potassium phosphate buffer (monobasic and dibasic potassium phosphate with water at ph 7.4), Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, beta-nicotinamide diamine phosphate (β-NADP) and hemin to get a 1.2 mL reaction. The reaction was then incubated for 1 hr at 37°C in a dark room, after which 1.2 mL of chloroform was added and the mixture vortexed for 15 seconds. The reaction was then frozen overnight, thawed and vortexed. The samples were centrifuged at 15,000 g for 10 minutes, resulting in a ring-like layer separating the supernatant from the lower half. The supernatant was discarded and the absorbance was measured at 464 and 530 nm in a spectrophotometer, using an extinction coefficient of 40 mM/cm for bilirubin. The resulting HO activity was expressed as nmoles of bilirubin/mg of kidney protein/hr (nmol bil/mg protein/hour).

2.4. Statistics. All variables were expressed as mean ± SEM. An unpaired Student's t-test was used to perform comparisons between two groups. The ANOVA test was employed to statistically compare two or more groups. Statistical

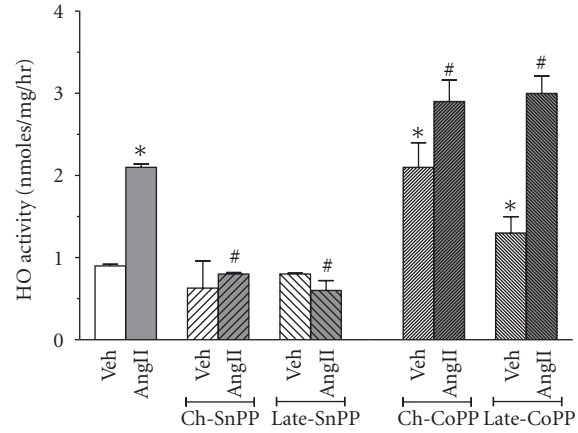

Figure 1: HO activity after chronic and late HO modulation. HO activity is determined by measuring the bilirubin formation in the following groups. Vehicle- (Veh-) treated ($n = 6$), SP-AngII-treated ($n = 6$), chronic-SnPP- (Ch-SnPP-) treated ($n = 6$), SP-AngII/Ch-SnPP-treated ($n = 6$), late-SnPP-treated ($n = 5$), SP-AngII/late-SnPP-treated ($n = 5$), chronic-CoPP- (Ch-CoPP-) treated ($n = 6$), SP-AngII/Ch-CoPP-treated ($n = 6$), late-CoPP-treated ($n = 5$), SP-AngII/late-CoPP-treated ($n = 6$) rats. $^{*}P < 0.05$ versus. control, $^{\#}P < 0.05$ versus. SP-AngII.

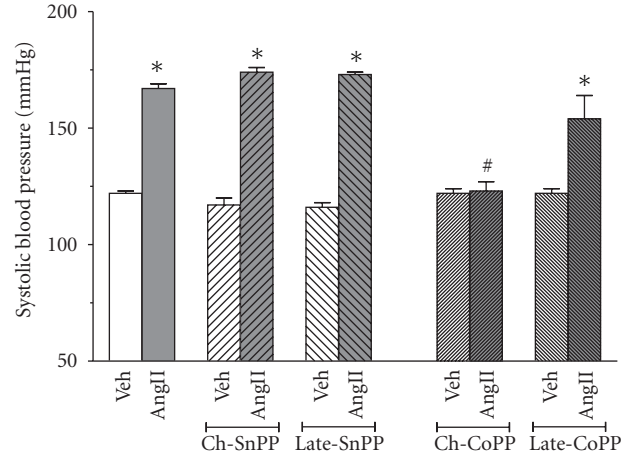

Figure 2: Effect of SP-Ang II and HO modulation on the SBP in the following groups Vehicle- (Veh-) treated ($n = 6$), SP-AngII-treated ($n = 6$), chronic-SnPP- (Ch-SnPP-) treated ($n = 6$), SP-AngII/Ch-SnPP-treated ($n = 6$), late-SnPP-treated ($n = 5$), SP-AngII/late-SnPP-treated ($n = 5$), chronic-CoPP- (Ch-CoPP-) treated ($n = 6$), SP-AngII/Ch-CoPP–treated ($n = 6$), Late CoPP-treated ($n = 5$), SP-AngII/late-CoPP-treated ($n = 6$) rats. $^{*}P < 0.05$ versus. control, $^{\#}P < 0.05$ versus. SP-AngII.

significance was established at a P value less than 0.05. We utilized the Instat software (GraphPad, v3.06; La Jolla, CA, USA) to analyze all data.

3. Results

3.1. HO Activity (Figure 1). We first examined whether our therapeutic protocols were effective in inhibiting or inducing HO activity. SP-AngII increased HO activity from 0.9 ± 0.01 to 2.1 ± 0.04 nmoles of bilirubin/mg protein/hour. Neither chronic nor late administration of SnPP decreased HO activity in control animals (0.63 ± 0.33 and 0.8 ± 0.01 nmoles of bilirubin/mg protein/hour, resp.) but both lowered HO activity in SP-AngII treated rats (from 2.1 ± 0.04 to 0.7 ± 0.02 and 0.7 ± 0.12 nmoles of bilirubin/mg protein/hour, resp.). Chronic and late induction of HO-1 with CoPP increased HO activity in control animals (from 0.9 ± 0.01 to 2.1 ± 0.3 and 1.3 ± 0.2 nmoles of bilirubin/mg protein/hour, resp.) and further elevated SP-AngII-induced HO activity (from 2.1 ± 0.04 to 3 ± 0.25 and 3 ± 0.21 nmoles of bilirubin/mg protein/hour, resp.).

3.2. Blood Pressure (Figure 2). Chronic infusion of Sp-Ang II increased the blood pressure from 122 ± 1 mm Hg to 167 ± 2 mm of Hg. Neither chronic nor late inhibition of HO augmented SP-AngII- induced HTN (167 ± 2 versus 174 ± 2 and 167 ± 2 versus 173 ± 1, resp.). On the other hand, chronic induction of HO-1 prevented the SP-AngII-induced elevation in SBP (SBP was 123 ± 4 mmHg after 2 weeks of SP-AngII). Late induction did not significantly blunt SP-AngII-induced HTN (154 ± 10 mmHg). Neither inhibition nor induction of HO altered blood pressure in vehicle-treated animals.

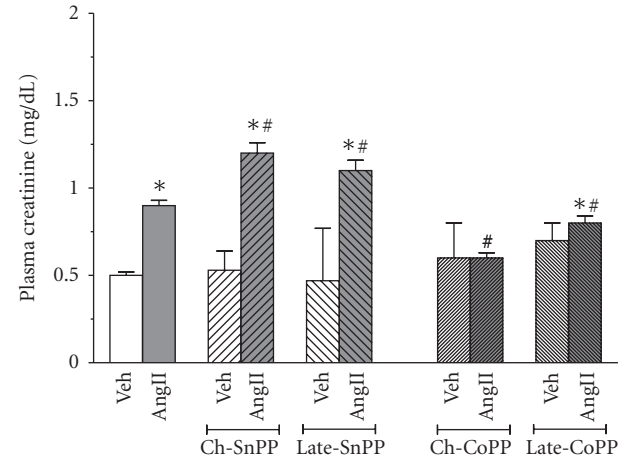

Figure 3: Effect of SP-Ang II and HO modulation on the plasma creatinine levels was determined in the following groups; Vehicle (Veh) treated ($n = 6$), SP-AngII-treated ($n = 6$), chronic-SnPP- (Ch-SnPP-) treated ($n = 6$), SP-AngII/Ch-SnPP-treated ($n = 6$), late SnPP-treated ($n = 5$), SP-AngII/late-SnPP-treated ($n = 5$), chronic-CoPP- (Ch-CoPP-) treated ($n = 6$), SP-AngII/Ch-CoPP–treated ($n = 6$), and late-CoPP-treated ($n = 5$), SP-AngII/late CoPP-treated ($n = 6$) rats. $^{*}P < 0.05$ versus. control, $^{\#}P < 0.05$ versus. SP-AngII.

3.3. Renal Function, Inflammation and Injury. SP-AngII increased plasma creatinine from 0.5 ± 0.02 mg/dL to 0.9 ± 0.03 mg/dL, suggesting that this dose of SP-AngII impairs renal function (Figure 3). Chronic inhibition of HO exacerbated SP-AngII-induced increases in plasma creatinine (1.2 ± 0.06 mg/dL). Late HO inhibition, despite having only a couple of days to exert its effect, also elevated SP-AngII-induced increases in plasma creatinine (1.1 ± 0.06 mg/dL).

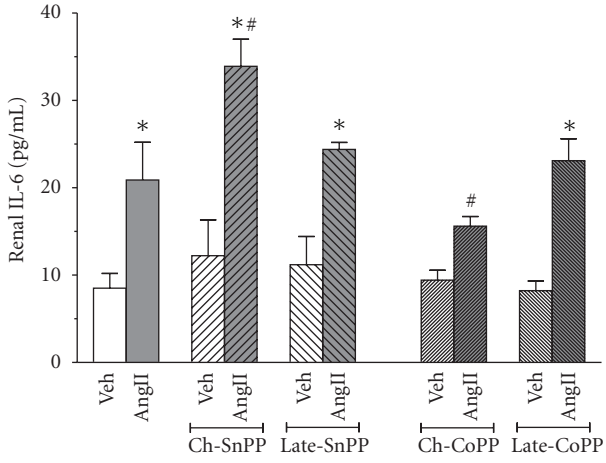

Figure 4: Effect of SP-Ang II and HO modulation on the inflammatory cytokine- IL-6 was estimated in the following groups; Vehicle (Veh) treated ($n = 6$), SP-AngII-treated ($n = 6$), chronic-SnPP- (Ch-SnPP-) treated ($n = 6$), SP-AngII/Ch-SnPP-treated ($n = 6$), late-SnPP-treated ($n = 5$), SP-AngII/late-SnPP-treated ($n = 5$), chronic-CoPP- (Ch-CoPP-) treated ($n = 6$), SP-AngII/Ch-CoPP–treated ($n = 6$), late-CoPP-treated ($n = 5$), SP-AngII/late CoPP-treated ($n = 6$) rats. $^{*}P < 0.05$ versus. control, $^{\#}P < 0.05$ versus. SP-AngII.

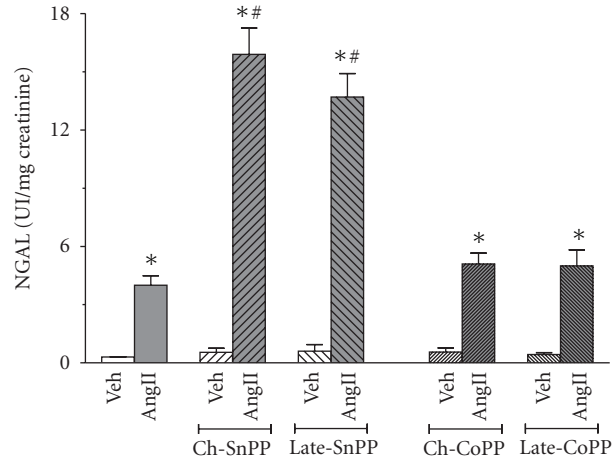

Figure 5: Effect of SP-Ang II and HO modulation on Urinary NGAL excretion was measured in the following groups; Vehicle-(Veh-) treated ($n = 6$), SP-AngII-treated ($n = 6$), chronic-SnPP-(Ch-SnPP-) treated ($n = 6$), SP-AngII/Ch-SnPP-treated ($n = 6$), late-SnPP-treated ($n = 5$), SP-AngII/late SnPP-treated ($n = 5$), chronic-CoPP- (Ch-CoPP-) treated ($n = 6$), SP-AngII/Ch-CoPP–treated ($n = 6$), late-CoPP-treated ($n = 5$), SP-AngII/Late-CoPP-treated ($n = 6$) rats. $^{*}P < 0.05$ versus. control, $^{\#}P < 0.05$ versus. SP-AngII.

As with blood pressure, chronic induction of HO-1 prevented the rise in plasma creatinine caused by SP-AngII (0.6 ± 0.03 mg/dL), whereas late induction of HO-1 blunted this increase (0.8 ± 0.04 mg/dL). Neither inhibition nor induction of HO altered plasma creatinine in vehicle-treated animals.

Renal inflammation was assessed by measuring IL-6 levels (Figure 4). SP-AngII increased IL-6 levels from 8.5 ± 1.7 pg/μg/mL to 20.9 ± 4.3 pg/μg/mL. Chronic HO inhibition accentuated SP-AngII-induced IL-6 levels (to 33.9 ± 3.1 pg/μg/mL). In contrast to plasma creatinine, late HO inhibition did not exacerbate the SP-AngII-induced IL-6 (24.4 ± 0.8 pg/μg/mL). Chronic induction of HO-1 did not completely block SP-AngII-induced increases in IL-6 but significantly blunted it (15.6 ± 1.1 pg/μg/mL), whereas late induction did not significantly alter IL-6 levels (23.1 ± 2.5 pg/μg/mL). Neither inhibition nor induction of HO altered IL-6 in vehicle-treated animals.

Renal damage was assessed by measuring the levels of the renal injury marker NGAL (Figure 5). SP-AngII increased NGAL from 0.3 ± 0.01 to 4 ± 0.49 UI/mg creatinine, thus suggesting that this dose of Ang II was inducing renal damage. Chronic HO inhibition markedly exacerbated SP-AngII-induced NGAL (to 15.9 ± 1.35 UI/mg creatinine). Late HO inhibition, despite having only a short duration to act, also increased SP-AngII-induced NGAL (to 13.7 ± 1.21 UI/mg creatinine). However, neither chronic nor late induction of HO-1 blunted the SP-AngII-induced NGAL elevation (5.1 ± 0.56 and 5 ± 0.82 UI/mg creatinine, resp.), which is in marked contrast to their effects on blood pressure, renal function and inflammation. This raises the possibility that SP-AngII-induced NGAL might not be always in tandem with co-existing renal injury. Hence we assessed additional markers of renal injury such as KIM-1 (biomarker of injury), cytochrome C (apoptosis) and TGF-β1(fibrosis). Since we were mainly interested in determining whether inducing HO-1 blunts frank injury, we only measured these parameters in the animals with chronic HO inhibition and induction. As shown in Figure 6(a), SP-AngII increased KIM-1 (from 54.5 ± 3.7 to 468.9 ± 120.6 pg/μg kidney protein) was increased by chronic HO inhibition (853.3 ± 145.1 pg/μg kidney protein), and blunted by chronic induction (140.8 ± 92.55 pg/μg kidney protein). This same pattern was seen with both cytochrome c and TGF-β1, except that SP-AngII induced increases in these parameters was completely blocked by chronic CoPP, (Figures 6(b) and 6(c), resp.).

4. Discussion

HO-1 is an important cytoprotectant which is induced in the kidney by oxidative stress, injury and certain hormones including AngII, and thus its role in modulating renal injury has been increasingly studied. Our laboratory has a long-standing interest in the interactions between AngII and HO in determining renal function and injury. In our recent study, we examined the effect of acutely inhibiting HO on renal hemodynamics in rats that were treated with very low doses of SP-AngII (50 ng/kg/min IV) [11]. We found that although renal HO-1 was not increased by this dose of SP-AngII, blocking it still modulated renal hemodynamics in SP-AngII-treated but not control rats. The present study is an extension of our previous one in that we now evaluated the impact of modulating HO activity on SP-AngII-induced renal inflammation and injury. We modulated HO activity by preemptively/chronically modulating the activity of HO,

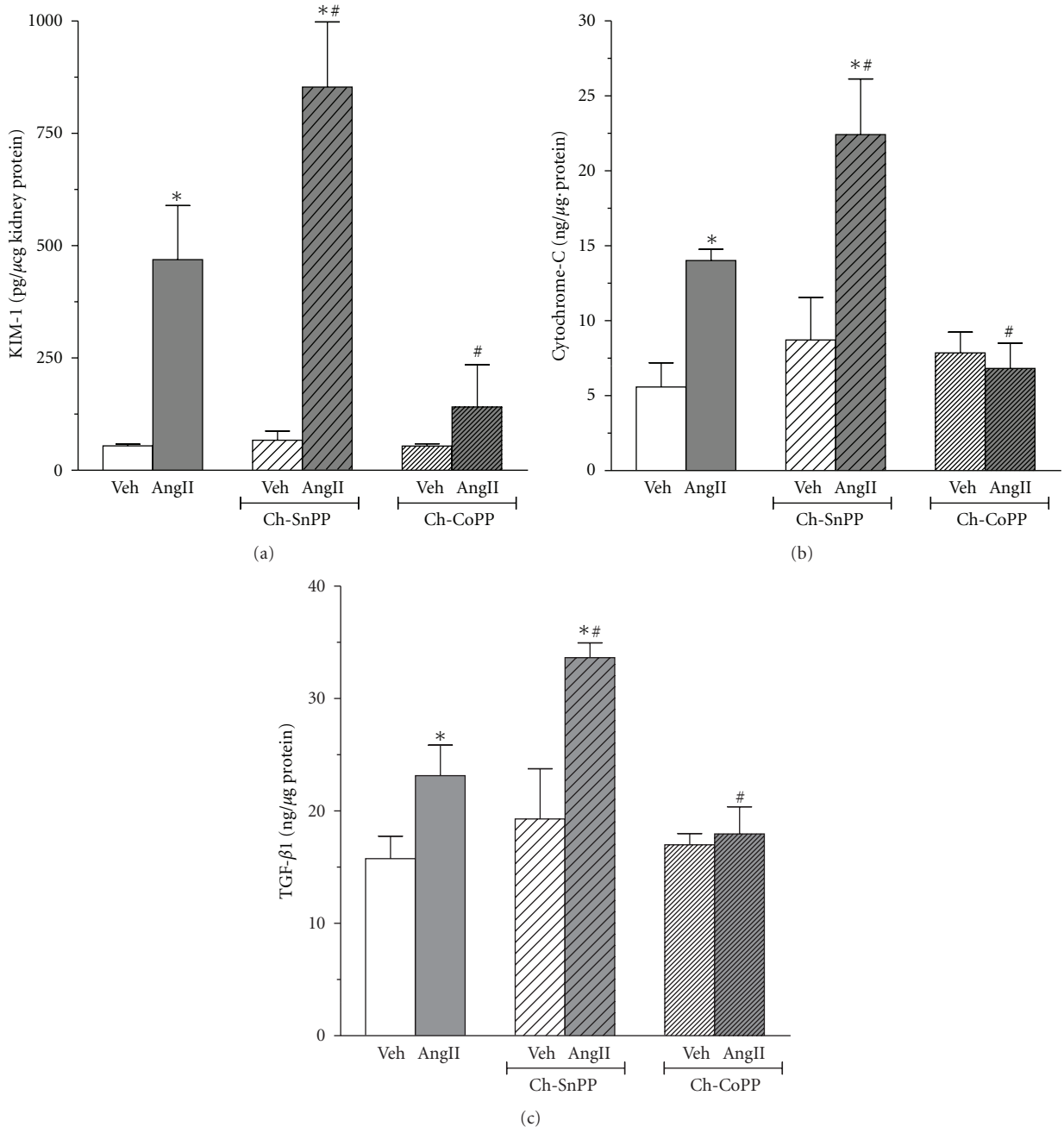

Figure 6: Effect of SP-Ang II and HO modulation on renal (a) KIM-1, (b) cytochrome c, and (c) TGF-β1 expression was estimated in the following groups; Vehicle (Veh) treated ($n = 6$), SP-AngII-treated ($n = 6$), Chronic SnPP (Ch-SnPP)-treated ($n = 6$), SP-AngII/Ch-SnPP-treated ($n = 6$), Chronic CoPP (Ch-CoPP)-treated ($n = 6$), SP-AngII/Ch-CoPP-treated ($n = 6$), $^{*}p < 0.05$ versus. Control, $^{\#}p < 0.05$ versus. SP-AngII.

and by reversing or enhancing its activity late in the course of SP-AngII-induced HTN. We found that chronic and late HO inhibition exacerbated SP-AngII-induced renal dysfunction without significantly increasing the blood pressure. We also found that chronic induction prevented SP-AngII-induced HTN as well as the majority of the parameters of renal inflammation and injury, whereas late-induction did not significantly reduce blood pressure, renal inflammation or NGAL, but improved plasma creatinine.

AngII is an important contributor to the pathogenesis of various forms of HTN and is also a major determinant in the progression of chronic kidney disease by virtue of its hemodynamic, proinflammatory, and profibrotic effects [15–17]. Indeed, numerous studies have demonstrated that the chronic infusion of AngII causes HTN, decreases GFR, while simultaneously inducing inflammatory signaling leading to renal injury, apoptosis and fibrosis [1, 2, 18]. However, most of these studies used very high pressor doses of AngII which

induce rapid and aggressive renal damage, regardless of the presence of any cytoprotection, and hence may not reflect the sequence of inflammation and injury in human disease. Our interests lie in studying the balance between the injurious and cytoprotective factors that are induced by more clinically relevant levels of AngII. In previous studies, we used doses of SP-AngII that increase oxidative stress and proinflammatory signaling, but do not consistently cause sustained HTN nor cause frank renal injury. In the present study our aim was to evaluate the modulatory effect of HO on SP-AngII-induced renal inflammation and injury. Thus we modified our SP-AngII model slightly to one that consistently increases blood pressure (it increased within 4 days) and leads to renal injury within the 2-week timeframe of our experiments, and HO-1 induction. Therefore, this model of SP-AngII is a valuable tool to examine the modulatory effects of HO on renal injury.

Various previous studies have found discrepant effects of HO inhibition on HTN [11, 14, 19]. Several studies have found that blocking HO results in an increase in blood pressure, whereas others show no change or even a decrease [11, 19]. Indeed, we previously found that HO blockade decreased mean arterial pressure (MAP) in anesthetized SP-AngII rats [11], which appeared to be due to a fall in cardiac output. The differences in blood pressure responses in the various studies may be due to differences in the species and protocols of AngII used. In the present study, we found that SnPP did not alter SBP in conscious SP-AngII rats, which at first glance appears to contradict our previous study. However, this variation may be due to the different SP-AngII models used, or to how blood pressure was measured. Indeed, the SnPP rats in the current study also tended to have a lower MAP when measured under anesthesia (data not shown).

Despite not altering the blood pressure, SnPP aggravated all the renal injury parameters suggesting that SP-AngII-induced HO is markedly attenuating the deleterious actions of AngII on the kidney. Interestingly, even late inhibition of HO activity caused a deleterious effect on renal function and NGAL levels. While, the mechanism by which late HO inhibition worsened renal function is likely due to the hemodynamic effects of HO inhibition [11], the mechanisms for the increased NGAL levels are unclear. Because NGAL does not represent cumulative injury, but rather ongoing and progressive renal injury [20], its increase may denote an accentuation in the rate of renal injury following late administration of SnPP that may be due to HO-inhibition or a direct renal toxic effect of SnPP. We speculate that the increase in NGAL was not emulated by changes in IL-6 because the relatively brief duration of HO inhibition was insufficient to detect worsening of renal inflammation. Altogether, our findings suggest that SP-AngII-induced HO tempers renal injury in a manner that is not reliant on lowering blood pressure.

Because HO-1 has profound renoprotective effects in a variety of renal diseases, there has been much interest in examining the therapeutic potential of inducing the HO-1 system in an attempt to ameliorate progression of renal injury. We therefore examined whether further induction of HO-1 during SP-AngII can further protect against SP-AngII-induced renal injury. We found that chronically inducing HO-1 completely blocked SP-AngII-induced HTN and real dysfunction. It also blunted the increase in renal inflammation, but surprisingly not the NGAL levels. These results were puzzling to us and therefore we further evaluated renal injury with other parameters; KIM-1, cytochrome c, and TGF-β1. We found that chronic HO-1 induction completely blocked SP-AngII-induced increases in all 3 parameters suggesting prevention of renal injury despite the high NGAL level. Although the reason for this discrepancy is unclear, it insinuates that under certain conditions NGAL may be induced independent of injury. However, we cannot discard the possibility that NGAL may simply be the earliest of the markers we measured to increase with injury.

In contrast to chronic induction, late HO-1 induction did not normalize blood pressure, nor did it alter IL-6 or NGAL. This was not unexpected as it seems unlikely that the brief duration of additional HO-1 induction would be sufficient to reverse the 12 days of SP-AngII-induced renal inflammation and injury. However, despite the lack of effect on blood pressure, late HO-1 induction significantly improved renal function, providing further evidence that under these conditions HO modulates renal but not systemic hemodynamics.

In summary, our results show that Sp-AngII-induced HO blunts the deleterious effects of SP-AngII on renal hemodynamics, inflammation and injury without significantly lowering blood pressure. Moreover, if induced early enough, HO may prevent SP-AngII-induced HTN and renal injury. Hence, we speculate that the activity of endogenous HO is an important determinant in the progression of SP-AngII-induced HTN and renal injury, and that at least part of its renoprotective effect is independent of its blood pressure lowering properties. Thus, conditions which lower HO activity may render the kidney more susceptible to injury [12, 13], while also raising the possibility of targeting HO-1 induction as a therapeutic measure to protect against renal injury.

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References

[1] T. Aizawa, N. Ishizaka, J. I. Taguchi et al., "Heme oxygenase-1 is upregulated in the kidney of angiotensin II-induced hypertensive rats: possible role in renoprotection," *Hypertension*, vol. 35, no. 3, pp. 800–806, 2000.

[2] E. N. Haugen, A. J. Croatt, and K. A. Nath, "Angiotensin ii induces renal oxidant stress in vivo and heme oxygenase-1 in vivo and in vitro," *Kidney International*, vol. 58, no. 1, pp. 144–152, 2000.

[3] N. G. Abraham and A. Kappas, "Pharmacological and clinical aspects of heme oxygenase," *Pharmacological Reviews*, vol. 60, no. 1, pp. 79–127, 2008.

[4] S. G. Hood, T. Cochrane, M. J. McKinley, and C. N. May, "Investigation of the mechanisms by which chronic infusion of

an acutely subpressor dose of angiotensin II induces hypertension," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 292, no. 5, pp. R1893–R1899, 2007.

[5] G. Simon, G. Abraham, and G. Cserep, "Pressor and subpressor angiotensin II administration. Two experimental models of hypertension," *American Journal of Hypertension*, vol. 8, no. 6, pp. 645–650, 1995.

[6] W. Wu, Y. Zhang, J. R. Ballew, G. Fink, and D. H. Wang, "Development of hypertension induced by subpressor infusion of angiotensin II: role of sensory nerves," *Hypertension*, vol. 36, no. 4, pp. 549–552, 2000.

[7] S. Rajagopalan, S. Kurz, T. Münzel et al., "Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: contribution to alterations of vasomotor tone," *Journal of Clinical Investigation*, vol. 97, no. 8, pp. 1916–1923, 1996.

[8] M. C. Ortiz, E. Sanabria, M. C. Manriquez, J. C. Romero, and L. A. Juncos, "Role of endothelin and isoprostanes in slow pressor responses to angiotensin II," *Hypertension*, vol. 37, no. 2, pp. 505–510, 2001.

[9] L. I. Pelaez, M. C. Manriquez, K. A. Nath, J. C. Romero, and L. A. Juncos, "Low-dose angiotensin II enhances pressor responses without causing sustained hypertension," *Hypertension*, vol. 42, no. 4, pp. 798–801, 2003.

[10] A. J. Brown, J. Casals-Stenzel, S. Gofford, A. F. Lever, and J. J. Morton, "Comparison of fast and slow pressor effects of angiotensin II in the conscious rat," *The American journal of physiology*, vol. 241, no. 3, pp. H381–388, 1981.

[11] K. A. Nath, M. C. Hernandez, A. J. Croatt, Z. S. Katusic, and L. A. Juncos, "Heme oxygenase activity as a determinant of the renal hemodynamic response to low-dose ANG II," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 299, no. 5, pp. R1183–R1191, 2010.

[12] C. Ronco, C. Y. Chionh, M. Haapio, N. S. Anavekar, A. House, and R. Bellomo, "The cardiorenal syndrome," *Blood Purification*, vol. 27, no. 1, pp. 114–126, 2009.

[13] R. A. Zager, L. A. Baltes, H. M. Sharma, and M. S. Jurkowitz, "Responses of the ischemic acute renal failure kidney to additional ischemic events," *Kidney International*, vol. 26, no. 5, pp. 689–700, 1984.

[14] T. Vera, S. Kelsen, L. L. Yanes, J. F. Reckelhoff, and D. E. Stec, "HO-1 induction lowers blood pressure and superoxide production in the renal medulla of angiotensin II hypertensive mice," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 292, no. 4, pp. R1472–R1478, 2007.

[15] M. E. Rosenberg, L. J. Smith, R. Correa-Rotter, and T. H. Hostetter, "The paradox of the renin-angiotensin system in chronic renal disease," *Kidney International*, vol. 45, no. 2, pp. 403–410, 1994.

[16] S. Anderson, "Physiologic actions and molecular expression of the renin-angiotensin system in the diabetic rat," *Mineral and Electrolyte Metabolism*, vol. 24, no. 6, pp. 406–411, 1998.

[17] R. A. Lafayette, G. Mayer, S. K. Park, and T. W. Meyer, "Angiotensin II receptor blockade limits glomerular injury in rats with reduced renal mass," *Journal of Clinical Investigation*, vol. 90, no. 3, pp. 766–771, 1992.

[18] T. Aizawa, N. Ishizaka, K. Kurokawa et al., "Different effects of angiotensin II and catecholamine on renal cell apoptosis and proliferation in rats," *Kidney International*, vol. 59, no. 2, pp. 645–653, 2001.

[19] F. K. Johnson, R. A. Johnson, W. Durante, K. E. Jackson, B. K. Stevenson, and K. J. Peyton, "Metabolic syndrome increases endogenous carbon monoxide production to promote hypertension and endothelial dysfunction in obese Zucker rats," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 290, no. 3, pp. R601–R608, 2006.

[20] D. Bolignano, A. Lacquaniti, G. Coppolino et al., "Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease," *Clinical Journal of the American Society of Nephrology*, vol. 4, no. 2, pp. 337–344, 2009.

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Review Article

Heme Oxygenase-1 Induction and Organic Nitrate Therapy: Beneficial Effects on Endothelial Dysfunction, Nitrate Tolerance, and Vascular Oxidative Stress

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Organic nitrates are a group of very effective anti-ischemic drugs. They are used for the treatment of patients with stable angina, acute myocardial infarction, and chronic congestive heart failure. A major therapeutic limitation inherent to organic nitrates is the development of tolerance, which occurs during chronic treatment with these agents, and this phenomenon is largely based on induction of oxidative stress with subsequent endothelial dysfunction. We therefore speculated that induction of heme oxygenase-1 (HO-1) could be an efficient strategy to overcome nitrate tolerance and the associated side effects. Indeed, we found that hemin cotreatment prevented the development of nitrate tolerance and vascular oxidative stress in response to chronic nitroglycerin therapy. Vice versa, pentaerithrityl tetranitrate (PETN), a nitrate that was previously reported to be devoid of adverse side effects, displayed tolerance and oxidative stress when the HO-1 pathway was blocked pharmacologically or genetically by using HO-$1^{+/-}$ mice. Recently, we identified activation of Nrf2 and HuR as a principle mechanism of HO-1 induction by PETN. With the present paper, we present and discuss our recent and previous findings on the role of HO-1 for the prevention of nitroglycerin-induced nitrate tolerance and for the beneficial effects of PETN therapy.

1. Organic Nitrate Therapy and Side Effects

Nitroglycerin (GTN) has been one of the most widely used anti-ischemic drugs for more than a century. Given acutely, organic nitrates are excellent agents for the treatment of stable effort angina, acute myocardial infarction, chronic congestive heart failure, pulmonary edema, and severe arterial hypertension (for review see [1, 2]). The chronic efficacy of nitrates, however, is blunted due to the development of nitrate tolerance and endothelial dysfunction, phenomena that are largely associated with increased vascular oxidative stress (for review see [1–5]). Oxidative stress was demonstrated to be a hallmark of most cardiovascular diseases [6]. The term oxidative stress defines a state with either increased formation of reactive oxygen and nitrogen species (RONS) and/or impaired cellular antioxidant defense system (e.g., downregulation of important antioxidant proteins) with subsequent depletion of low-molecular-weight antioxidants and a shift in the cellular redox balance. The central role of the endothelium for the regulation of vascular tone makes it a vulnerable target for RONS which can interfere at many positions with the NO/cGMP signaling cascade [7].

It is well established that most organic nitrates cause nitrate tolerance and/or cross-tolerance to endothelium-dependent vasodilators (e.g., acetylcholine) [8–11]. The first report on a role for oxidative stress in the development of nitrate tolerance was published in 1995 by Münzel and coworkers for nitroglycerin therapy [12]. These authors found that superoxide levels were twofold higher in aortic

segments from nitrate tolerant vessels with intact endothelium. Based on these findings, they suspected that the enhanced levels of superoxide in nitroglycerin tolerant vessels might contribute not only to nitroglycerin tolerance, but also to cross-tolerance to 3-morpholinosydnonimine (Sin-1) and endogenous NO production stimulated by acetylcholine. To test this hypothesis, they examined the effects of bovine Cu, Zn-superoxide dismutase (SOD) entrapped in pH sensitive liposomes. In nitroglycerin-tolerant aortic segments with endothelium, liposomal SOD markedly enhanced the relaxations evoked by nitroglycerin, Sin-1, and acetylcholine. The source of RONS formation in the setting of nitrate tolerance was first found to be NADH oxidase. This finding was mainly based on the observation that the superoxide signal was most pronounced in the presence of NADH and that it was located in the particulate and not cytosolic fraction [13]. More compelling data came from the observation that the protein kinase C inhibition effectively suppressed nitroglycerin-induced vascular RONS formation and vasoconstrictor supersensitivity in tolerant vessels, keeping in mind that protein kinase C activates NADPH oxidase [14, 15].

Since nitroglycerin is thought to release NO and induce superoxide formation simultaneously, the formation of peroxynitrite from the reaction of NO and superoxide could be expected. Indeed, some studies have reported on increased levels of tyrosine-nitrated proteins, which is a marker for increased peroxynitrite formation in tissue from nitrate-tolerant animals [16]. We could also identify higher concentrations of nitrated prostacyclin synthase and decreased prostacyclin levels in these animals [17]. Indirect proof for a role of peroxynitrite for nitrate tolerance came from the observation that hydralazine, which efficiently improves nitrate tolerance, is a powerful peroxynitrite scavenger and inhibitor of protein tyrosine nitration [18]. Moreover, authentic or in situ generated (Sin-1-derived) peroxynitrite was most efficient in inhibiting the bioactivating enzyme of nitroglycerin [19]. In addition, three independent reports provided data that peroxynitrite plays a central role in the development and pathogenesis of nitrate tolerance [20–22].

The concept of NAD(P)H oxidase-driven RONS formation as the most important source of oxidative stress in nitrate tolerance was accepted for almost 10 years. In 2004, we reported for the first time on mitochondrial ROS formation in nitroglycerin induced tolerance [23], although bioactivation of nitroglycerin by mitochondrial aldehyde dehydrogenase (ALDH-2) was already reported 2 years earlier [24]. Despite the fact that the harmful effects of organic nitrates on mitochondria have already been described in the 1960s by Needleman and coworkers (mitochondrial swelling, thiol depletion, and impaired respiration) [25, 26], it took more than 40 years to reveal the pivotal role of mitochondria in nitroglycerin toxicity [23, 24, 27]. To test this hypothesis, we used mice with heterozygous Mn-SOD deficiency (Mn-SOD$^{+/-}$), which is the mitochondrial isoform of superoxide dismutases [28, 29]. Nitroglycerin-driven vascular and mitochondrial ROS formation was increased in Mn-SOD$^{+/-}$ mice, and, vice versa, the ALDH-2 activity in these samples was decreased by nitroglycerin in a more pronounced manner. Moreover, nitroglycerin potency was significantly impaired in response to low-dose nitroglycerin *in vivo* treatment indicating the development of nitrate tolerance by this low dose in Mn-SOD$^{+/-}$ mice but not in wild-type controls. The detrimental role of mitochondrial RONS formation for the development of GTN-induced nitrate tolerance was further supported by a subsequent report on the prevention of GTN side effects by the mitochondria-targeted antioxidant mitoquinone (mitoQ) [30].

2. Effects of HO-1 Induction and Suppression on Nitrate Tolerance and Oxidative Stress

With a previous study, we demonstrated that chronic nitroglycerin (GTN) therapy results in impaired vasodilatory potency of GTN (nitrate tolerance) and of the endothelium-dependent vasodilator acetylcholine (endothelial dysfunction) as well as increased vascular and mitochondrial RONS formation [31]. Since another organic nitrate (PETN) was previously described to be devoid of nitrate tolerance and induction of oxidative stress due to induction of the heme oxygenase-1 (HO-1) system [35, 36], we hypothesized that pharmacological activation of the HO-1 system may be suitable to prevent GTN-dependent side effects. In deed, cotreatment of GTN-infused rats with the potent HO-1 inducer hemin completely prevented nitrate tolerance (restored the GTN-dependent relaxation), restored NO/cGMP signaling, increased the activity of the GTN bioactivating enzyme ALDH-2, and suppressed the mitochondrial RONS formation (Figures 1(a)–1(c)) [31]. The HO-1 product bilirubin suppressed GTN-induced RONS formation in isolated heart mitochondria (Figure 1(d)). To test the essential role of HO-1 for the tolerance devoid action of PETN, we cotreated PETN-infused rats with the HO-1 suppressor apigenin and observed a tolerance-like phenomenon displaying impaired PETN-dependent relaxation, disturbed NO/cGMP signaling, and increased mitochondrial RONS formation (Figures 1(e)-1(f)) [31].

These observations are in good accordance with previous reports on HO-1 induction by statins [37, 38] and prevention of nitrate tolerance in GTN-infused experimental animals [39] as well as human individuals [40, 41]. The role of HO-1 for prevention of organic nitrate induced tolerance, endothelial dysfunction, and oxidative stress is further supported by observations that the HO-1 product bilirubin efficiently scavenged GTN-induced RONS (most probably peroxynitrite) formation in isolated mitochondria [29, 31]. Likewise, the HO-1 products bilirubin and carbon monoxide as well as PETN increased the expression of the GTP-cyclohydrolase-1 (GCH-1), the most important enzyme for *de novo* synthesis of tetrahydrobiopterin (BH_4), an essential cofactor for endothelial NO synthase (eNOS) function [34, 42]. In contrast, GTN *in vivo* therapy decreased the expression of the GCH-1 [43]. Since BH_4 levels are directly linked to eNOS activity and endothelial function [44, 45] and GCH-1 is oxidatively degraded by activation of the proteasome26S [46], activation of antioxidant pathways by induction of HO-1 may represent an attractive explanation for the tolerance and endothelial dysfunction devoid profile

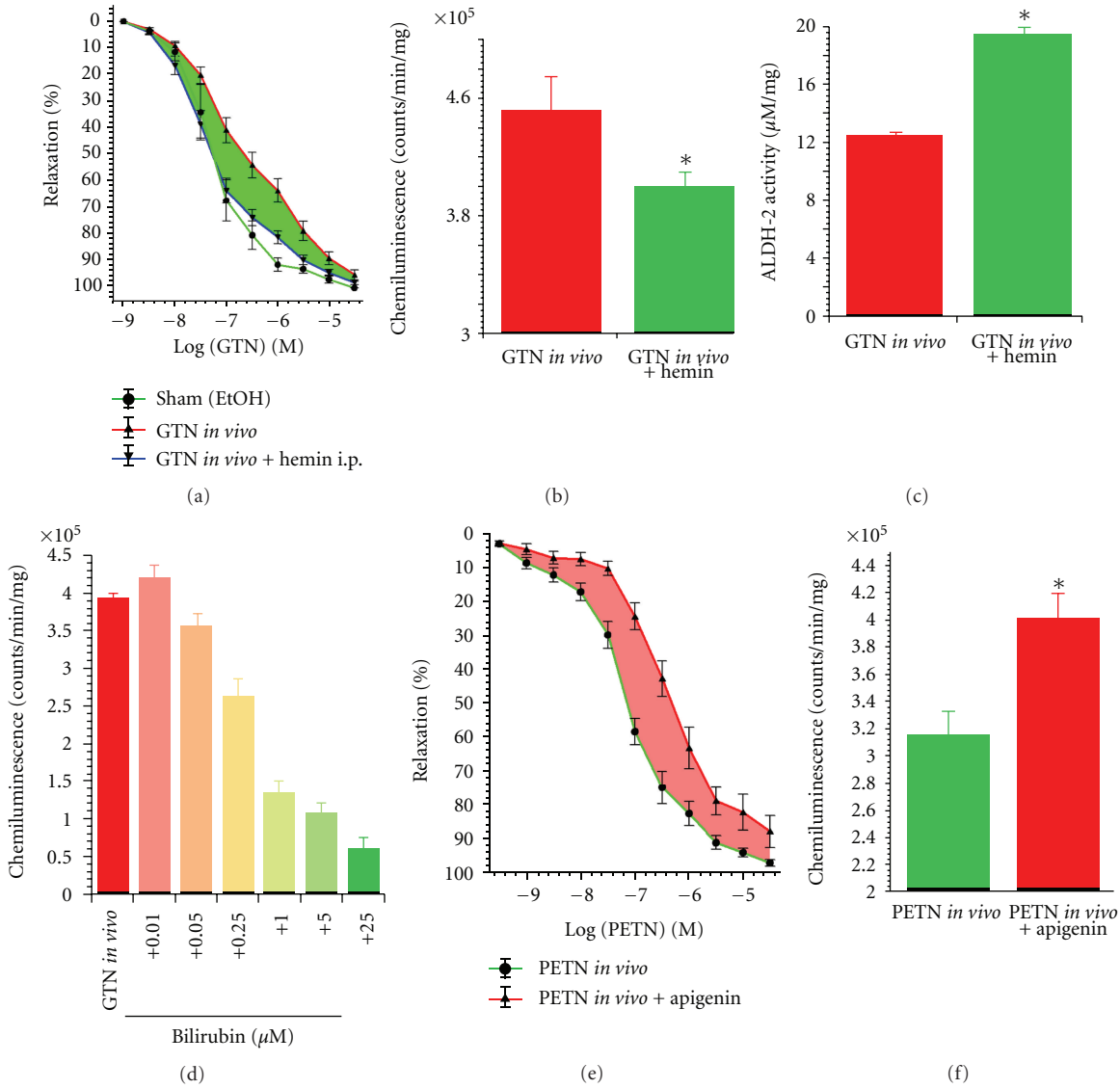

Figure 1: Effects of the HO-1 inducer hemin on GTN-induced tolerance and effects of the HO-1 suppressor apigenin on PETN side effects. Hemin (25 mg/kg) was administrated by single i.p. injection on day 3 of GTN treatment (6.6 μg/kg/min for 4 days via s.c. infusion) and markedly improved vascular GTN responsiveness (see area between curves) as demonstrated by isometric tension studies (a), a significant decrease in mitochondrial ROS formation (b), and improvement of mitochondrial ALDH-2 activity (c). Effects of bolus bilirubin on ROS formation in isolated heart mitochondria from GTN *in vivo* treated rats were determined by L-012 (100 μM) ECL in the presence of 2.5 mM succinate and bilirubin (0–25 μM) (d). Apigenin (10 mg/kg/d) was coinfused over 4d together with PETN (10.5 μg/kg/min for 4 days via s.c. infusion). Apigenin cotreatment decreased PETN vasodilator potency (see area between curves) and induced a tolerance-like right shift in the PETN concentration-relaxation-curve (E). This observation was accompanied by increased mitochondrial ROS formation (F). Data are mean ± SEM of n = 8–12 (a), 40 (b), 6–18 (c), 4–6 (d), 9–11 (e), and 28–43 (f) independent experiments. $^*P < 0.05$ versus GTN or PETN treatment. Modified from [31].

of chronic PETN therapy and the undesired side effects of most other organic nitrates (but most pronounced for GTN treatment). Similar observations were made for extracellular superoxide dismutase (ecSOD), a downstream target of HO-1 and its products [47], which is upregulated by PETN [34, 48] but not by isosorbide-5-mononitrate (ISMN). Moreover, it was recently reported that HO-1 plays a significant role in the maintenance of soluble guanylyl cyclase (sGC) in a reduced heme state providing another important function for HO-1 in the regulatory pathways of vascular tone [49]. This novel beneficial property of HO-1 could also significantly improve vascular dysfunction in the setting of nitrate tolerance. These observations underline the distinct properties of organic nitrates, and these drugs do not represent a class with homogeneous effects [50] but also challenge the traditional assumption that all organic nitrates release the same vasoactive species (nitric oxide)—an assumption that was already challenged for GTN in 2003 [51] (and reviewed in [1, 5]).

In addition, previous work has demonstrated that organic nitrates have distinct effects on the function and survival of circulating angiogenic cells (formally known as

endothelial progenitor cells) [52, 53]. These studies showed that isosorbide dinitrate in contrast to PETN impair the migration and incorporation activities of these circulating angiogenic cells in an experimental model of myocardial infarction, whereas GTN *in vitro* exposure increased apoptosis while decreasing phenotypic differentiation, migration, and mitochondrial dehydrogenase activity in these cells. In a subsequent study, Lin et al. investigated the involvement of heme oxygenase-1 for the related neovascularization process by hematopoietic stem cells and endothelial progenitor cells in the infarcted area [54]. Thum et al. have shown that the impaired function of these circulating angiogenic cells is based on oxidative stress as envisaged in the setting of diabetes, leading to eNOS uncoupling, which was improved by antioxidants (e.g., superoxide dismutase) [55]. These findings underline the importance of maintaining the BH_4 levels to prevent eNOS uncoupling and the role of HO-1 for this antioxidant mechanism via increase in GCH-1 expression by carbon monoxide and bilirubin as outlined above. This concept is further supported by protective effects of folic acid (a precursor of BH_4) on impaired endothelial function in GTN-treated healthy volunteers [56], and decreased BH_4 levels in GTN-treated rabbits were restored by cotherapy with pioglitazone [57]. It should be noted that another group found no association between aortic BH_4 content and eNOS function in response to GTN *ex vivo* and *in vivo* treatment [58].

Finally, it should be mentioned that oxidative stress in response to organic nitrates may also be protective by a process called ischemic preconditioning (IPC) [59–61]. Recently, the involvement of HO-1 in organic nitrate-mediated IPC was proposed [59, 62] as an explanation for the sustained IPC protective effect under chronic PETN therapy but loss of this beneficial effect under chronic GTN therapy. This is in accordance with the accepted view that HO-1 plays a role in IPC [63, 64].

3. Molecular Proof of a Role of HO-1 for the Tolerance-Devoid Profile of PETN by Using HO-$1^{+/-}$ Mice

The role of HO-1 as the antioxidative principle of PETN was elucidated by 3-key experiments aiming to prove this hypothesis at a molecular basis [32]. The first experimental setup consisted of the treatment of control (HO-$1^{+/+}$) and partially deficient (HO-$1^{+/-}$) mice with PETN. In HO-$1^{+/-}$ but not HO-$1^{+/+}$ mice, PETN infusion induced desensitization to PETN-induced vasorelaxation (envisaged by impaired vasodilator potency of the drug in isolated aortic segments) and increased mitochondrial ROS formation (Figures 2(a) and 2(b)), demonstrating nitrate tolerance to PETN in a setting of HO-1 deficiency. The second approach was on the basis of HO-1 induction by the known inducer of this enzyme, hemin, which improved angiotensin-II-(high-dose) dependent endothelial dysfunction and prevented activation of NADPH oxidase in HO-$1^{+/+}$ mice (Figures 2(c) and 2(d)). The third experiment demonstrated that PETN did not improve endothelial dysfunction and cardiac oxidative stress in angiotensin-II-(low-dose) treated HO-$1^{+/-}$ mice but further impaired vascular function and increased ROS formation in this setting (Figure 2(e) and 2(f)). It is somewhat surprising that already heterozygous deficiency in HO-1 leads to complete loss of the beneficial and protective effects of PETN but may also underline how essential the upregulation of HO-1 is to prevent nitrate tolerance, endothelial dysfunction, and oxidative stress under chronic therapy with organic nitrates. It would be of great clinical importance to study the vasodilatory potency of GTN and development of nitrate tolerance under chronic GTN therapy in human individuals with Morbus Meulengracht (Gilbert's syndrome) to translate our preclinical data from bench to bedside and to further explore the therapeutical potential of HO-1 induction to overcome the side effects of GTN therapy. Since human subjects with hyperbilirubinemia (e.g., new born with mild jaundice or patients with Gilbert's syndrome) have a better prognosis and have a significant lower cardiovascular risk [65], it may be hypothesized that they will also display a lower degree of nitrate tolerance in response to chronic GTN therapy.

The beneficial effects of HO-1 induction on mitochondrial RONS formation may be attributed to the presence of HO-1 in mitochondria [66] and the improvement of mitochondrial biogenesis as well as suppression of doxorubicin cardiotoxicity [67]. Recent report also suggests that targeting HO-1 to mitochondria can prevent inflammation-triggered mitochondrial oxidative stress and apoptosis [68]. Since the side effects of chronic GTN therapy are mainly based on adverse regulation of mitochondrial function such as the inhibition of mitochondrial aldehyde dehydrogenase (ALDH-2) [23], increase in mitochondrial oxidative stress [23, 27], increase in cellular apoptosis [53], the induction of a mitochondrial antioxidant principle (HO-1) would be most effective to prevent GTN-induced tolerance and endothelial dysfunction. This concept was supported by previous reports on the improvement of GTN side effects by a mitochondria-targeted antioxidant (mitoQ) [30], by aggravation of GTN toxicity by partial deficiency in the mitochondrial superoxide dismutase (Mn-SOD) [28], and by interference of blockers of the mitochondrial pores (cyclosporine A and glibenclamide) with the crosstalk between cytosolic and mitochondrial sources of RONS [69]. Vice versa, induction of HO-1 may explain the beneficial profile of PETN [70]. This concept is summarized in Figure 3 and was previously published [31]. To describe the content of this scheme briefly: *In vivo* treatment with PETN is devoid of tolerance and endothelial dysfunction induction in response to chronic *in vivo* treatment. In contrast to GTN, PETN does not increase vascular oxidative stress and therefore does not interfere with its bioactivation by ALDH-2. A likely explanation for this beneficial property of PETN is the induction of the antioxidant enzyme HO-1 and subsequent increases in the expression of ferritin as well as other protective downstream mechanisms not shown here (e.g., ecSOD, GCH-1, and sGC) in vascular tissue but also in the heart. This favorable characteristics of PETN may also explain why therapy with GTN but not PETN causes tolerance and stimulates ROS production in human subjects [9, 71].

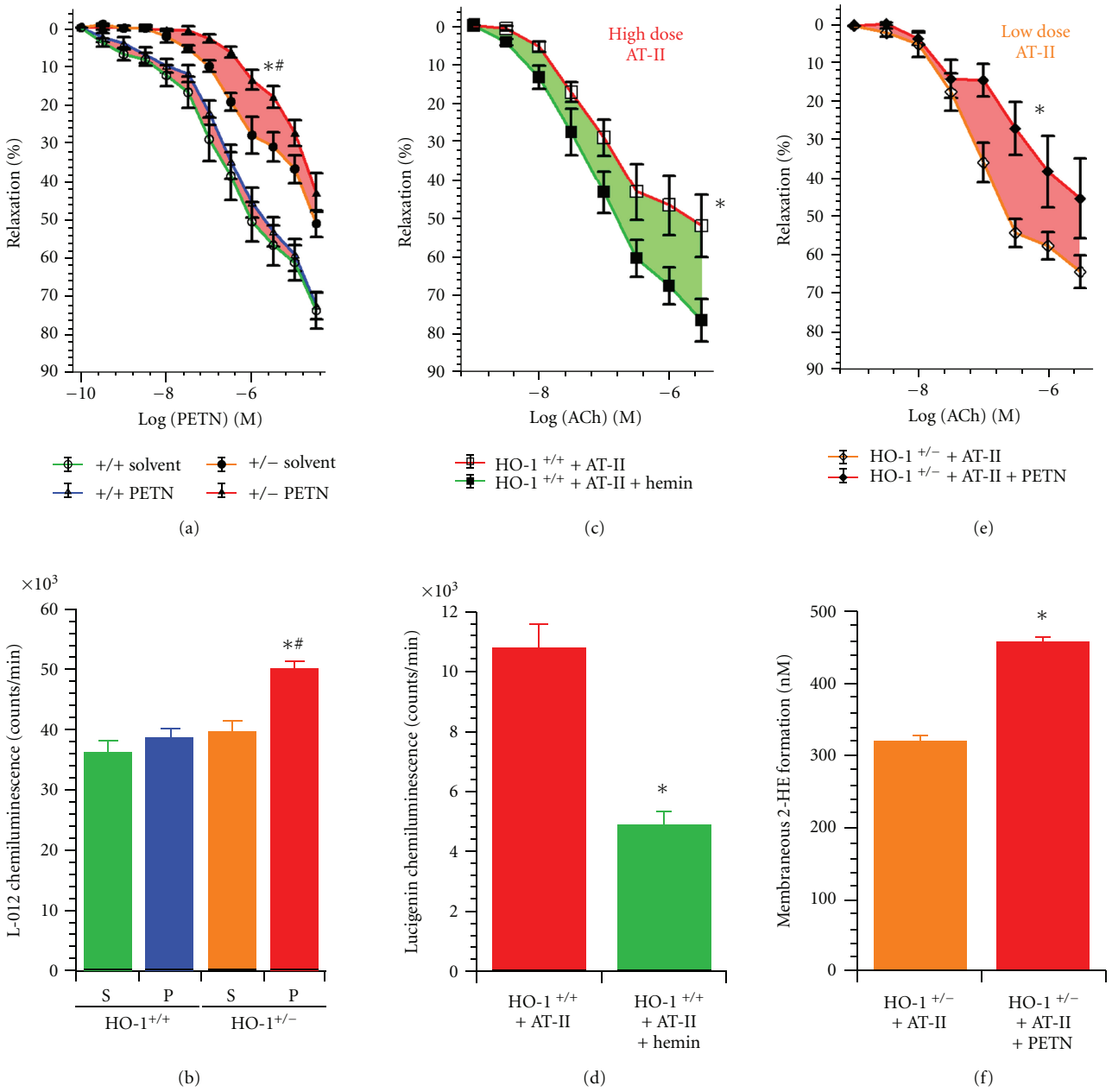

Figure 2: Effects of HO-1 deficiency versus HO-1 induction on vascular improvement by pentaerithrityl tetranitrate (PETN). (a, b) PETN treatment (75 mg/kg/d for 4d) had no effect on PETN potency (PETN-induced relaxation) in aorta from control mice (HO-$1^{+/+}$) but caused nitrate tolerance (see area between curves) in aorta from mice with partial HO-1 deficiency (HO-$1^{+/-}$). In accordance, cardiac mitochondrial ROS formation (L-012 ECL) was increased in PETN-treated HO-$1^{+/-}$ mice. $P < 0.05$: *versus HO-$1^{+/+}$/DMSO; #versus HO-$1^{+/-}$/DMSO. S: solvent; P: PETN-treated. (c, d) Hemin (25 mg/kg i.p.)-triggered HO-1 induction improved high-dose AT-II (1 mg/kg/d for 7d)-induced endothelial dysfunction (ACh-response) in aorta (see area between curves) and NADPH oxidase activity in heart (lucigenin ECL) from control mice (HO-$1^{+/+}$). $P < 0.05$: *versus AT-II-treated HO-$1^{+/+}$/DMSO. (e, f) PETN (75 mg/kg/d for 7d) failed to prevent endothelial dysfunction (ACh-response) induced by low-dose AT-II (0.1 mg/kg/d for 7d) in aorta from HO-$1^{+/-}$ mice (see area between curves). In accordance, PETN did not improve NADPH oxidase activity (2-HE formation by HPLC analysis) in cardiac samples from AT-II-treated HO-$1^{+/-}$ mice. $P < 0.05$: *versus AT-II-treated HO-$1^{+/-}$/DMSO. All data are mean ± SEM of aortic rings and hearts from 4-5 animals/group. Modified from [32].

4. Effects of Organic Nitrates on Gene Expression

There are several examples in the literature showing a transcriptional as well as posttranscriptional modulation of gene expression by nitric oxide (NO) [72–75]. The activity of several transcription factors like NF-κB, AP1 [74], or NRF2 [76] as well as RNA-binding protein like HuR [77] has been shown to be modulated by NO (directly or indirectly via cGMP). Organic nitrates are believed to be indirect

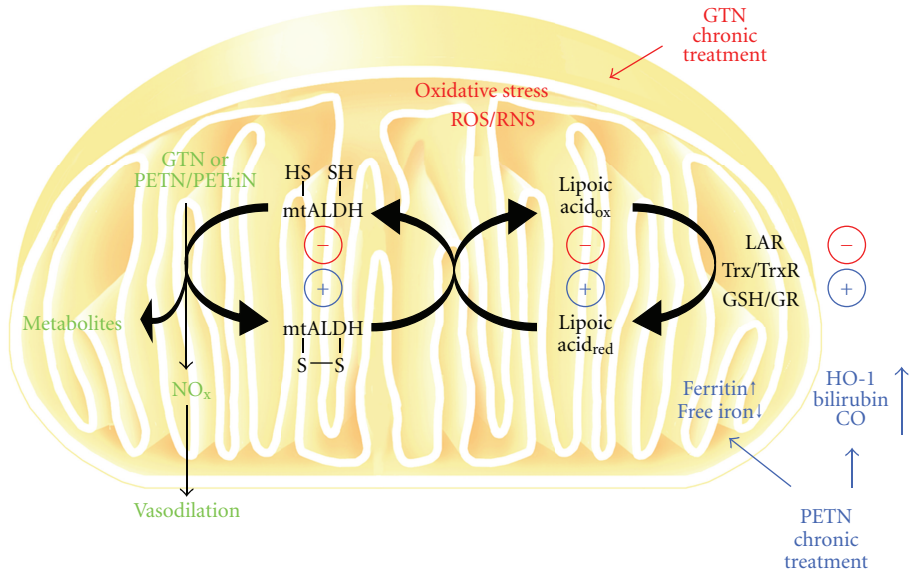

Figure 3: Scheme illustrating the mechanisms underlying the oxidative stress concept of nitrate tolerance in response to GTN treatment and the mechanisms underlying the beneficial vascular effects in response to PETN. PETN and GTN are bioactivated by mitochondrial ALDH (ALDH-2) yielding 1,2-glyceryl dinitrate and PETriN, respectively, as well as a yet undefined nitrogen species (NO_x, probably nitrite) that undergoes further reduction by the mitochondrial respiratory chain or acidic disproportionation to form an activator of sGC (probably nitrico xide). GTN treatment induces mitochondrial reactive oxygen and nitrogen species formation (ROS/RNS). These ROS/RNS in turn inhibit the GTN bioactivation process by inactivation of ALDH-2 or by inhibiting the repair system of the ALDH-2, which includes lipoic acid, as well as a reductase system depending on the NADH or NADPH (lipoicacid reductase (LAR), thioredoxin/thioredoxin reductase (Trx/TrxR) or glutathione/glutathione reductase (GSH/GR). In contrast to GTN, PETN provides potent antioxidative effects by inducing HO-1 and ferritin, which in turn decrease ROS levels and therefore protect the ALDH-2 from ROS mediated inactivation. Adapted from [31].

NO-donors. Therefore, it seems very likely that treatment with organic nitrates may have implications on the expression of multiple genes. GTN has been described to enhance the expression of c-fos, COX-2, Bcl2, and nNOS in brain nuclei [78–81], to reduce beta-catenin expression in colon cancer cells [82], and to reduce NOX1, NOX2, NOX4 and ALDH2-expression in rat aorta and rat smooth muscle cells [83]. Using microarray analysis, Wang et al. described changes of the expression of 290 genes in the aortas of rats treated with GTN for 8 h [84]. Analyzing the gene expression in the hearts treated for 4 days with GTN, the authors described expressional changes of more than 500 genes [85].

There are also some reports about the expressional effects of PETN [29, 31, 35, 36, 62]. PETN (but not GTN) has been shown to enhance the expression of the antioxidant genes HO-1 and ferritin heavy chain (FeHc) in human endothelial cells [29, 35, 36, 62] and rat aorta [31]. In microarray experiments, the authors showed that PETN modulated the expression of more than 1200 genes in the hearts of rats treated with PETN for 4 days [85].

5. Molecular Mechanisms Involved in the Regulation of Gene Expression by Organic Nitrates

The comparison of the 5′-flanking sequences of the HO-1 gene (promoter, 10 kb) in different species (rat, mouse, rhesus macaque, chimpanzee, and human) displays regions with very high homology between these species ("evolutionary conserved regions," ECRs; see Figure 4). These ECR are likely to be involved in the regulation of the HO-1 promoter activity. Bioinformatic analyses show that these ECR contain the binding sites of transcription factors (e.g., NRF2) known to regulate HO-1 promoter activity in different mammalian cell systems (e.g., macrophages, fibroblasts, smooth muscle cells [33]) after various stimuli. In human SH-SY5Y neuroblastoma [86] or rat vascular smooth muscle cells [87], the enhancement of the HO-1 expression by different NO donors DETA-NO has been shown to depend on the expression of NRF2.

To analyze the molecular mechanisms of the regulation of the HO-1 expression by organic nitrates, the authors cloned a 11 kb fragment of the human HO-1 promoter into a luciferase reporter gene construct. This construct was stably transfected into human epithelial DLD-1 cells (DLD1-HO-11kb-Prom). These cells were treated for 8 h with PETN, ISDN, ISMN, or GTN (or the respective solvents, dimethyl sulfoxide DMSO, H_2O, ethanol EtOH). Cell extracts were prepared, and luciferase activity was measured. As shown in Figure 5, only PETN was able to enhance the promoter activity of the human HO-1 gene. Therefore, the enhancement of HO-1 expression by PETN seems to depend at least partly on PETN-induced enhancement of the HO-1 promoter. A similar comparison of the effects of different organic nitrates on HO-1 induction was recently published [34].

To analyze the involvement of the transcription factor NRF2 in this PETN-mediated enhancement of HO-1

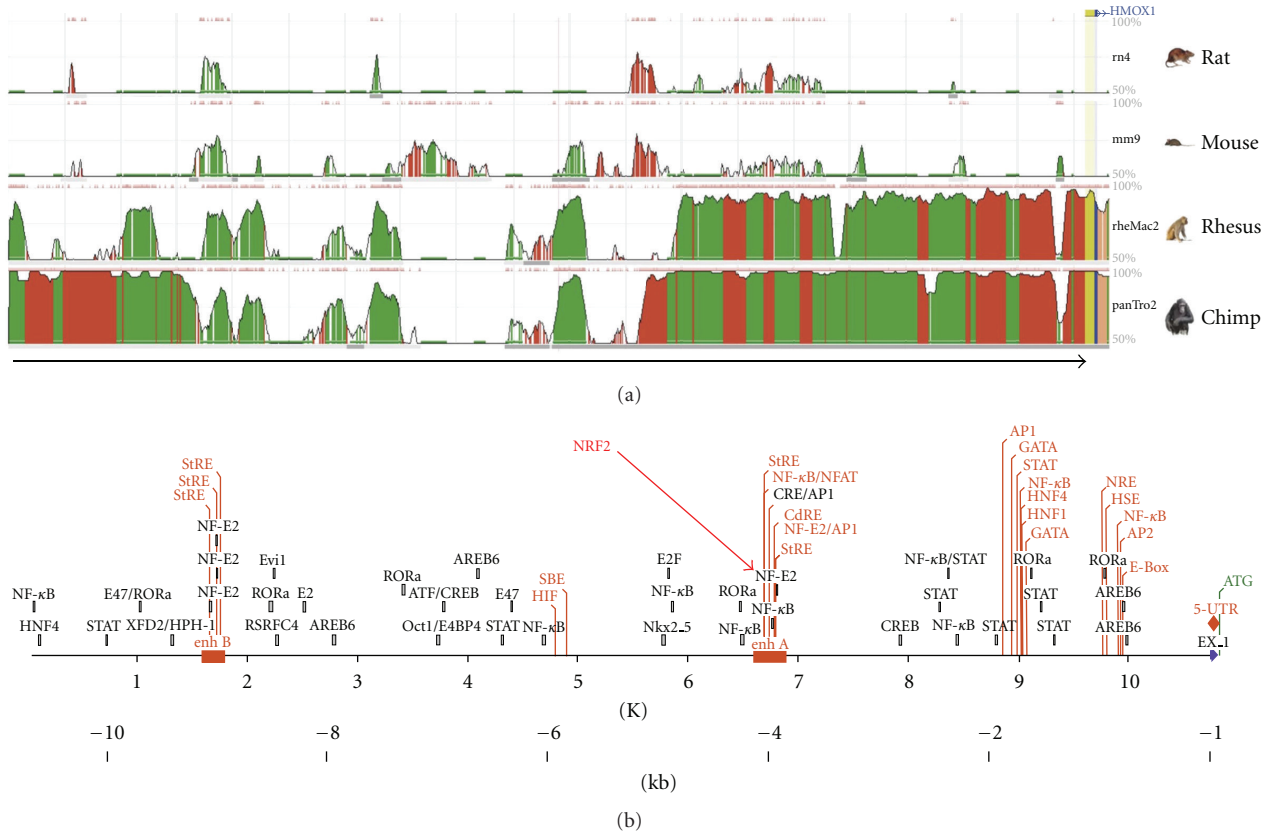

Figure 4: Comparison of the HO-1 promoter sequences of different species. (a) Comparison of the 5′-flanking sequences (10 kb) of the rat, mouse, rhesus macaque, chimpanzee, and human (arrow) HO-1 gene using the ECR-Browser software (http://ecrbrowser.dcode.org/). The search area was 10 bp, and the minimal homology was 80%. The height of the curves ($50\% < X < 100\%$) indicates the homology (red: intergenic regions, green: single repeats, yellow: untranslated regions of the RNA (UTR), blue: exon, salmon: intron, pink ECR, above). (b) Map of transcription factor binding sites (TFBS) in the human HO-1 promoter. TFBS labeled in red have been verified experimentally in different cells systems (see also [33]).

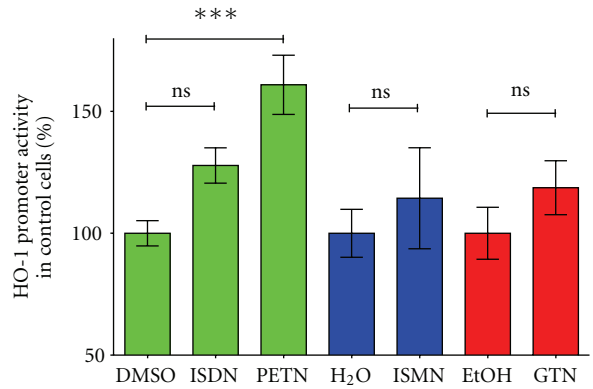

Figure 5: Effects of organic nitrates on the human HO-1 promoter activity. DLD1-HO-11kb-Prom cells were treated for 8 h with isosorbide dinitrate ISDN, PETN, isosorbide-5-mononitrate ISMN, or GTN at a concentration of 50 μM or the respective solvent (DMSO, H_2O, EtOH). Extracts were prepared, and luciferase activity and protein content were measured, and luciferase activity was normalized to the protein content. Shown (mean ± SEM; $n = 8$–10) are the normalized luciferase activity values. The normalized luciferase activity of the cell treated with solvent was set to 100%. (***= $P < 0.001$, ns: not significant different from solvent-treated cells.) These data were partly published in [34].

promoter activity, NRF2-knockdown experiments using a specific anti-NRF2 siRNA were performed. These experiments clearly showed that the PETN-mediated enhancement of the human HO-1 promoter activity depends on the NRF2 expression (see Figure 6) [34]. Therefore, it is likely to speculate that the NO generated by PETN activates NRF2 which in turn binds to the HO-1 promoter and enhances transcription.

Beside transcriptional regulation, the modulation of expression of HO-1 has been shown to depend on post-transcriptional mechanisms [77, 88–92]. Posttranscriptional regulation of mRNA stability and translatability mostly depends on sequences found in the 3′-untranslated sequence (3′-UTR) of the mRNAs [93, 94]. Analysis of the 3′-UTR sequences of the HO-1 mRNA of different species (rat, mouse, rhesus macaque, chimpanzee, and human, see Figure 7) reveals evolutionary conserved regions (ECR). In the 3′-UTR sequences of all species, AU-rich elements (AREs) is highly conserved. AREs have been shown to be the binding sites of RNA, binding proteins like HuR or KSRP, which stabilize or destabilize the mRNAs. In a recent paper, the NO-dependent stabilization of the HO-1 mRNA in murine fibroblasts was shown to depend on the RNA-binding protein HuR [77].

To analyze the effects of organic nitrates on the HO-1 mRNA stability, the authors cloned the 3′-UTR sequence of the human HO-1 mRNA behind the luciferase reporter gene (pGL3-Control-HO-1-3-UTR). Stable human endothelial cells (EA.hy 926) were transiently transfected with this construct and treated with GTN or PETN (see Figure 8). PETN but not GTN enhanced luciferase activity in the transfected cells indicating a PETN-dependent stabilization of the HO-1 mRNA (unpublished data, Hartmut Kleinert).

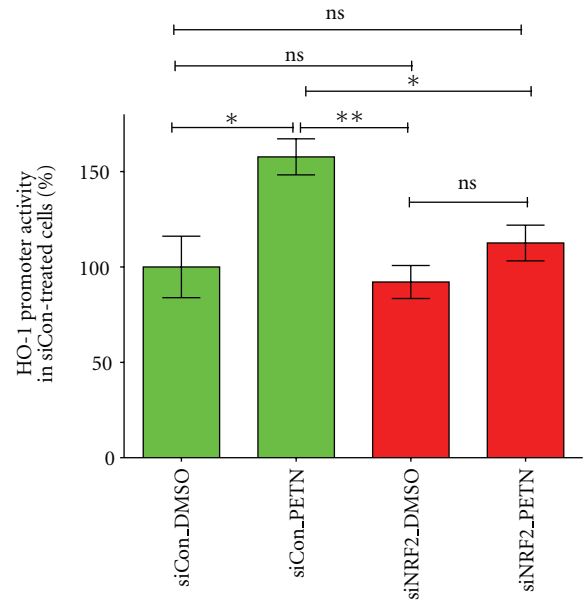

Figure 6: PETN-induced enhancement of the human HO-1 promoter activity depends on NRF2. DLD1-HO-11kb-Prom cells were transfected with a specific anti-NRF2 siRNA (siNRF2) or a nonrelated control siRNA (siCon). After 48 h, the cells were incubated with PETN or the solvent DMSO. Extracts were prepared and luciferase activity and protein content were measured, and luciferase activity was normalized to the protein content. Shown (mean ± SEM; $n = 5$-6) are the normalized luciferase activity values. The normalized luciferase activity of the cell treated with solvent and siCon was set to 100%. (**= $P < 0.01$, *= $P < 0.05$, ns: not significant different to DMSO and siCon-treated cells. Modified from [34].

6. Conclusions and Clinical Implications

Organic nitrates like GTN or PETN seem to have marked distinct pharmacological properties and side effects [50], translating to different therapeutic profiles and clinical properties (e.g., induction or lack of nitrate tolerance) [9], which may be at least in part explained by their different effects on HO-1 gene expression [2]. This may be attributed to the different amounts of bioactive NO generated from these compounds. As GTN markedly enhances ROS production in cells, only small amounts of bioactive NO are produced. In contrast, PETN incubation results in decent amount of bioactive NO (or a related species such as S-nitrosothiols or heme-NO) resulting in enhanced NRF2 binding to the HO-1 promoter and HuR binding to the HO-1 mRNA. Thereby, PETN enhances HO-1 expression both by transcriptional and posttranscriptional effects (see Figure 9). Since HO-1 and its products may directly regulate other genes [47], distinct modulation of HO-1 expression by different organic nitrates may also explain differential regulation of gene expression by organic nitrates in general (e.g., GTN versus PETN) [85].

First clinical evidence for the importance of HO-1 to overcome nitrate tolerance is based on the observation that statin therapy (well-known inducers of HO-1) was able to prevent nitrate tolerance in human subjects [40, 41]. Despite the fact that published preclinical data clearly show that an organic nitrate with HO-1 inducing properties such as PETN has less side effects [31] and even has beneficial effects on experimental hypertension [32], diabetes [34], and atherosclerosis [95], these findings still lack molecular proof in human subjects to increase the clinical importance of this concept. A proof of concept study aiming to demonstrate that individuals with Morbus Meulengracht (Gilbert's syndrome), displaying increased HO-1 activity and bilirubin levels, are devoid of tolerance, endothelial dysfunction and oxidative stress in response to nitroglycerin (GTN) therapy could provide a new therapeutic option to overcome these undesired side effects of GTN treatment.

Authors' Contribution

A. Daiber and H. Kleinert wrote the first draft, prepared the figures, and designed the original research. M. Oelze, P. Wenzel, F. Bollmann, and A. Pautz commented on subsequent drafts and have performed the original experiments. All authors have approved the paper.

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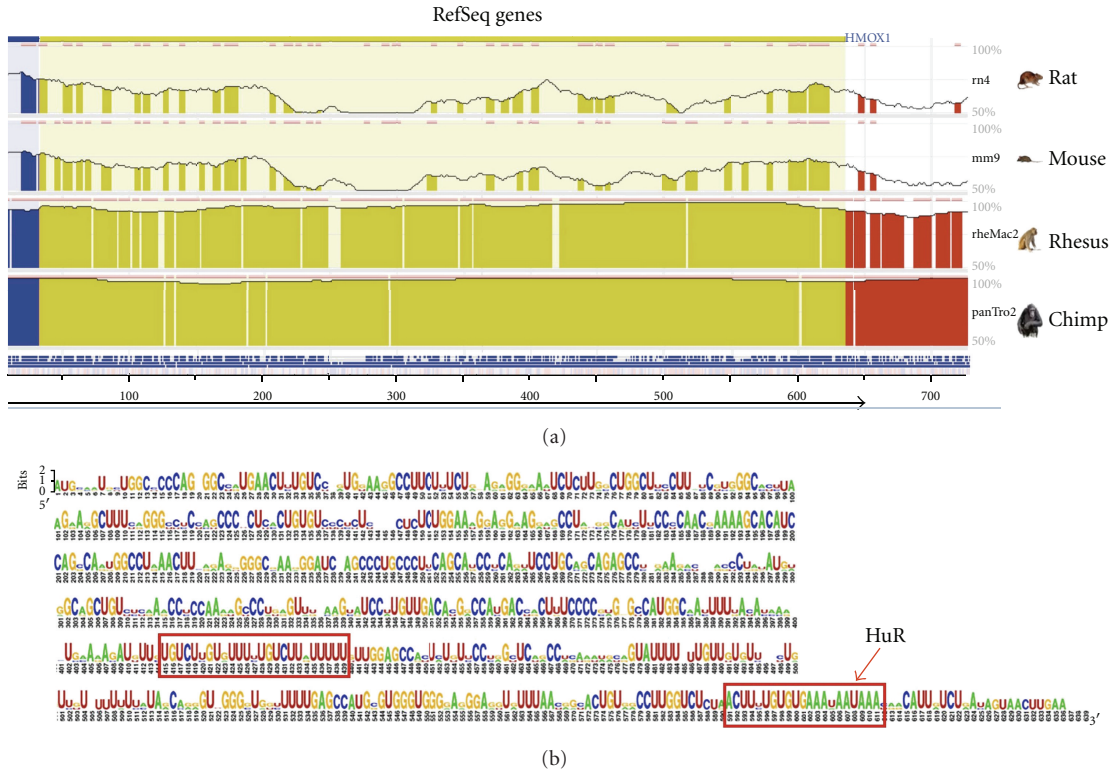

Figure 7: Comparison of the 3′-UTR of the HO-1 mRNA of different species. (a) The 3′-UTR sequences of the HO-1 mRNA from rat, mouse, rhesus macaque, chimpanzee and human was compared using the ECR-Browser software. The search area was 10 bp and the minimal homology was 80%. The height of the curves ($50\% < X < 100\%$) indicates the homology (red = intergenic regions, yellow = untranslated regions of the RNA (UTR), blue = exon, pink ECR, above). (b) Using the software RNALogo (http://rnalogo.mbc.nctu.edu.tw/createlogo.html) a consensus sequence of all 5 3′-UTR sequences was generated. The height of the letters indicate the frequency of the appearance of this base. AREs are marked by a red box. A putative HuR binding site is indicated.

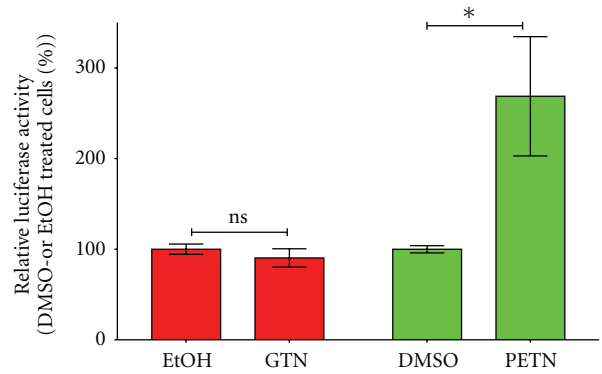

Figure 8: Post-transcriptional regulation of the human HO-1 expression. Human endothelial EA.hy 926 cells were transiently transfected with pGL3-Control-HO-1-3-UTR and pRenilla (normalization of transfection efficiency). After 24h the cells were treated with 50 μM nitroglycerin (GTN) or PETN (or the solvents ethanol [EtOH] or DMSO) for 6h. Extracts were prepared and luciferase and Renilla activity were determined. The luciferase activity was normalized to the renilla activity. Shown (mean ± SEM; $n = 6$–8) are the normalized luciferase activity values. The normalized luciferase activity of the cell treated with solvent were set to 100% (*= $P < 0.05$; ns = not significant versus EtOH- or. DMSO treated cells).

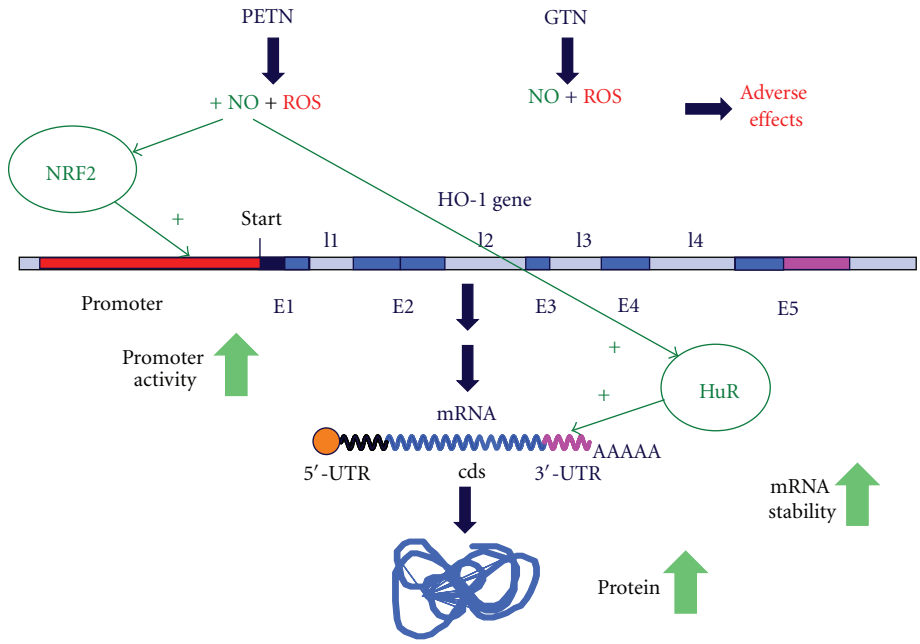

Figure 9: Molecular mechanisms of PETN-mediated enhancement of HO-1 expression. The high amounts of bioactive NO generated from PETN (but not GTN) activate the transcription factor NRF2 and thereby enhance the HO-1 promoter activity. In addition, the interaction of the stabilizing RNA binding protein HuR with the 3′-UTR of the HO-1 mRNA is enhanced. Both effects result in an enhancement of HO-1 expression (E: exon, I: intron).

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References

[1] T. Munzel, A. Daiber, and A. Mulsch, "Explaining the phenomenon of nitrate tolerance," *Circulation Research*, vol. 97, no. 7, pp. 618–628, 2005.

[2] T. Munzel, A. Daiber, and T. Gori, "Nitrate therapy: new aspects concerning molecular action and tolerance," *Circulation*, vol. 123, no. 19, pp. 2132–2144, 2011.

[3] A. Daiber, T. Munzel, and T. Gori, "Organic nitrates and nitrate tolerance—state of the art and future developments," *Advances in Pharmacology*, vol. 60, pp. 177–227, 2010.

[4] A. Daiber, M. Oelze, P. Wenzel et al., "Nitrate tolerance as a model of vascular dysfunction: roles for mitochondrial aldehyde dehydrogenase and mitochondrial oxidative stress," *Pharmacological Reports*, vol. 61, no. 1, pp. 33–48, 2009.

[5] A. Daiber, P. Wenzel, M. Oelze, and T. Munzel, "New insights into bioactivation of organic nitrates, nitrate tolerance and cross-tolerance," *Clinical Research in Cardiology*, vol. 97, no. 1, pp. 12–20, 2008.

[6] K. K. Griendling and G. A. FitzGerald, "Oxidative stress and cardiovascular injury: part II: animal and human studies," *Circulation*, vol. 108, no. 17, pp. 2034–2040, 2003.

[7] A. Daiber and T. Münzel, *Oxidativer Stress, Redoxregulation und NO-Bioverfügbarkeit—Experimentelle und Klinische Aspekte*, Steinkopff Verlag Darmstadt, Darmstadt, Germany, 2006.

[8] E. Schulz, N. Tsilimingas, R. Rinze et al., "Functional and biochemical analysis of endothelial (Dys)function and NO/cGMP signaling in human blood vessels with and without nitroglycerin pretreatment," *Circulation*, vol. 105, no. 10, pp. 1170–1175, 2002.

[9] T. Gori, A. Al-Hesayen, C. Jolliffe, and J. D. Parker, "Comparison of the effects of pentaerythritol tetranitrate and nitroglycerin on endothelium-dependent vasorelaxation in male volunteers," *American Journal of Cardiology*, vol. 91, no. 11, pp. 1392–1394, 2003.

[10] M. Sekiya, M. Sato, J. Funada, T. Ohtani, H. Akutsu, and K. Watanabe, "Effects of the long-term administration of nicorandil on vascular endothelial function and the progression of arteriosclerosis," *Journal of Cardiovascular Pharmacology*, vol. 46, no. 1, pp. 63–67, 2005.

[11] G. R. Thomas, J. M. DiFabio, T. Gori, and J. D. Parker, "Once daily therapy with isosorbide-5-mononitrate causes endothelial dysfunction in humans. Evidence of a free-radical-mediated mechanism," *Journal of the American College of Cardiology*, vol. 49, no. 12, pp. 1289–1295, 2007.

[12] T. Munzel, H. Sayegh, B. A. Freeman, M. M. Tarpey, and D. G. Harrison, "Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance," *Journal of Clinical Investigation*, vol. 95, no. 1, pp. 187–194, 1995.

[13] T. Munzel, S. Kurz, S. Rajagopalan et al., "Hydralazine prevents nitroglycerin tolerance by inhibiting activation of a membrane-bound NADH oxidase: a new action for an old drug," *Journal of Clinical Investigation*, vol. 98, no. 6, pp. 1465–1470, 1996.

[14] T. Munzel and D. G. Harrison, "Evidence for a role of oxygen derived free radicals and protein kinase C in nitrate tolerance," *Journal of Molecular Medicine*, vol. 75, no. 11-12, pp. 891–900, 1997.

[15] T. Munzel, A. Giaid, S. Kurz, D. J. Stewart, and D. G. Harrison, "Evidence for a role of endothelin 1 and protein kinase C in nitroglycerin tolerance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 11, pp. 5244–5248, 1995.

[16] A. Warnholtz, H. Mollnau, T. Heitzer et al., "Adverse effects of nitroglycerin treatment on endothelial function, vascular nitrotyrosine levels and cGMP-dependent protein kinase activity in hyperlipidemic Watanabe rabbits," *Journal of the American College of Cardiology*, vol. 40, no. 7, pp. 1356–1363, 2002.

[17] U. Hink, M. Oelze, P. Kolb et al., "Role for peroxynitrite in the inhibition of prostacyclin synthase in nitrate tolerance," *Journal of the American College of Cardiology*, vol. 42, no. 10, pp. 1826–1834, 2003.

[18] A. Daiber, M. Oelze, M. Coldewey et al., "Hydralazine is a powerful inhibitor of peroxynitrite formation as a possible explanation for its beneficial effects on prognosis in patients with congestive heart failure," *Biochemical and Biophysical Research Communications*, vol. 338, no. 4, pp. 1865–1874, 2005.

[19] P. Wenzel, U. Hink, M. Oelze et al., "Role of reduced lipoic acid in the redox regulation of mitochondrial aldehyde dehydrogenase (ALDH-2) activity: implications for mitochondrial oxidative stress and nitrate tolerance," *Journal of Biological Chemistry*, vol. 282, no. 1, pp. 792–799, 2007.

[20] Q. Fan, F. Gao, L. Zhang, T. A. Christopher, B. L. Lopez, and X. L. Ma, "Nitrate tolerance aggravates postischemic myocardial apoptosis and impairs cardiac functional recovery after ischemia," *Apoptosis*, vol. 10, no. 6, pp. 1235–1242, 2005.

[21] G. Abou-Mohamed, J. A. Johnson, L. Jin et al., "Roles of superoxide, peroxynitrite, and protein kinase C in the development of tolerance to nitroglycerin," *Journal of Pharmacology and Experimental Therapeutics*, vol. 308, no. 1, pp. 289–299, 2004.

[22] M. J. Mihm, C. M. Coyle, L. Jing, and J. A. Bauer, "Vascular peroxynitrite formation during organic nitrate tolerance," *Journal of Pharmacology and Experimental Therapeutics*, vol. 291, no. 1, pp. 194–198, 1999.

[23] K. Sydow, A. Daiber, M. Oelze et al., "Central role of mitochondrial aldehyde dehydrogenase and reactive oxygen species in nitroglycerin tolerance and cross-tolerance," *Journal of Clinical Investigation*, vol. 113, no. 3, pp. 482–489, 2004.

[24] Z. Chen, J. Zhang, and J. S. Stamler, "Identification of the enzymatic mechanism of nitroglycerin bioactivation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 12, pp. 8306–8311, 2002.

[25] P. Needleman and F. E. Hunter Jr., "Effects of organic nitrates on mitochondrial respiration and swelling: possible correlations with the mechanism of pharmacologic action," *Molecular Pharmacology*, vol. 2, no. 2, pp. 134–143, 1966.

[26] B. Jakschik and P. Needleman, "Sulfhydryl reactivity of organic nitrates: biochemical basis for inhibition of glyceraldehyde p dehydrogenase and monoamine oxidase," *Biochemical and Biophysical Research Communications*, vol. 53, no. 2, pp. 539–544, 1973.

[27] A. Daiber, M. Oelze, M. Coldewey et al., "Oxidative stress and mitochondrial aldehyde dehydrogenase activity: a comparison of pentaerythritol tetranitrate with other organic nitrates," *Molecular Pharmacology*, vol. 66, no. 6, pp. 1372–1382, 2004.

[28] A. Daiber, M. Oelze, S. Sulyok et al., "Heterozygous deficiency of manganese superoxide dismutase in mice (Mn-SOD+/-): a novel approach to assess the role of oxidative stress for the development of nitrate tolerance," *Molecular Pharmacology*, vol. 68, no. 3, pp. 579–588, 2005.

[29] H. Mollnau, P. Wenzel, M. Oelze et al., "Mitochondrial oxidative stress and nitrate tolerance—comparison of nitroglycerin and pentaerithrityl tetranitrate in Mn-SOD+/- mice," *BMC Cardiovascular Disorders*, vol. 6, article 44, 2006.

[30] J. V. Esplugues, M. Rocha, C. Nunez et al., "Complex I dysfunction and tolerance to nitroglycerin: an approach based on mitochondrial-targeted antioxidants," *Circulation Research*, vol. 99, no. 10, pp. 1067–1075, 2006.

[31] P. Wenzel, M. Oelze, M. Coldewey et al., "Heme oxygenase-1: a novel key player in the development of tolerance in response to organic nitrates," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 8, pp. 1729–1735, 2007.

[32] S. Schuhmacher, P. Wenzel, E. Schulz et al., "Pentaerythritol tetranitrate improves angiotensin II-induced vascular dysfunction via induction of heme oxygenase-1," *Hypertension*, vol. 55, no. 4, pp. 897–904, 2010.

[33] J. Alam and J. L. Cook, "How many transcription factors does it take to turn on the heme oxygenase-1 gene?" *American Journal of Respiratory Cell and Molecular Biology*, vol. 36, no. 2, pp. 166–174, 2007.

[34] S. Schuhmacher, M. Oelze, F. Bollmann et al., "Vascular dysfunction in experimental diabetes is improved by pentaerithrityl tetranitrate but not isosorbide-5-mononitrate therapy," *Diabetes*, vol. 60, no. 10, pp. 2608–2616, 2011.

[35] S. Oberle, A. Abate, N. Grosser et al., "Heme oxygenase-1 induction may explain the antioxidant profile of pentaerythrityl trinitrate," *Biochemical and Biophysical Research Communications*, vol. 290, no. 5, pp. 1539–1544, 2002.

[36] S. Oberle, P. Schwartz, A. Abate, and H. Schroder, "The antioxidant defense protein ferritin is a novel and specific target for pentaerithrityl tetranitrate in endothelial cells," *Biochemical and Biophysical Research Communications*, vol. 261, no. 1, pp. 28–34, 1999.

[37] N. Grosser, A. Hemmerle, G. Berndt et al., "The antioxidant defense protein heme oxygenase 1 is a novel target for statins in endothelial cells," *Free Radical Biology and Medicine*, vol. 37, no. 12, pp. 2064–2071, 2004.

[38] F. Gueler, J. K. Park, S. Rong et al., "Statins attenuate ischemia-reperfusion injury by inducing heme oxygenase-1 in infiltrating macrophages," *American Journal of Pathology*, vol. 170, no. 4, pp. 1192–1199, 2007.

[39] D. Fontaine, A. Otto, J. Fontaine, and G. Berkenboom, "Prevention of nitrate tolerance by long-term treatment with statins," *Cardiovascular Drugs and Therapy*, vol. 17, no. 2, pp. 123–128, 2003.

[40] T. Inoue, K. Takayanagi, T. Hayashi, and S. Morooka, "Fluvastatin attenuates nitrate tolerance in patients with ischemic heart disease complicating hypercholesterolemia," *International Journal of Cardiology*, vol. 90, no. 2-3, pp. 181–188, 2003.

[41] A. Liuni, M. C. Luca, G. Di Stolfo et al., "Coadministration of atorvastatin prevents nitroglycerin-induced endothelial dysfunction and nitrate tolerance in healthy humans," *Journal of the American College of Cardiology*, vol. 57, no. 1, pp. 93–98, 2011.

[42] T. Jansen, M. Hortmann, M. Oelze et al., "Conversion of biliverdin to bilirubin by biliverdin reductase contributes to endothelial cell protection by heme oxygenase-1-evidence for direct and indirect antioxidant actions of bilirubin," *Journal of*

Molecular and Cellular Cardiology, vol. 49, no. 2, pp. 186–195, 2010.

[43] M. Knorr, M. Hausding, S. Kroller-Schuhmacher et al., "Nitroglycerin-induced endothelial dysfunction and tolerance involve adverse phosphorylation and S-glutathionylation of endothelial nitric oxide synthase: beneficial effects of therapy with the AT1 receptor blocker telmisartan," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 10, pp. 2223–2231, 2011.

[44] N. J. Alp, S. Mussa, J. Khoo et al., "Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression," *Journal of Clinical Investigation*, vol. 112, no. 5, pp. 725–735, 2003.

[45] C. Antoniades, C. Cunnington, A. Antonopoulos et al., "Induction of vascular GTP-cyclohydrolase i and endogenous tetrahydrobiopterin synthesis protect against inflammation-induced endothelial dysfunction in human atherosclerosis," *Circulation*, vol. 124, no. 17, pp. 1860–1870, 2011.

[46] J. Xu, Y. Wu, P. Song, M. Zhang, S. Wang, and M. H. Zou, "Proteasome-dependent degradation of guanosine 5′-triphosphate cyclohydrolase I causes tetrahydrobiopterin deficiency in diabetes mellitus," *Circulation*, vol. 116, no. 8, pp. 944–953, 2007.

[47] N. G. Abraham and A. Kappas, "Pharmacological and clinical aspects of heme oxygenase," *Pharmacological Reviews*, vol. 60, no. 1, pp. 79–127, 2008.

[48] M. Oppermann, V. Balz, V. Adams et al., "Pharmacological induction of vascular extracellular superoxide dismutase expression in vivo," *Journal of Cellular and Molecular Medicine*, vol. 13, no. 7, pp. 1271–1278, 2009.

[49] A. W. Jones, W. Durante, and R. J. Korthuis, "Heme oxygenase-1 deficiency leads to alteration of soluble guanylate cyclase redox regulation," *Journal of Pharmacology and Experimental Therapeutics*, vol. 335, no. 1, pp. 85–91, 2010.

[50] T. Gori and A. Daiber, "Non-hemodynamic effects of organic nitrates and the distinctive characteristics of pentaerithrityl tetranitrate," *American Journal of Cardiovascular Drugs*, vol. 9, no. 1, pp. 7–15, 2009.

[51] A. L. Kleschyov, M. Oelze, A. Daiber et al., "Does nitric oxide mediate the vasodilator activity of nitroglycerin?" *Circulation Research*, vol. 93, no. 9, pp. e104–112, 2003.

[52] T. Thum, D. Fraccarollo, S. Thum et al., "Differential effects of organic nitrates on endothelial progenitor cells are determined by oxidative stress," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 4, pp. 748–754, 2007.

[53] J. M. DiFabio, G. R. Thomas, L. Zucco et al., "Nitroglycerin attenuates human endothelial progenitor cell differentiation, function, and survival," *Journal of Pharmacology and Experimental Therapeutics*, vol. 318, no. 1, pp. 117–123, 2006.

[54] H. H. Lin, Y. H. Chen, P. F. Chang, Y. T. Lee, S. F. Yet, and L. Y. Chau, "Heme oxygenase-1 promotes neovascularization in ischemic heart by coinduction of VEGF and SDF-1," *Journal of Molecular and Cellular Cardiology*, vol. 45, no. 1, pp. 44–55, 2008.

[55] T. Thum, D. Fraccarollo, M. Schultheiss et al., "Endothelial nitric oxide synthase uncoupling impairs endothelial progenitor cell mobilization and function in diabetes," *Diabetes*, vol. 56, no. 3, pp. 666–674, 2007.

[56] T. Gori, J. M. Burstein, S. Ahmed et al., "Folic acid prevents nitroglycerin-induced nitric oxide synthase dysfunction and nitrate tolerance: a human in vivo study," *Circulation*, vol. 104, no. 10, pp. 1119–1123, 2001.

[57] H. Ikejima, T. Imanishi, H. Tsujioka et al., "Effect of pioglitazone on nitroglycerin-induced impairment of nitric oxide bioavailability by a catheter-type nitric oxide sensor," *Circulation Journal*, vol. 72, no. 6, pp. 998–1002, 2008.

[58] K. Schmidt, M. Rehn, H. Stessel, G. Wolkart, and B. Mayer, "Evidence against tetrahydrobiopterin depletion of vascular tissue exposed to nitric oxide/superoxide or nitroglycerin," *Free Radical Biology and Medicine*, vol. 48, no. 1, pp. 145–152, 2010.

[59] T. Gori, S. Dragoni, G. Di Stolfo et al., "Tolerance to nitroglycerin-induced preconditioning of the endothelium: a human in vivo study," *American Journal of Physiology*, vol. 298, no. 2, pp. H340–H345, 2010.

[60] T. Gori, M. Lisi, and S. Forconi, "Ischemia and reperfusion: the endothelial perspective. A radical view," *Clinical Hemorheology and Microcirculation*, vol. 35, no. 1-2, pp. 31–34, 2006.

[61] T. Gori and J. D. Parker, "Nitrate-induced toxicity and preconditioning. A rationale for reconsidering the use of these drugs," *Journal of the American College of Cardiology*, vol. 52, no. 4, pp. 251–254, 2008.

[62] S. Dragoni, T. Gori, M. Lisi et al., "Pentaerythrityl tetranitrate and nitroglycerin, but not isosorbide mononitrate, prevent endothelial dysfunction induced by ischemia and reperfusion," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 9, pp. 1955–1959, 2007.

[63] D. K. Das and N. Maulik, "Cardiac genomic response following preconditioning stimulus," *Cardiovascular Research*, vol. 70, no. 2, pp. 254–263, 2006.

[64] G. Jancso, B. Cserepes, B. Gasz et al., "Expression and protective role of heme oxygenase-1 in delayed myocardial preconditioning," *Annals of the New York Academy of Sciences*, vol. 1095, pp. 251–261, 2007.

[65] L. Vitek and H. A. Schwertner, "The heme catabolic pathway and its protective effects on oxidative stress-mediated diseases," *Advances in Clinical Chemistry*, vol. 43, pp. 1–57, 2007.

[66] D. P. Converso, C. Taille, M. C. Carreras, A. Jaitovich, J. J. Poderoso, and J. Boczkowski, "HO-1 is located in liver mitochondria and modulates mitochondrial heme content and metabolism," *The FASEB Journal*, vol. 20, no. 8, pp. 1236–1238, 2006.

[67] H. B. Suliman, M. S. Carraway, A. S. Ali, C. M. Reynolds, K. E. Welty-Wolf, and C. A. Piantadosi, "The CO/HO system reverses inhibition of mitochondrial biogenesis and prevents murine doxorubicin cardiomyopathy," *Journal of Clinical Investigation*, vol. 117, no. 12, pp. 3730–3741, 2007.

[68] S. Bindu, C. Pal, S. Dey et al., "Translocation of heme oxygenase-1 to mitochondria is a novel cytoprotective mechanism against non-steroidal anti-inflammatory drug-induced mitochondrial oxidative stress, apoptosis, and gastric mucosal injury," *Journal of Biological Chemistry*, vol. 286, no. 45, pp. 39387–39402, 2011.

[69] P. Wenzel, H. Mollnau, M. Oelze et al., "First evidence for a crosstalk between mitochondrial and NADPH oxidase-derived reactive oxygen species in nitroglycerin-triggered vascular dysfunction," *Antioxidants and Redox Signaling*, vol. 10, no. 8, pp. 1435–1447, 2008.

[70] A. Daiber and T. Munzel, "Characterization of the antioxidant properties of pentaerithrityl tetranitrate (PETN)-induction of the intrinsic antioxidative system heme oxygenase-1 (HO-1)," *Methods in Molecular Biology*, vol. 594, pp. 311–326, 2010.

[71] U. Jurt, T. Gori, A. Ravandi, S. Babaei, P. Zeman, and J. D. Parker, "Differential effects of pentaerythritol tetranitrate and nitroglycerin on the development of tolerance and evidence of lipid peroxidation: a human in vivo study," *Journal of the*

American College of Cardiology, vol. 38, no. 3, pp. 854–859, 2001.

[72] J. Pfeilschifter and K. F. Beck, "Nitric oxide and gene expression," in *Nitric Oxide, Cell Signaling, and Gene Expression*, E. Cadenas and S. Lamas, Eds., pp. 327–348, Taylor & Francis, Boca Raton, Fla, USA, 2005.

[73] T. Thum and J. Bauersachs, "Microarray-based gene expression profiling to elucidate cellular responses to nitric oxide-a review from an analytical and biomedical point of view," *Journal of Chromatography B*, vol. 851, no. 1-2, pp. 3–11, 2007.

[74] C. Bogdan, "Nitric oxide and the regulation of gene expression," *Trends in Cell Biology*, vol. 11, no. 2, pp. 66–75, 2001.

[75] R. B. Pilz and D. E. Casteel, "Regulation of gene expression by cyclic GMP," *Circulation Research*, vol. 93, no. 11, pp. 1034–1046, 2003.

[76] B. J. Buckley, Z. M. Marshall, and A. R. Whorton, "Nitric oxide stimulates Nrf2 nuclear translocation in vascular endothelium," *Biochemical and Biophysical Research Communications*, vol. 307, no. 4, pp. 973–979, 2003.

[77] Y. Kuwano, A. Rabinovic, S. Srikantan, M. Gorospe, and B. Demple, "Analysis of nitric oxide-stabilized mRNAs in human fibroblasts reveals HuR-dependent heme oxygenase 1 upregulation," *Molecular and Cellular Biology*, vol. 29, no. 10, pp. 2622–2635, 2009.

[78] C. Tassorelli and S. A. Joseph, "NADPH-diaphorase activity and Fos expression in brain nuclei following nitroglycerin administration," *Brain Research*, vol. 695, no. 1, pp. 37–44, 1995.

[79] C. Tassorelli, R. Greco, M. T. Armentero, F. Blandini, G. Sandrini, and G. Nappi, "A role for brain cyclooxygenase-2 and prostaglandin-E2 in migraine: effects of nitroglycerin," *International Review of Neurobiology*, vol. 82, pp. 373–382, 2007.

[80] R. Greco, D. Amantea, F. Blandini et al., "Neuroprotective effect of nitroglycerin in a rodent model of ischemic stroke: evaluation of Bcl-2 expression," *International Review of Neurobiology*, vol. 82, pp. 423–435, 2007.

[81] C. Suwattanasophon, P. Phansuwan-Pujito, and A. Srikiatkhachorn, "5-HT1B/1D serotonin receptor agonist attenuates nitroglycerin-evoked nitric oxide synthase expression in trigeminal pathway," *Cephalalgia*, vol. 23, no. 8, pp. 825–832, 2003.

[82] L. Prevotat, R. Filomenko, E. Solary, J. F. Jeannin, and A. Bettaieb, "Nitric oxide-induced down-regulation of β-catenin in colon cancer cells by a proteasome-independent specific pathway," *Gastroenterology*, vol. 131, no. 4, pp. 1142–1152, 2006.

[83] K. Szocs, B. Lassegue, P. Wenzel et al., "Increased superoxide production in nitrate tolerance is associated with NAD(P)H oxidase and aldehyde dehydrogenase 2 downregulation," *Journal of Molecular and Cellular Cardiology*, vol. 42, no. 6, pp. 1111–1118, 2007.

[84] E. Q. Wang, W. I. Lee, D. Brazeau, and H. L. Fung, "cDNA microarray analysis of vascular gene expression after nitric oxide donor infusions in rats: implications for nitrate tolerance mechanisms," *AAPS PharmSci*, vol. 4, no. 2, article E10, 2002.

[85] A. Pautz, P. Rauschkolb, N. Schmidt et al., "Effects of nitroglycerin or pentaerithrityl tetranitrate treatment on the gene expression in rat hearts: evidence for cardiotoxic and cardioprotective effects," *Physiological Genomics*, vol. 38, no. 2, pp. 176–185, 2009.

[86] S. Dhakshinamoorthy and A. G. Porter, "Nitric oxide-induced transcriptional up-regulation of protective genes by Nrf2 via the antioxidant response element counteracts apoptosis of neuroblastoma cells," *Journal of Biological Chemistry*, vol. 279, no. 19, pp. 20096–20107, 2004.

[87] X. M. Liu, K. J. Peyton, D. Ensenat et al., "Nitric oxide stimulates heme oxygenase-1 gene transcription via the Nrf2/ARE complex to promote vascular smooth muscle cell survival," *Cardiovascular Research*, vol. 75, no. 2, pp. 381–389, 2007.

[88] C. L. Hartsfield, J. Alam, J. L. Cook, and A. M. Choi, "Regulation of heme oxygenase-1 gene expression in vascular smooth muscle cells by nitric oxide," *American Journal of Physiology*, vol. 273, no. 5, part 1, pp. L980–L988, 1997.

[89] C. Bouton and B. Demple, "Nitric oxide-inducible expression of heme oxygenase-1 in human cells. Translation-independent stabilization of the mRNA and evidence for direct action of nitric oxide," *Journal of Biological Chemistry*, vol. 275, no. 42, pp. 32688–32693, 2000.

[90] V. Leautaud and B. Demple, "Regulation of heme oxygenase-1 mRNA deadenylation and turnover in NIH3T3 cells by nitrosative or alkylation stress," *BMC Molecular Biology*, vol. 8, article 116, 2007.

[91] U. Hinkelmann, N. Grosser, K. Erdmann, H. Schroder, and S. Immenschuh, "Simvastatin-dependent up-regulation of heme oxygenase-1 via mRNA stabilization in human endothelial cells," *European Journal of Pharmaceutical Sciences*, vol. 41, no. 1, pp. 118–124, 2010.

[92] J. D. Beckman, C. Chen, J. Nguyen et al., "Regulation of heme oxygenase-1 protein expression by miR-377 in combination with miR-217," *Journal of Biological Chemistry*, vol. 286, no. 5, pp. 3194–3202, 2011.

[93] C. M. Misquitta, V. R. Iyer, E. S. Werstiuk, and A. K. Grover, "The role of 3′-untranslated region (3′-UTR) mediated mRNA stability in cardiovascular pathophysiology," *Molecular and Cellular Biochemistry*, vol. 224, no. 1-2, pp. 53–67, 2001.

[94] K. S. Khabar, "The AU-rich transcriptome: more than interferons and cytokines, and its role in disease," *Journal of Interferon and Cytokine Research*, vol. 25, no. 1, pp. 1–10, 2005.

[95] A. Hacker, S. Muller, W. Meyer, and G. Kojda, "The nitric oxide donor pentaerythritol tetranitrate can preserve endothelial function in established atherosclerosis," *British Journal of Pharmacology*, vol. 132, no. 8, pp. 1707–1714, 2001.

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Clinical Study

Role of HO/CO in the Control of Peripheral Circulation in Humans

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Experimental studies show that the heme oxygenase/carbon monoxide system (HO/CO) plays an important role in the homeostasis of circulation and in the pathophysiology of hypertension. No data are available on its role in the control of peripheral circulation in humans. We evaluated the effects of inhibition of HO with stannous mesoporphyrin IX (SnMP) (200 μM) locally administered by iontophoresis, on human skin blood flow, evaluated by laser-Doppler flowmetry, in the presence and absence of nitric oxide synthase (NOS) inhibition with L-NG-Nitroarginine methyl ester (L-NAME) (100 μM). We also evaluated the effect of HO inhibition on vasodilatation induced by acetylcholine (ACh) and vasoconstriction caused by noradrenaline (NA). SnMP and L-NAME caused a similar 20–25% decrease in skin flow. After nitric oxide (NO) inhibition with L-NAME, HO inhibition with SnMP caused a further 20% decrease in skin perfusion. SnMP decreased vasodilatation induced by ACh by about 70%, while it did not affect vasoconstriction to NA. In conclusion, HO/CO participates in the control of peripheral circulation, independently from NO, and is involved in vasodilatation to ACh.

1. Introduction

Carbon monoxide (CO) is physiologically produced in the arterial wall by the action of heme oxygenase (HO) on heme. HO exists in two isoforms: the inducible HO-1, and the constitutive HO-2 [1, 2].

CO, generated in endothelial and smooth muscle layers of blood vessels by HO, modulates vascular tone, by inducing relaxation of vascular smooth muscle cells. Smooth muscle cell relaxation is obtained by stimulating soluble guanylyl cyclase (sGC) and by opening large-conductance calcium-activated K^+ channels (BK-Ca) [3]. CO acts on smooth muscle cells through mechanisms that are also involved in the action of other vasoactive substances, such as nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) [4, 5]. CO also inhibits the cytochrome P450-dependent monooxygenase system, with a decrease in 20-hydroxyeicosatetraenoic acid (20-HETE), which sustains contractile tone by inhibiting potassium channels [4]. In normal rats, inhibition of HO with chromium mesoporphyrin impairs ACh-induced vasorelaxation only when cyclooxygenase, NO synthase, and sGC are all inhibited, while it has no effect in baseline condition [3]. Nonetheless, HO inhibition increases peripheral vascular resistance and blood pressure in experimental animals, but no data are available in humans [2, 4].

Aim of this study was to evaluate whether the HO/CO system participates in the control of peripheral circulation in humans. We have chosen skin circulation as it is involved in the control of peripheral resistance and can be estimated by the laser-Doppler technique, which allows to evaluate not only skin flow but also the local effects of vasoactive substances and inhibitors administered locally by iontophoresis.

2. Patients and Methods

The study was performed in 20 normal volunteers, age 24–50 yr, 12 males and 8 females. An informed consent was

obtained from each subject, and the study was approved by the institutional local ethical committee.

After at least a 4-hour fast and 10 min lying on a bed at 21–24 C, a laser Doppler probe was applied on the dorsal face of the 2nd phalanx of the 3rd finger and skin flow, expressed as arbitrary units (PFUs), was measured for 5 minutes. The average value was considered as the basal flow.

After baseline measurement, the HO inhibitor stannous mesoporphyrin IX (SnMP) (200 μM) (Frontiers Scientific, Logan, UT, USA) was applied by iontophoresis (four 15-second infusions at 0.06 mA) in the same skin area and its effects were evaluated, while another probe was measuring flow in another finger of the other hand in order to exclude changes of flow not due to the inhibitor. The effect of the NOS inhibitor, L-NG-Nitroarginine methyl ester (L-NAME) (100 μM) (Sigma Chemicals, St. Louis, MO, USA), similarly applied by iontophoresis, was then evaluated on another finger. The HO inhibitor SnMP (200 μM) was then applied together with L-NAME and the effect of the two inhibitors was measured.

The effects of noradrenaline (NA) (10 mMol) (Sigma Chemicals, St. Louis, MO, USA) (four 15-second infusions at 0.06 mA) and of the endothelium-dependent vasodilator, acetylcholine (ACh) (11 mMol) (Sigma Chemicals, St. Louis, MO, USA) (seven 15-second infusions at 0.06 mA, with 45-sec intervals), applied by iontophoresis, were evaluated in different subjects, in the presence or absence of HO inhibition with SnMP.

Laser Doppler flowmetry gives a semiquantitative assessment of microvascular blood perfusion, reflecting perfusion of capillaries, arterioles, venules, and dermal vascular plexa [6]. Measurements were done using a PeriFlux laser Doppler flowmeter (LDF) (Periflux system 5000, Perimed, Jarfalla/Stockholm, Sweden). Iontophoresis allows transdermal delivery of polar compounds by means of a small electrical current. The delivery is done in the same area where blood perfusion is measured, allowing the assessment of microvascular reactivity when blood perfusion is measured simultaneously.

2.1. Statistical Analysis. Results were shown as mean ± SD. Differences among groups were analyzed by Anova and unpaired Student's t-test. Statistical significance was set at $P < 0.05$.

3. Results

Skin flow under basal condition was 23 ± 6 PFU and was stable during the whole experiment, as demonstrated on the control finger. Inhibition of HO with SnMP, administered locally by iontophoresis in the same skin area where the flow probe was positioned, caused a 20% decrease in skin flow (Figure 1). A similar decrease was shown after NOS inhibition with L-NAME (Figure 1), also given locally by iontophoresis on a different finger. Inhibition of SnMP after L-NAME almost doubled the decrease in flow (Figure 1). NA, administered locally by iontophoresis, caused a 42% reduction in skin flow (Figure 2), while ACh caused a 290% increase (Figure 3). Inhibition of HO with SnMP, or NOS with L-NAME, did not affect the response to NA (Figure 2), while both of them reduced vasodilatation to ACh by about 70% (Figure 3).

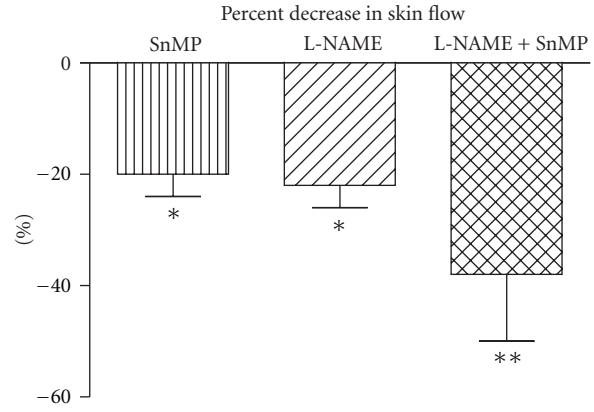

Figure 1: Effect of heme oxygenase inhibition with stannous mesoporphyrin IX (SnMP) (200 μM), of nitric oxide synthase inhibition with L-NG-Nitroarginine methyl ester (L-NAME) (100 μM), and of both, on skin blood flow measured by laser Doppler flowmetry. SnMP and L-NAME were administered locally by iontophoresis. $^*P < 0.05$ versus baseline; $^{**}P < 0.05$ versus L-NAME.

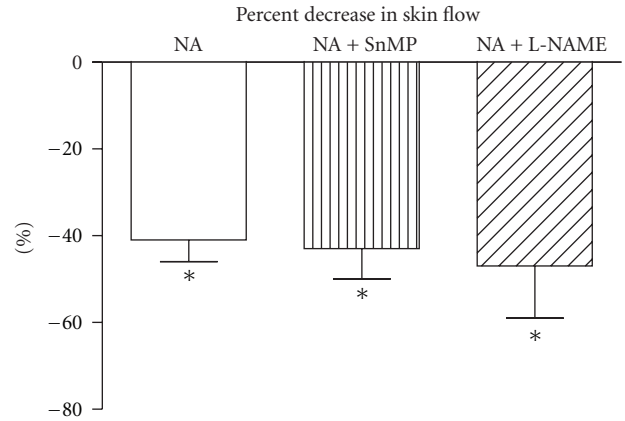

Figure 2: Effect of heme oxygenase inhibition with stannous mesoporphyrin IX (SnMP) (200 μM) and nitric oxide synthase inhibition with L-NG-Nitroarginine methyl ester (L-NAME) (100 μM) on the vasoconstricting effect of noradrenaline (NA) on skin blood flow measured by laser Doppler flowmetry. NA, SnMP, and L-NAME were locally administered by iontophoresis. $^*P < 0.05$ versus baseline.

4. Discussion

The results of this study show for the first time a role of the HO/CO system in the control of peripheral circulation in humans.

Skin blood flow is the result of a balance between vasoconstricting and vasodilating systems. NO is considered the most important endothelium-dependent vasodilator of the skin microcirculation and is released in response to ACh and shear stress. Its synthesis from L-arginine is inhibited by

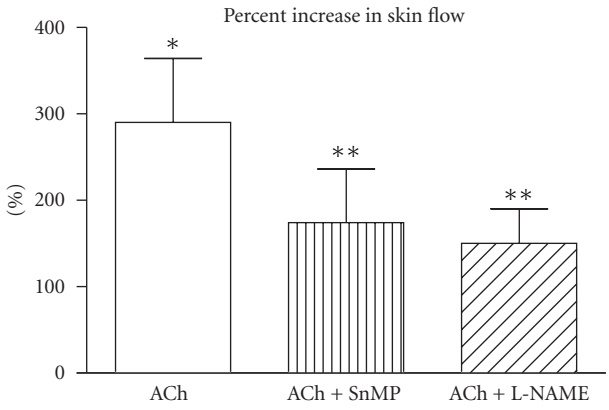

Figure 3: Effect of heme oxygenase inhibition with stannous mesoporphyrin IX (SnMP) (200 μM) and nitric oxide synthase inhibition with L-NG-Nitroarginine methyl ester (L-NAME) (100 μM) on vasodilatation induced by acethylcholine (ACh) in skin circulation evaluated by laser Doppler flowmetry. ACh, SnMP, and L-NAME were locally administered by iontophoresis. $^{*}P < 0.05$ versus baseline; $^{**}P < 0.05$ versus Ach.

L-NMMA or L-NAME. Kvandal et al. [6] have shown that intraarterial infusion of L-NMMA causes a 20% reduction in skin flow, which could be reversed by l-arginine. L-NMMA; however, it did not affect skin perfusion raised by ACh. Our results, although obtained with a different technique, confirm that inhibition of NOS causes a reduction in skin flow. Furthermore, we showed that NOS inhibition reduced the vasodilating affect of ACh by about 70%. This difference in results is probably related to the different method of administration of the NOS inhibitor, which was given locally, by iontophoresis in our subjects, and intraarterially in Kvandal's study. It is, in fact, possible that the local administration allows to obtain higher concentrations and, thus, a higher effect due to a higher inhibition of NOS, considering that the intraarterial infusion cannot be done using high doses to avoid the systemic effects. Thus, our results confirm that cutaneous vascular tone is regulated by NO.

Treatment of normotensive rats with HO inhibitors causes elevation of peripheral vascular resistance and blood pressure, suggesting that endogenous CO subserves a vasodepressor function [7]. The same conclusion was derived from reports that treatment of hypertensive rats with HO inducers or substrates reduces blood pressure via a heme oxygenase-dependent mechanism [8–11]. Also, observations that heme elicits HO-dependent vasodilation in isolated gracilis muscle arterioles and tail arteries suggested that CO of vascular origin can be a mediator of vasodilatory mechanisms.

In our normal subjects, basal skin flow was reduced by SnMP, even in the presence of NOS inhibition with L-NAME. Thus, it is conceivable that CO participates in the physiologic control of tone in the peripheral circulation and may also be involved in pathophysiology. CO-induced cell signaling has been proposed to occur via sGC activation, although CO is far less effective at activating sGC than is NO [2, 5]. CO also activates BK-Ca channels in smooth muscle cells from a variety of different vascular beds, including cerebral and tail arteries [12]. In isolated vascular smooth muscle cells, CO-induced BK-Ca channel activation is not blocked by inhibitors of sGC and is not reproduced by other products of HO-mediated heme metabolism. Thus, vascular smooth muscle cell HO-derived CO, or exogenous CO, activates arterial smooth muscle cell BK-Ca channels either directly or via interaction with channel-associated regulatory elements. Furthermore, NO and CO activate vascular smooth muscle cell BK-Ca channels via distinct mechanisms that involve effects on different channel subunits [2, 3, 5], thus explaining the additive effect of NOS and HO inhibition on skin flow.

The effect of ACh was also blunted by HO inhibition, suggesting that the NO-independent and prostacyclin-independent component of vasodilatation to ACh is mediated, at least in part, by the HO/CO system.

We have previously shown that HO inhibition in mesenteric microvessels from normal rats, pretreated with indomethacin, L-NAME, and also the cGMP inhibitor 1H-[1, 2, 4]oxadiazolo[4, 3-a] quinoxalin-1-one (ODQ), causes a decrease in the vasodilating effect of ACh [3]. This effect was similar to that of iberiotoxin, a BK-Ca channels inhibitor. Thus, it is conceivable that the HO/CO system participates in the homeostasis of vascular tone by stimulating BK-Ca channels, although it is well known that other mechanisms are possible, like inhibition of 20HETE synthesis, decrease of ROS [4]. HO inhibition did not increase the vasoconstricting response to NA. This last result is similar to what shown in the rat mesenteric circulation [13]. It is possible that an increased response to NA by HO inhibition is masked by an increase in NO, prostacyclin, or other vasodilators, but there has also to be considered the possibility that the degree of vasoconstriction induced by NA was the highest which could be obtained with a vasoconstrictor (a mean of 42%) in the skin and, thus, any increase could not be demonstrated. On the contrary, when HO is induced, like in cirrhosis or after transfection with the HO-1 gene [13], the hyporeactivity to vasoconstrictors can be reversed by inhibition of HO.

The results of this study may have therapeutic implications. While inhibition of HO may be useful for treatment of vasodilatation and hyperdynamic circulation, like in sepsis or cirrhosis, in conditions of increased peripheral resistance, like hypertension, or of vasoconstriction, like angina or ischemic arteriopathy, HO induction or supplementation of CO could be employed.

In conclusion, in normal physiological conditions, in humans, the HO/CO system participates in the control of peripheral circulation, besides NO, and also mediates, in part, the endothelium-dependent vasodilatation to ACh.

Abbreviations

CO: Carbon monoxide
HO: Heme oxygenase
NO: Nitric oxide
L-NAME: L-NG-Nitroarginine methyl ester
NOS: Nitric oxide synthase
SnMP: Stannous mesoporphyrin IX
Ach: Acetylcholine
NA: Noradrenaline.

References

[1] C. W. Leffler, H. Parfenova, and J. H. Jaggar, "Carbon monoxide as an endogenous vascular modulator," *American Journal of Physiology*, vol. 301, no. 1, pp. H1–H11, 2011.

[2] L. Wu and R. Wang, "Carbon monoxide: endogenous production, physiological functions, and pharmacological applications," *Pharmacological Reviews*, vol. 57, no. 4, pp. 585–630, 2005.

[3] M. Bolognesi, D. Sacerdoti, A. Piva et al., "Carbon monoxide-mediated activation of large-conductance calcium-activated potassium channels contributes to mesenteric vasodilatation in cirrhotic rats," *Journal of Pharmacology and Experimental Therapeutics*, vol. 321, no. 1, pp. 187–194, 2007.

[4] N. G. Abraham and A. Kappas, "Heme oxygenase and the cardiovascular-renal system," *Free Radical Biology and Medicine*, vol. 39, no. 1, pp. 1–25, 2005.

[5] G. Edwards, M. Félétou, and A. H. Weston, "Endothelium-derived hyperpolarising factors and associated pathways: a synopsis," *Pflügers Archiv*, vol. 459, no. 6, pp. 863–879, 2010.

[6] P. Kvandal, A. Stefanovska, M. Veber, H. D. Kvermmo, and K. A. Kirkebøen, "Regulation of human cutaneous circulation evaluated by laser Doppler flowmetry, iontophoresis, and spectral analysis: importance of nitric oxide and prostaglandines," *Microvascular Research*, vol. 65, no. 3, pp. 160–171, 2003.

[7] R. A. Johnson, M. Lavesa, B. Askari, N. G. Abraham, and A. Nasjletti, "A heme oxygenase product, presumably carbon monoxide, mediates a vasodepressor function in rats," *Hypertension*, vol. 25, no. 2, pp. 166–169, 1995.

[8] D. Sacerdoti, B. Escalante, N. G. Abraham, J. C. McGiff, R. D. Levere, and M. L. Schwartzman, "Treatment with tin prevents the development of hypertension in spontaneously hypertensive rats," *Science*, vol. 243, no. 4889, pp. 388–390, 1989.

[9] B. Escalante, D. Sacerdoti, M. M. Davidian, M. Laniado-Schwartzman, and J. C. McGiff, "Chronic treatment with tin normalizes blood pressure in spontaneously hypertensive rats," *Hypertension*, vol. 17, no. 6, part 1, pp. 776–779, 1991.

[10] R. A. Johnson, M. Lavesa, K. Deseyn, M. J. Scholer, and A. Nasjletti, "Heme oxygenase substrates acutely lower blood pressure in hypertensive rats," *American Journal of Physiology*, vol. 271, no. 3, pp. H1132–H1138, 1996.

[11] F. Kozma, R. A. Johnson, and A. Nasjletti, "Role of carbon monoxide in heme-induced vasodilation," *European Journal of Pharmacology*, vol. 323, no. 2-3, pp. R1–R2, 1997.

[12] R. Wang, Z. Wang, and L. Wu, "Carbon monoxide-induced vasorelaxation and the underlying mechanisms," *British Journal of Pharmacology*, vol. 121, no. 5, pp. 927–934, 1997.

[13] D. Sacerdoti, N. G. Abraham, A. O. Oyekan, L. Yang, A. Gatta, and J. C. McGiff, "Role of the heme oxygenases in abnormalities of the mesenteric circulation in cirrhotic rats," *Journal of Pharmacology and Experimental Therapeutics*, vol. 308, no. 2, pp. 636–643, 2004.

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Research Article

Induction of Hemeoxygenase-1 Reduces Renal Oxidative Stress and Inflammation in Diabetic Spontaneously Hypertensive Rats

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The renoprotective mechanisms of hemeoxygenase-1 (HO-1) in diabetic nephropathy remain to be investigated. We hypothesize that HO-1 protects the kidney from diabetic insult via lowering renal oxidative stress and inflammation. We used control and diabetic SHR with or without HO-1 inducer cobalt protoporphyrin (CoPP) treatment for 6 weeks. Urinary albumin excretion levels were significantly elevated in diabetic SHR compared to control and CoPP significantly attenuated albumin excretion. Immuno-histochemical analysis revealed an elevation in TGF-β staining together with increased urinary collagen excretion in diabetic versus control SHR, both of which were reduced with CoPP treatment. Renal oxidative stress markers were greater in diabetic SHR and reduced with CoPP treatment. The increase in renal oxidative stress was associated with an elevation in renal inflammation in diabetic SHR. CoPP treatment also significantly attenuated the markers of renal inflammation in diabetic SHR. In vitro inhibition of HO with stannous mesoporphyrin (SnMP) increased glomerular NADPH oxidase activity and inflammation and blocked the anti-oxidant and anti-inflammatory effects of CoPP. These data suggest that the reduction of renal injury in diabetic SHR upon induction of HO-1 are associated with decreased renal oxidative stress and inflammation, implicating the role of HO-1 induction as a future treatment of diabetic nephropathy.

1. Introduction

The incidence of diabetes mellitus has dramatically increased worldwide [1, 2]. One of the major complications of diabetes is the progression of renal injury, affecting approximately 35% of type 1 and type 2 diabetic patients, which often leads to end-stage renal disease. Diabetes is often associated with an elevation in blood pressure which is known to worsen renal function [3–5]. Accordingly, we induced diabetes in spontaneously hypertensive rats (SHR) in the current study as a genetic model of essential hypertension to address the effects of diabetes on a hypertensive background.

Increased oxidative stress has been implicated in the pathogenesis of diabetes and hypertension [6, 7]. NADPH oxidase, the major source of superoxide production in the vasculature, is known to activate numerous inflammatory cytokines [8]. NADPH oxidase has been shown to be activated in the kidney of diabetic animal models, with enhanced expression in the glomerulus and distal tubules [9–11]. NADPH oxidase-derived reactive oxygen species increase renal hypertrophy and fibronectin expression in streptozotocin-induced type 1 diabetic rats [11, 12] as well as exacerbate the damage in glomerular basement membrane and slit diaphragm [10, 13]. Collectively, these data suggest that NADPH oxidase-derived superoxide contributes to the progression of diabetic-induced renal injury.

Clinically, inflammatory processes in the kidney also contribute to the progression of nephropathy in patients with type 1 diabetes and in diabetic animal models [14–19]. Diabetic renal injury is an inflammatory disease characterized by monocyte infiltration at every stage of the disease progression with chemokines driving the recruitment of inflammatory cells into renal compartments [15, 18]. Kidney of diabetic humans and experimental animal models both show increased macrophage infiltration and overproduction of leukocyte adhesion molecules [14–19].

Activated inflammatory cells further exacerbate cytokine release leading to enhanced fibrosis, matrix deposition, and progressive renal injury. Moreover, oxidative stress has been demonstrated to modulate expression of many inflammatory genes in diabetes, including cell adhesion molecules (CAMs) and monocyte chemoattractant protein (MCP-1). Taken together, these data support a role of immune response in the progression of diabetic renal injury [11, 20].

Heme catabolism is primarily driven by hemeoxygenase (HO) generating biliverdin, iron, and carbon monoxide [21, 22]. There are two isoenzymes of HO: inducible HO-1 and constitutive HO-2 which accounts for most HO activity in the normal state [21, 22]. Studies have shown that HO-1 is upregulated in response to oxidative stress, ischemia, and inflammation [21, 22]. Induction of HO-1 also reduces blood pressure and inflammation in experimental models of diabetes and hypertension suggesting that HO-1 induction may protect the diabetic kidney via inhibition of oxidative stress and inflammation [23–26].

Previous studies have suggested a role for hyperglycemia in increasing oxidative stress and inflammation in diabetic animal models [27, 28]; however, most of the studied diabetic animal models remained normotensive. Because diabetic nephropathy is characterized by increased albuminuria with an elevation in blood pressure and decline in renal function, the coexistence of hypertension and diabetes in the current animal model is expected to worsen the degree of renal injury and more accurately reflect the clinical picture of diabetic nephropathy. The current study tests the hypothesis that HO-1 induction-mediated decreases in renal injury are associated with decreases in renal oxidative stress and inflammation in diabetic SHR.

2. Materials and Methods

All procedures with animals were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals and Georgia Health Sciences University guidelines. Eleven-week-old male SHR (Charles River, MA) were used to induce diabetes by a single injection of streptozotocin (Sigma, MO; 65 mg/kg i.v dissolved in 0.1 M citrate buffer) and control SHR only received 0.1 M citrate buffer injection. The blood glucose levels of these rats were maintained within 400–500 mg/dL via the use of sustained release insulin implants (s.c, Lanshin, Canada), and blood glucose levels were tested weekly using a glucometer. Normal control and diabetic SHR rats were randomized to receive either vehicle (0.1 M NaOH, pH 8.3) or the HO-1 inducer cobalt protoporphyrin (CoPP, 5.0 mg/100 g body weight s.c) weekly for six weeks after induction of diabetes (n = 8/group). Systolic blood pressure was recorded weekly using the tail cuff method (IITC Life Science, Woodland Hills, CA) [25]. Rats were placed in metabolic cages (Nalgene Corp. Rochester, NY) for 24-hour urine collection at the end of the experiment. Urinary creatinine (Cayman Chemical, Ann Arbor, MI), albumin, and collagen (Exocell, Philadelphia, PA) excretion levels were determined as indices of renal injury. Urinary thiobarbituric acid reactive substances (TBARs, Cayman Chemical, Ann Arbor, MI), and 8-hydroxy deoxyguanosine (*8-OHdG,* Northwest, WA) excretion levels were assessed as markers of oxidative stress.

2.1. Homogenization of the Renal Cortex for Protein Expression Using Western Blotting Analysis. Renal cortical samples were homogenized in RIPA buffer supplemented with inhibitors for proteases and phosphatases as previously described [25]. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA). Cortical samples were separated by SDS-PAGE as previously described [25]. Gels were then transferred onto nitrocellulose membranes. The primary antibodies used were: rabbit HO-1, HO-2 (EMD Biosciences, San Diego, CA), and mouse β-actin (Sigma, St. Louis, MO). These antibodies were detected with a horseradish peroxidase-conjugated secondary antibody and ECL chemiluminescence (Amersham BioSciences, Buckinghamshire, UK). Intensity of immunoreactivity was measured by densitometry, and β-actin was used to verify equal loading of protein.

2.2. Renal NADPH Oxidase Activity. NADPH activity was measured in cortical samples by lucigenin chemiluminescence using 35 μg protein in the presence of NADPH (100 μM) and lucigenin (5 μM) as previously described [29] and average sample counts (cpm) were normalized to μg protein.

2.3. Renal MCP-1, HO-1, and sICAM-1 Assays. HO-1 activity was measured in renal cortical samples using a commercially available ELISA according to manufacturer's instructions (Enzo Life Sciences Inc., Farmingdale, NY). Renal cortical MCP-1 levels were assessed using a commercially available ELISA according to manufacturer's instructions (BD Biosciences, Bedford, MA). Renal soluble ICAM-1 levels (sICAM-1) were also determined using a commercially available ELISA according to manufacturer's instructions (R&D Systems, Minneapolis, MN).

2.4. Renal Histopathology. In a separate set of rats (n = 5/group), kidneys were perfused with 10% formalin solution and were then paraffin embedded and cut into 4- to 5-μm sections. Kidney sections were used for immunohistochemical evaluation of CD68 to assess monocyte/macrophage infiltration (ED-1 staining) and CD3 to assess T-cell infiltration as previously described [30]. Ten microscopic images of the kidney cortex per rat were randomly taken at $\times$200 magnification, and CD68-positive and CD3-positive cells were counted by a blinded reviewer experienced in analysis. The number of positive cells per millimeter squared was calculated and averaged for each group. Additional kidney sections were immunohistochemically stained with TGF-β antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and staining intensity was evaluated at $\times$200 and $\times$400 magnification power, respectively. Masson's trichrome staining of kidney sections was also used to assess the amount of collagen deposition, $\times$200 magnification.

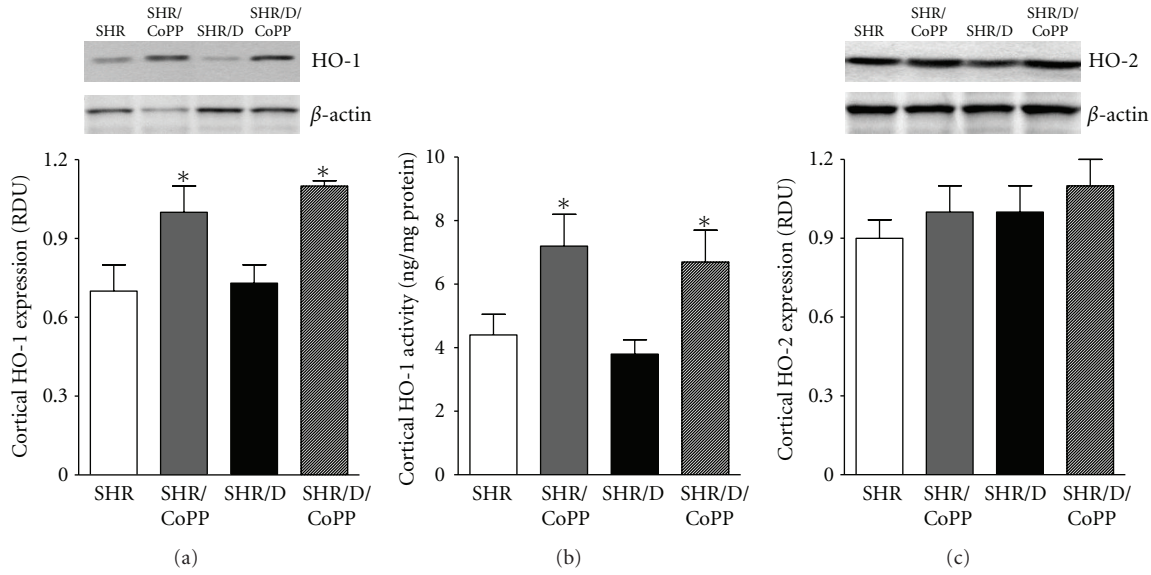

Figure 1: Renal cortical HO-1 expression relative to β-actin (a), HO-1 activity (b), and HO-2 expression relative to β-actin (c) in control and diabetic (D) SHR with or without CoPP treatment ($n = 6$, *indicates significant difference from control SHR and $^\#$indicates significant difference from diabetic SHR).

2.5. Isolation of Glomeruli. Glomeruli were isolated as previously described [39] by a gradual sieving technique from control and diabetic SHR and incubated for 2 hours at 37°C with the HO inhibitor stannous mesoporphyrin (SnMP, 20 mM), CoPP (10 mM), or both SnMP and CoPP (n = 4/group). Glomerular NADPH activity was determined by lucigenin method, and glomerular P-ERK/ERK ratio was also assessed by Western blotting using antibodies from cell-signaling technology (Beverly, MA).

2.6. Data Analyses. Statistical analyses were performed using Prism software (GraphPad, San Diego, CA, USA). Data were reported as means ± SEM and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test ($P < 0.05$ was considered significant).

3. Results

As shown in Figures 1(a) and 1(b), induction of diabetes with streptozotocin did not significantly change renal HO-1 activity or expression in SHR. However, CoPP treatment significantly elevated renal HO-1 expression and activity in both control and diabetic SHR ($P < 0.05$). There was no difference in renal HO-2 expression among all rat groups (Figure 1(c)). Induction of diabetes did not significantly change systolic blood pressure in SHR (209 ± 4 versus 200 ± 4 mmHg) although blood glucose levels were significantly elevated compared to control SHR (507 ± 43 versus 205 ± 15 mg/dL). CoPP treatment reduced blood pressure (187 ± 2 mmHg) and blood glucose (425 ± 47 mg/dL) in diabetic SHR; however, blood glucose and blood pressure remained significantly higher than control SHR.

3.1. Renal Injury. We assessed urinary albumin and creatinine excretion levels as markers of renal injury. Control SHR had a significantly higher level of albuminuria than normotensive WKY (1.0 ± 0.2 versus 0.35 ± 0.05 mg/day, $P < 0.05$). As shown in Figure 2(a), diabetic SHR exhibited a significant increase in albuminuria after 6 weeks of induction of diabetes compared to control SHR (6.5 ± 0.6 versus 1.0 ± 0.2 mg/day, resp., $P < 0.05$). CoPP treatment lowered albuminuria in control SHR (0.6 ± 0.1 mg/day) and significantly attenuated the elevation in albuminuria in diabetic SHR (2.2 ± 0.6, $P < 0.05$). Similarly, creatinine excretion was significantly elevated in diabetic SHR compared to control and was reduced with CoPP treatment (Figure 2(b)).

The progression of renal injury in diabetic SHR was associated with renal vascular remodeling and increased extracellular matrix deposition and fibrosis as manifested by greater collagen deposition (blue staining, Figure 3(a)) and enhanced TGF-β levels (red staining, Figure 3(b)) in diabetic SHR. The increase in collagen deposition was also associated with an elevation in urinary collagen excretion in diabetic SHR compared to control SHR (Figure 3(c)). Induction of HO-1 with CoPP reduced collagen deposition and TGF-β staining and significantly lowered urinary collagen excretion in diabetic SHR (Figure 3).

3.2. NADPH Oxidase and Oxidative Stress. Oxidative stress has been shown to play a role in the pathogenesis of diabetic-induced renal injury, and NADPH oxidase is the main source of superoxide production in diabetes [10, 11]. Consistent with these observations, renal cortical NADPH oxidase activity was significantly elevated in diabetic SHR compared to control SHR (Figure 4(a)). The increase in NADPH oxidase activity was also associated with elevation in the oxidative stress markers TBARs and *8-OHdG* excretion levels in diabetic versus control SHR (Figures 4(b) and 4(c)). Induction of HO-1 with CoPP inhibited NADPH oxidase activity and reduced excretion levels of oxidative

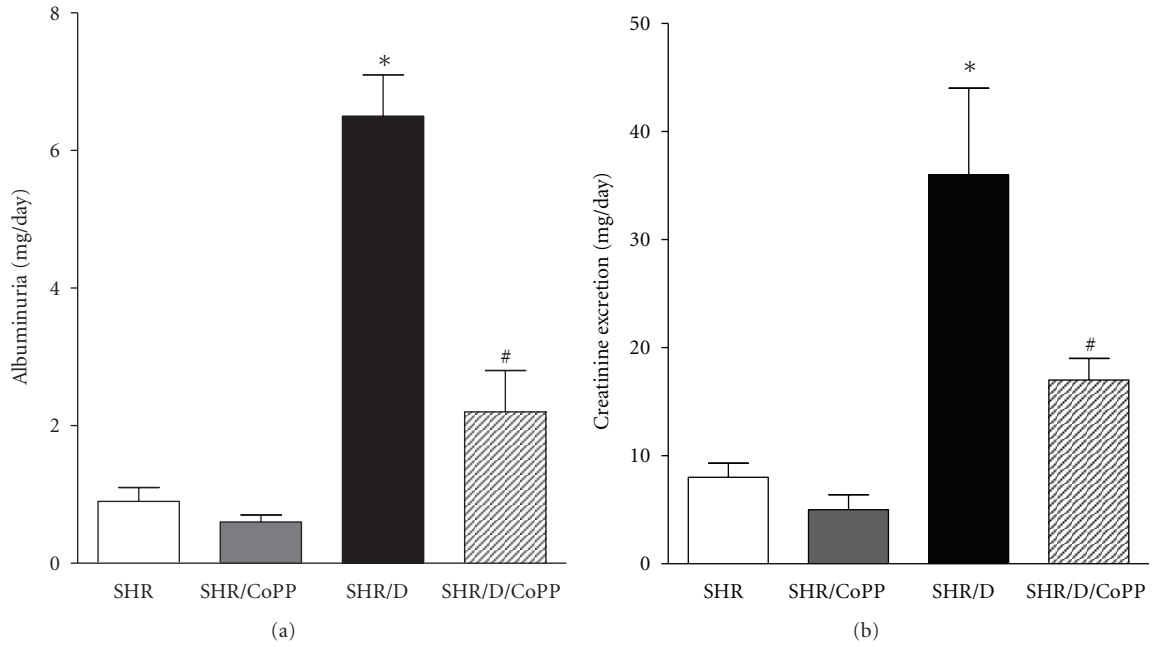

Figure 2: Urinary albuminuria (a) and creatinine excretion (b) in control and diabetic SHR with or without CoPP ($n = 8$, *indicates significant difference from control SHR and #indicates significant difference from diabetic SHR).

stress markers in diabetic SHR (Figure 4). Plasma TBARs were also elevated in diabetic versus control SHR (39 ± 8 versus $26 \pm 4\,\mu$M) and were reduced with CoPP treatment in diabetic and control SHR (26 ± 5 & $13 \pm 4\,\mu$M, resp.) suggesting that induction of HO-1 lowers renal as well as systemic oxidative stress levels.

3.3. Renal Inflammation. Because diabetic renal injury is characterized by leukocyte infiltration at every stage of the disease progression [14, 18], kidneys were processed for immunohistochemical quantification of macrophage (CD68) and T-cell (CD3) infiltration. Macrophage infiltration was significantly greater in diabetic versus control SHR and induction of HO-1 with CoPP significantly attenuated the increase in macrophage infiltration in diabetic SHR (Figure 5(a)). There was no difference in T-cell infiltration between all groups (Figure 5(b)).

We have recently shown that the NFκB inflammatory signaling pathway plays a crucial role in the progression of diabetic renal injury via the activation of proinflammatory molecules such as MCP-1 [31]. Consistent with this observation, renal cortical MCP-1 levels were significantly elevated in diabetic SHR compared to control SHR (129 ± 10 versus 101 ± 3 pg/mg protein, $P < 0.05$), and levels were reduced with CoPP treatment in diabetic SHR (90 ± 10 pg/mg protein, Figure 6(a)). Similarly, renal sICAM-1 levels were significantly elevated in diabetic SHR compared to control SHR and reduced with CoPP treatment (Figure 6(b)).

3.4. In Vitro Inhibition of HO in Isolated Glomeruli. Glomerular NADPH oxidase was significantly elevated in diabetic versus control SHR and incubation of isolated glomeruli from diabetic SHR with CoPP reduced NADPH oxidase activity (Figure 7(a)). Treatment of isolated glomeruli from diabetic SHR with the HO inhibitor SnMP further increased NADPH oxidase and prevented the ability of CoPP to reduce NADPH oxidase activity (Figure 7(a)). Previous studies demonstrated that MAPK activation is involved in the secretion of proinflammatory cytokines [32, 33] and increased ERK phoshorylation could be an indicative of renal inflammation during diabetes [34]. In our study, CoPP treatment significantly inhibited hyperglycemia-induced ERK phosphorylation in glomeruli isolated from diabetic SHR, and this effect was also prevented with SnMP treatment (Figure 7(b)).

4. Discussion

The current study provides evidence that HO-1 induction mitigates renal injury and inflammation in type 1 diabetic SHR as a model in which diabetes coexists with hypertension to exaggerate the progression of renal injury. Induction of HO-1 attenuated the elevation in albuminuria and creatinine excretion in diabetic SHR and decreased renal fibrosis and extracellular matrix deposition in the kidney of diabetic SHR as evidence by decreased kidney TGF-β and collagen and decreased collagen excretion in diabetic SHR. It is now widely acceptable that oxidative stress and inflammatory cytokines play a crucial role in the progression of diabetic renal injury. Interestingly, HO-1 induction inhibited NADPH oxidase activation and reduced markers of oxidative stress in diabetic SHR. HO-1 induction also reduced kidney macrophage infiltration and attenuated renal MCP-1 and sICAM-1 levels in diabetic SHR. Inhibition of HO with SnMP negated the

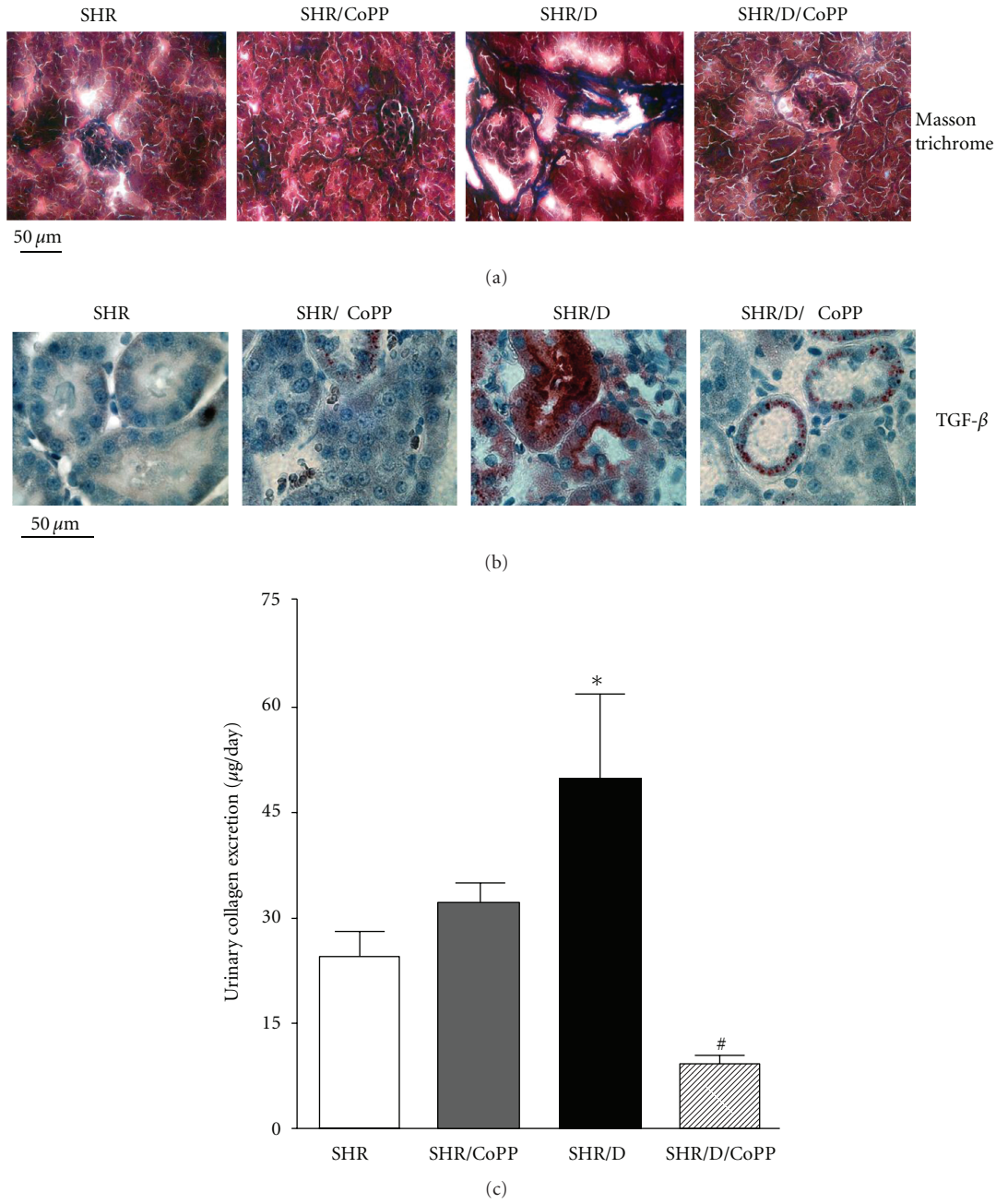

Figure 3: Representative images of Masson's trichrome staining (a) and immunohistochemical staining of TGF-β (b) in kidney sections from control and diabetic SHR with or without CoPP treatment ($n = 5$). (c) is urinary collagen excretion in control and diabetic SHR with or without CoPP treatment ($n = 8$, *indicates significant difference from control SHR and #indicates significant difference from diabetic SHR).

protective effect of CoPP on NADPH oxidase activation and ERK phosphorylation in isolated glomeruli from diabetic SHR. These findings suggest that induction of HO-1 could function to protect the kidney from diabetes-induced renal injury. We postulate that the renoprotective effects of HO-1 induction could be linked to inhibition of renal NADPH oxidase-derived oxidative stress and inflammation in diabetic SHR.

The potential renoprotective mechanisms of HO-1 induction remain to be explored. Induction of HO-1 has been shown to decrease blood pressure in experimental hypertensive and diabetic animal models including SHR [24–26]. Consistent with the previous findings, HO-1 induction lowered blood pressure in control and diabetic SHR; however, this is unlikely to be the sole renoprotective mechanism as the blood pressure of CoPP-treated SHR remained very high. Besides, a blood pressure lowering effect, induction of HO-1 with CoPP has also previously been shown to reduce fasting blood glucose and plasma levels of inflammatory cytokines in obese male and female mice suggesting the

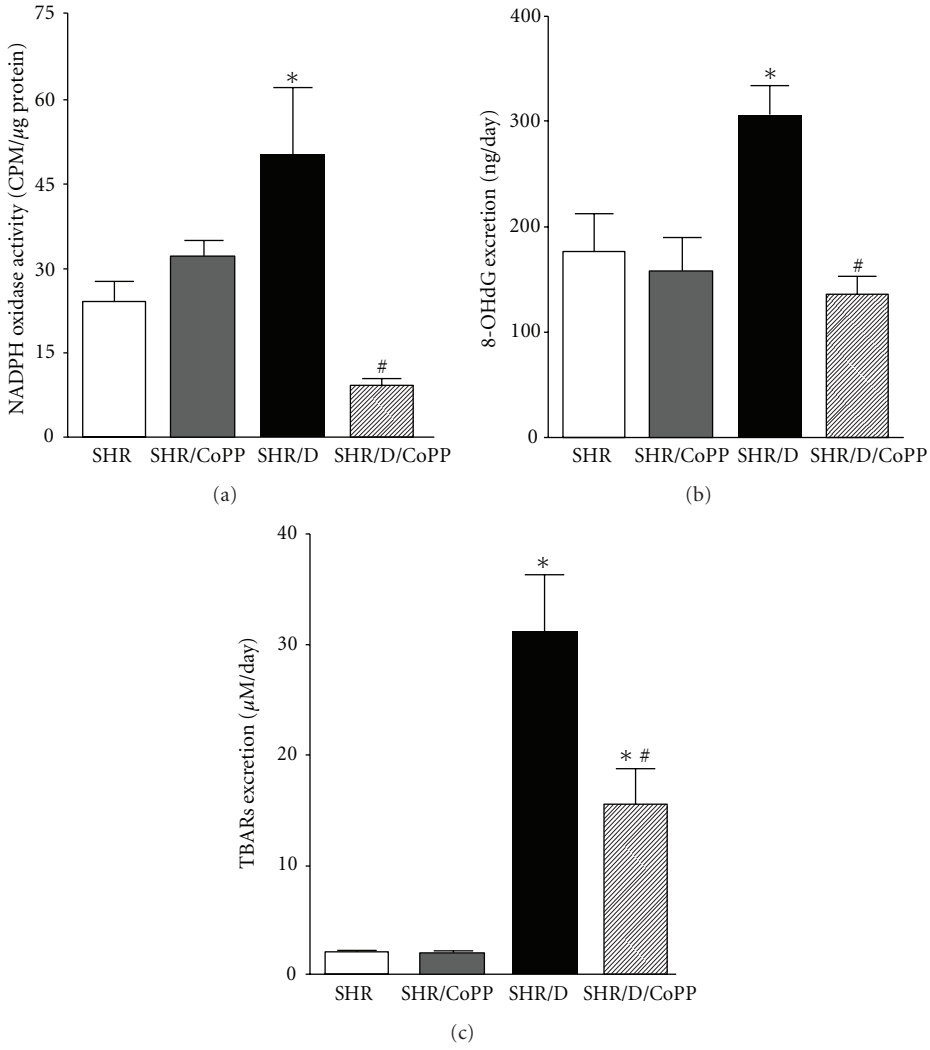

Figure 4: Cortical NADPH oxidase activity (a), urinary thiobarbituric acid reactive substances (TBARs) and urinary *8-hydroxy deoxyguanosine (8-OHdG)* excretion levels (b) and (c), respectively, in control and diabetic SHR with or without CoPP treatment ($n = 8$, *indicates significant difference from control SHR, #indicates significant difference from diabetic SHR).

potential beneficial effects of HO-1 in treating not only hypertension, but also the metabolic consequences of obesity such as insulin resistance and dyslipidemia [23]. In support of this hypothesis, HO-1 upregulation has been shown to improve insulin sensitivity and glucose metabolism in SHR [35] which could be another potential renoprotective mechanism in diabetes. Consistent with these findings, induction of HO-1 with CoPP in this current study decreased blood glucose levels in control and diabetic SHR; however, it is unlikely to be the only mechanism of CoPP-induced kidney protection as blood glucose levels in CoPP-treated diabetic SHR remained significantly higher than control SHR. Overall, the hypotensive and hypoglycemic effects of HO-1 induction could contribute, in part, to the renal protection against diabetic insult.

HO-1 has been implicated in the modulation of renal injury in hypertensive animal models. For example, induction of HO-1 with hemin has also been shown by others to attenuate proteinuria and tubular atrophy in salt-sensitive angiotensin II hypertension [36]. Hemin also ameliorated renal injury in angiotensin II hypertension as it prevented the decrease in glomerular filtration rate and reduced proteinuria [37]. In SHR, we have recently shown that induction of HO-1 with CoPP reduced proteinuria when compared to Wistar Kyoto rats (WKY), whereas inhibition of HO with stannous mesoporphyrin further increased blood pressure and proteinuria and blocked the ability of CoPP to reduce blood pressure and proteinuria in SHR. In diabetes, HO-1 could also play a role in preserving renal function and morphology. For example, induction of diabetes with streptozotocin produced a marked degree of renal impairment in HO-2 knockout mice compared to control [38]. Furthermore, induction of HO-1 with CoPP prevented the elevation in plasma creatinine levels and acute tubular damage in diabetic HO-2 knockout mice whereas inhibition of HO with tin mesoporphyrin exacerbated the increase

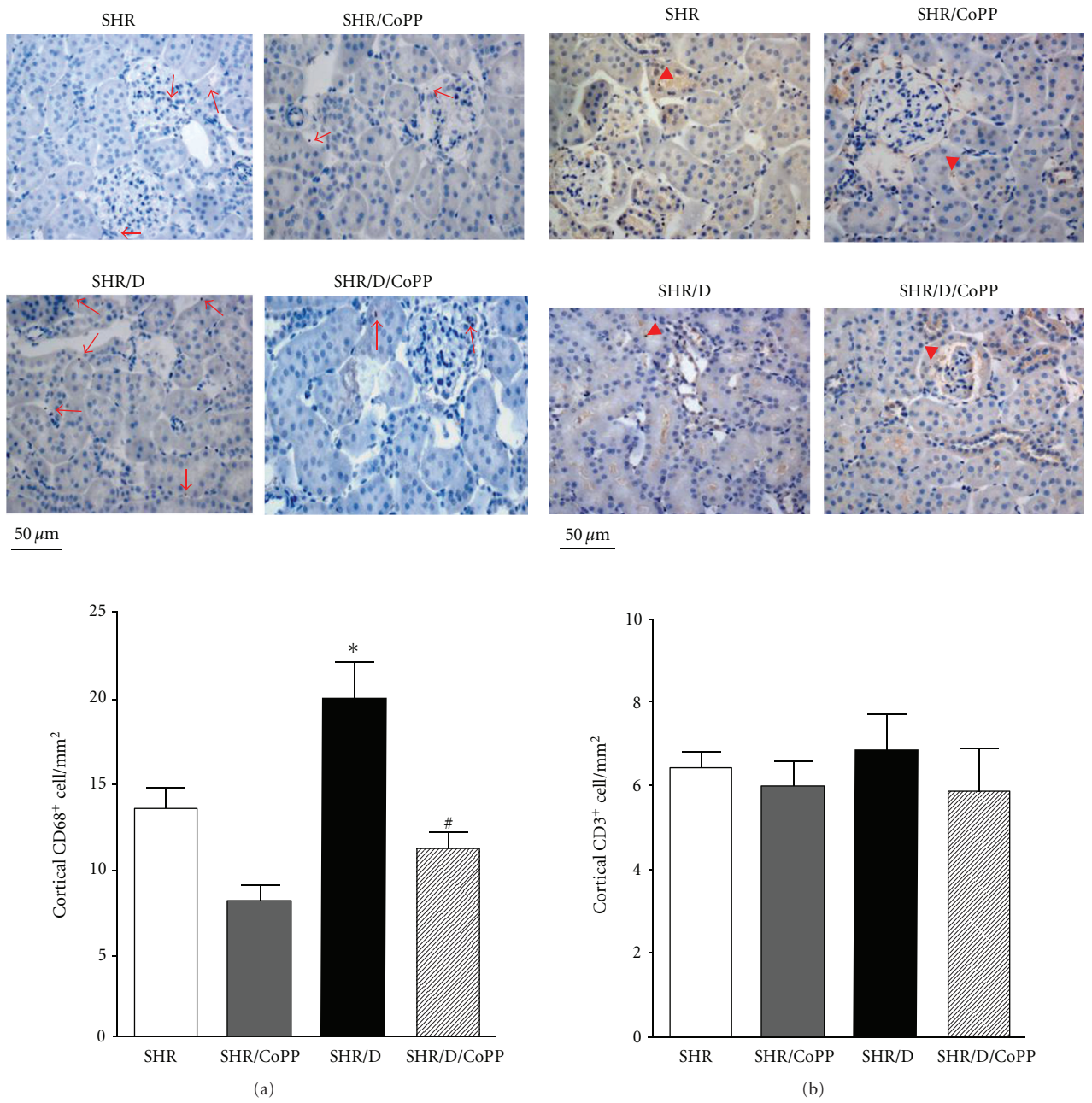

Figure 5: Representative images and average number of CD68-positive and CD3-positive cells (C) to assess monocytes/macrophages and T-cell infiltration, respectively, per 1 mm^2 in the kidney cortex of control and diabetic SHR with or without CoPP treatment ($n = 5$, *indicates significant difference from control SHR, #indicates significant difference from diabetic SHR).

in plasma creatinine and tubular damage in diabetic HO-2 knockout mice [38]. Consistent with these observations, the coexistence of hypertension and diabetes in diabetic SHR exaggerated the degree of renal injury as manifested by increased albumin and creatinine excretion and induction of HO-1 with CoPP reduced these changes.

Although induction of diabetes with streptozotocin in Sprague Dawley rats does not have extensive fibrosis as detected by histological staining [28], Saleh et al. recently demonstrated that glomerular TGF-β, an early marker of fibrosis increased in streptozotocin-induced diabetic rats [39]. Others have shown that that overexpression of glomerular TGF-β1 in diabetes contributes to glomerular basement membrane thickening and fibrosis [40], and inhibition of TGF-β prevents kidney fibrosis in experimental diabetes [41] suggesting an important role of TGF-β in the progression of kidney fibrosis during diabetes. Previous studies have demonstrated that induction of HO-1 with hemin reduced the overexpression of osteopontin and TGF-β, the hallmarks of tubulointerstitial injury in salt-sensitive

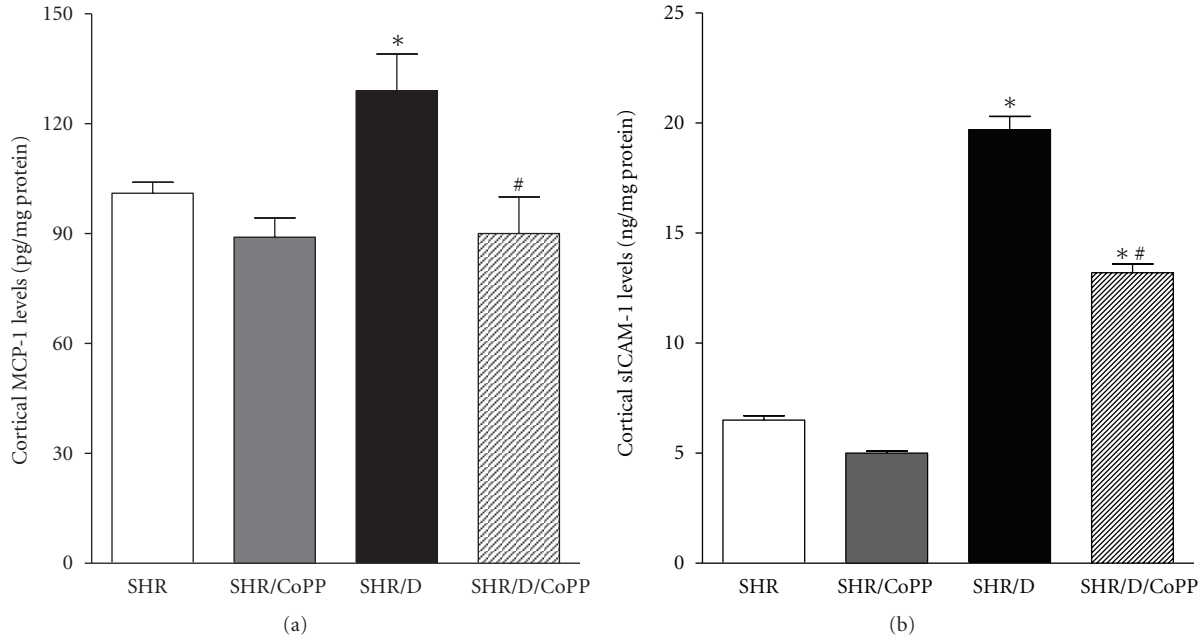

Figure 6: Renal cortical MCP-1 (a) and sICAM-1 (b) levels in control and diabetic SHR with or without CoPP treatment ($n = 8$, * indicates significant difference from control SHR, # indicates significant difference from diabetic SHR).

angiotensin II hypertension suggesting a role of HO-1 induction against renal fibrosis in hypertension [36]. In our study, the coexistence of hypertension and diabetes in diabetic SHR also exaggerated the degree of renal fibrosis as reflected by increased renal TGF-β1 staining, collagen IV deposition, and urinary collagen excretion compared to control SHR. The incidence of renal fibrosis was significantly reduced with CoPP treatment suggesting that HO-1 induction protects the kidney from diabetic-induced renal damage and fibrosis.

Increased oxidative stress is involved in the development of diabetic renal injury, and overexpression of HO-1 has previously been shown to decrease oxidative stress in diabetic animals [38, 42, 43]. Thus, the induction of HO-1 could provide cellular protection against oxidative insult during diabetes. In SHR, elevated oxidative stress and inflammatory markers not only accentuate oxidative damage but also impair the insulin signaling [35]. In streptozotocin-induced diabetic SHR, upregulation of HO-1 with stannous chloride was associated with a concomitant decrease in renal superoxide levels [24]. HO-1 upregulation by CoPP attenuated diabetic injury in nonobese diabetic (NOD) mice, an animal model for type 1 diabetes, and this was associated with decreases in blood glucose and pancreatic superoxide [44]. HO-1 induction also reduces aortic superoxide generation via decreased NADPH oxidase activation in apolipoprotein E-deficient mice [45]. Consistent with previous reports, our study showed that induction of HO-1 decreased renal cortical NADPH oxidase activity and urinary TBARs and 8-OHdG excretion levels in diabetic SHR. These data support the conclusion that HO-1 induction inhibits renal NADPH oxidase activity and reduces markers of oxidative stress, which could be a mechanism to protect the kidney against diabetic-induced renal injury.

Clinically, inflammatory processes contribute to the progression of renal injury in patients with type 1 diabetes [14, 46]. MCP-1 and ICAM-1 have been identified as key players in monocyte/macrophage infiltration and leukocyte adhesion in diabetic animal models [47, 48]. Many factors contribute to the increase in ICAM-1 production during diabetes including hyperglycemia, shear stress, advanced glycation end products, and oxidative stress [46]. Blocking ICAM-1 signaling abrogated the infiltration of macrophages in kidneys from diabetic rats and decreased glomerular hypertrophy and interstitial fibrosis in ICAM-1-deficient mice [48, 49] indicating a potential role of ICAM-1 in the progression of renal injury during diabetes. MCP-1 is also a potent chemoattractant for monocytes/macrophages and increased MCP-1 production was associated with macrophage infiltration in the kidney of diabetic patients [50]. MCP-1 is involved in the progression of kidney injury in response to many factors such as high glucose, oxidative stress, and interleukin-1 [27, 51]. MCP-1-deficiency or blocking MCP-1 receptor in mice reduced kidney macrophage accumulation and decreased renal injury in diabetes [16, 52] underscoring the importance of this pathway in the pathogenesis of diabetic renal injury. We have previously shown that induction of HO-1 with CoPP decreased MCP-1 excretion, whereas inhibition of HO with stannous mesoporphyrin blocked the ability of CoPP to decrease MCP-1 in SHR [25]. In our current study, induction of HO-1 with CoPP decreased the activation of renal MCP-1 and sICAM-1 together with decreased kidney macrophage, but not T-cell infiltration in diabetic SHR. Similarly, isolated glomeruli from diabetic SHR had a significant elevation in NADPH oxidase activity and ERK phosphorylation, and these effects were reduced with CoPP treatment and

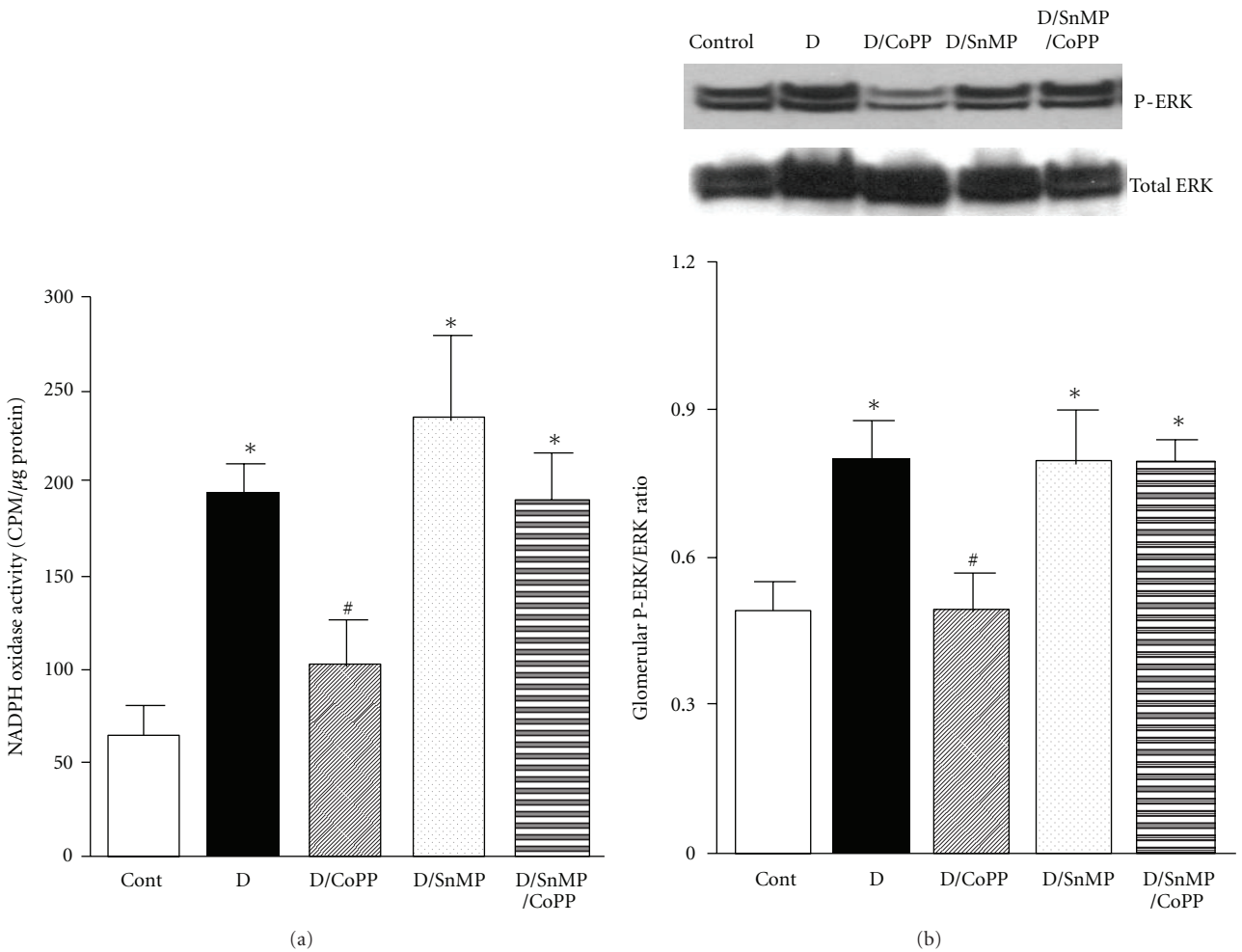

Figure 7: NADPH oxidase activity (a) and P-ERK/ERK ratio (b) in glomeruli isolated from control and diabetic SHR and incubated with or without CoPP and/or SnMP for 2 hours at 37°C ($n = 4$, Cont is an abbreviation for control SHR and D is an abbreviation for diabetic SHR, *indicates significant difference from control SHR, #indicates significant difference from diabetic SHR).

prevented by HO inhibition with SnMP. These data suggest that HO-1 upregulation reduces renal inflammation in diabetic SHR which could be an additional mechanism protecting the kidneys from diabetic insults.

In summary, HO-1 induction improves renal damage and decreases fibrosis in diabetic SHR. Based on data in the literature and our own studies, we postulate that hyperglycemia increases NADPH oxidase-induced oxidative stress which enhances the activation of proinflammatory cytokines stimulating immune cell infiltration and further increasing oxidative stress thereby exacerbating renal injury and fibrosis. The study highlights the potential therapeutic benefit of HO-1 induction to protect the kidney from diabetic renal injury via antioxidant and anti-inflammatory properties.

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References

[1] G. P. Forlenza and M. Rewers, "The epidemic of type 1 diabetes: what is it telling us?" *Current Opinion in Endocrinology, Diabetes and Obesity*, vol. 18, no. 4, pp. 248–251, 2011.

[2] Y. C. Wang, K. McPherson, T. Marsh, S. L. Gortmaker, and M. Brown, "Health and economic burden of the projected obesity trends in the USA and the UK," *The Lancet*, vol. 378, no. 9793, pp. 815–825, 2011.

[3] A. Barnett, "Prevention of loss of renal function over time in patients with diabetic nephropathy," *American Journal of Medicine*, vol. 119, no. 5, pp. S40–S47, 2006.

[4] C. Luft and H. Haller, "Hypertension-induced renal injury: is mechanically mediated interstitial inflammation involved?" *Nephrology Dialysis Transplantation*, vol. 10, no. 1, pp. 9–11, 1995.

[5] E. Ritz, R. E. Schmieder, and C. A. Pollock, "Renal protection in diabetes: lessons from ONTARGET®," *Cardiovascular Diabetology*, vol. 9, article 60, 2010.

[6] L. J. Dominguez, A. Galioto, A. Pineo et al., "Age, homocysteine, and oxidative stress: relation to hypertension and type 2 diabetes mellitus," *Journal of the American College of Nutrition*, vol. 29, no. 1, pp. 1–6, 2010.

[7] T. M. Paravicini and R. M. Touyz, "NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities," *Diabetes Care*, vol. 31, pp. S170–S180, 2008.

[8] S. Arora, R. Vaishya, P. K. Dabla, and B. Singh, "NAD(P)H oxidases in coronary artery disease," *Advances in Clinical Chemistry*, vol. 50, pp. 65–86, 2010.

[9] J.-Y. Moon, M. Tanimoto, T. Gohda et al., "Attenuating effect of angiotensin-(1-7) on angiotensin II-mediated NAD(P)H oxidase activation in type 2 diabetic nephropathy of KK-Ay/Ta mice," *American Journal of Physiology*, vol. 300, no. 6, pp. F1271–F1282, 2011.

[10] M. Satoh, S. Fujimoto, Y. Haruna et al., "NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy," *American Journal of Physiology*, vol. 288, no. 6, pp. F1144–F1152, 2005.

[11] A. Tojo, K. Asaba, and M. L. Onozato, "Suppressing renal NADPH oxidase to treat diabetic nephropathy," *Expert Opinion on Therapeutic Targets*, vol. 11, no. 8, pp. 1011–1018, 2007.

[12] Y. Gorin, K. Block, J. Hernandez et al., "Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney," *Journal of Biological Chemistry*, vol. 280, no. 47, pp. 39616–39626, 2005.

[13] N. Kashihara, Y. Haruna, V. K. Kondeti, and Y. S. Kanwar, "Oxidative stress in diabetic nephropathy," *Current Medicinal Chemistry*, vol. 17, no. 34, pp. 4256–4269, 2010.

[14] F. Y. Chow, D. J. Nikolic-Paterson, R. C. Atkins, and G. H. Tesch, "Macrophages in streptozotocin-induced diabetic nephropathy: potential role in renal fibrosis," *Nephrology Dialysis Transplantation*, vol. 19, no. 12, pp. 2987–2996, 2004.

[15] F. Y. Chow, D. J. Nikolic-Paterson, F. Y. Ma, E. Ozols, B. J. Rollins, and G. H. Tesch, "Monocyte chemoattractant protein-1-induced tissue inflammation is critical for the development of renal injury but not type 2 diabetes in obese db/db mice," *Diabetologia*, vol. 50, no. 2, pp. 471–480, 2007.

[16] F. Y. Chow, D. J. Nikolic-Paterson, E. Ozols, R. C. Atkins, B. J. Rollin, and G. H. Tesch, "Monocyte chemoattractant protein-1 promotes the development of diabetic renal injury in streptozotocin-treated mice," *Kidney International*, vol. 69, no. 1, pp. 73–80, 2006.

[17] F. Y. Chow, D. J. Nikolic-Paterson, E. Ozols, R. C. Atkins, and G. H. Tesch, "Intercellular adhesion molecule-1 deficiency is protective against nephropathy in type 2 diabetic db/db mice," *Journal of the American Society of Nephrology*, vol. 16, no. 6, pp. 1711–1722, 2005.

[18] D. Nguyen, F. Ping, W. Mu, P. Hill, R. C. Atkins, and S. J. Chadban, "Macrophage accumulation in human progressive diabetic nephropathy," *Nephrology*, vol. 11, no. 3, pp. 226–231, 2006.

[19] A. Rivero, C. Mora, M. Muros, J. García, H. Herrera, and J. F. Navarro-González, "Pathogenic perspectives for the role of inflammation in diabetic nephropathy," *Clinical Science*, vol. 116, no. 6, pp. 479–492, 2009.

[20] G. H. Tesch and A. K.H. Lim, "Recent insights into diabetic renal injury from the db/db mouse model of type 2 diabetic nephropathy," *American Journal of Physiology*, vol. 300, no. 2, pp. F301–F310, 2011.

[21] N. G. Abraham, J. Cao, D. Sacerdoti, X. Li, and G. Drummond, "Heme oxygenase: the key to renal function regulation," *American Journal of Physiology*, vol. 297, no. 5, pp. F1137–F1152, 2009.

[22] K. A. Nath, "Heme oxygenase-1: a provenance for cytoprotective pathways in the kidney and other tissues," *Kidney International*, vol. 70, no. 3, pp. 432–443, 2006.

[23] A. Burgess, M. Li, L. Vanella et al., "Adipocyte heme oxygenase-1 induction attenuates metabolic syndrome in both male and female obese mice," *Hypertension*, vol. 56, no. 6, pp. 1124–1130, 2010.

[24] J. Cao, G. Drummond, K. Inoue, K. Sodhi, X. Y. Li, and S. Omura, "Upregulation of heme oxygenase-1 combined with increased adiponectin lowers blood pressure in diabetic spontaneously hypertensive rats through a reduction in endothelial cell dysfunction, apoptosis and oxidative stress," *International Journal of Molecular Sciences*, vol. 9, no. 12, pp. 2388–2406, 2008.

[25] A. A. Elmarakby, J. Faulkner, S. P. Posey, and J. C. Sullivan, "Induction of hemeoxygenase-1 attenuates the hypertension and renal inflammation in spontaneously hypertensive rats," *Pharmacological Research*, vol. 62, no. 5, pp. 400–407, 2010.

[26] A. Jadhav, E. Torlakovic, and J. F. Ndisang, "Hemin therapy attenuates kidney injury in deoxycorticosterone acetate-salt hypertensive rats," *American Journal of Physiology*, vol. 296, no. 3, pp. F521–F534, 2009.

[27] M. A. Saleh, E. I. Boesen, J. S. Pollock, V. J. Savin, and D. M. Pollock, "Endothelin receptor A-specific stimulation of glomerular inflammation and injury in a streptozotocin-induced rat model of diabetes," *Diabetologia*, vol. 54, pp. 979–988, 2011.

[28] J. M. Sasser, J. C. Sullivan, J. L. Hobbs et al., "Endothelin A receptor blockade reduces diabetic renal injury via an anti-inflammatory mechanism," *Journal of the American Society of Nephrology*, vol. 18, no. 1, pp. 143–154, 2007.

[29] M. S. Mozaffari, B. Baban, J. Y. Liu, W. Abebe, J. C. Sullivan, and A. El-Marakby, "Mitochondrial complex I and NAD(P)H oxidase are major sources of exacerbated oxidative stress in pressure-overloaded ischemic-reperfused hearts," *Basic Research in Cardiology*, vol. 106, no. 2, pp. 287–297, 2011.

[30] J. C. Sullivan, K. Bhatia, T. Yamamoto, and A. A. Elmarakby, "Angiotensin (1-7) receptor antagonism equalizes angiotensin II-induced hypertension in male and female spontaneously hypertensive rats," *Hypertension*, vol. 56, no. 4, pp. 658–666, 2010.

[31] A. A. Elmarakby, J. Faulkner, M. Al-Shabrawey, M.-H. Wang, K. R. Maddipati, and J. D. Imig, "Deletion of soluble epoxide hydrolase gene improves renal endothelial function and reduces renal inflammation and injury in streptozotocin-induced type 1 diabetes," *American Journal of Physiology*, vol. 301, no. 5, pp. R1307–R1317, 2011.

[32] K. Nakajima, Y. Tohyama, S. Kohsaka, and T. Kurihara, "Protein kinase Cα requirement in the activation of p38 mitogen-activated protein kinase, which is linked to the induction of tumor necrosis factor α in lipopolysaccharide-stimulated microglia," *Neurochemistry International*, vol. 44, no. 4, pp. 205–214, 2004.

[33] C. H. Yeh, L. Sturgis, J. Haidacher et al., "Requirement for p38 and p44/p42 mitogen-activated protein kinases in RAGE-mediated nuclear factor-κB transcriptional activation and cytokine secretion," *Diabetes*, vol. 50, no. 6, pp. 1495–1504, 2001.

[34] A. A. Elmarakby, A. S. Ibrahim, J. Faulkner, M. S. Mozaffari, G. I. Liou, and R. Abdelsayed, "Tyrosine kinase inhibitor, genistein, reduces renal inflammation and injury in streptozotocin-induced diabetic mice," *Vascular Pharmacology*, vol. 55, no. 5-6, pp. 149–156, 2011.

[35] J. F. Ndisang, N. Lane, N. Syed, and A. Jadhav, "Up-regulating the heme oxygenase system with hemin improves insulin sensitivity and glucose metabolism in adult spontaneously hypertensive rats," *Endocrinology*, vol. 151, no. 2, pp. 549–560, 2010.

[36] A. Pradhan, M. Umezu, and M. Fukagawa, "Heme-oxygenase upregulation ameliorates angiotensin II-induced tubulointerstitial injury and salt-sensitive hypertension," *American Journal of Nephrology*, vol. 26, no. 6, pp. 552–561, 2007.

[37] T. Aizawa, N. Ishizaka, J. I. Taguchi et al., "Heme oxygenase-1 is upregulated in the kidney of angiotensin II-induced hypertensive rats: possible role in renoprotection," *Hypertension*, vol. 35, no. 3, pp. 800–806, 2000.

[38] A. I. Goodman, P. N. Chander, R. Rezzani et al., "Heme oxygenase-2 deficiency contributes to diabetes-mediated increase in superoxide anion and renal dysfunction," *Journal of the American Society of Nephrology*, vol. 17, no. 4, pp. 1073–1081, 2006.

[39] M. A. Saleh, J. S. Pollock, and D. M. Pollock, "Distinct actions of endothelin A-selective versus combined endothelin A/B receptor antagonists in early diabetic kidney disease," *Journal of Pharmacology and Experimental Therapeutics*, vol. 338, no. 1, pp. 263–270, 2011.

[40] S. Krag, J. R. Nyengaard, and L. Wogensen, "Combined effects of moderately elevated blood glucose and locally produced TGF-β1 on glomerular morphology and renal collagen production," *Nephrology Dialysis Transplantation*, vol. 22, no. 9, pp. 2485–2496, 2007.

[41] S. Chen, M. C. Iglesias-de la Cruz, B. Jim, S. W. Hong, M. Isono, and F. N. Ziyadeh, "Reversibility of established diabetic glomerulopathy by anti-TGF-β antibodies in db/db mice," *Biochemical and Biophysical Research Communications*, vol. 300, no. 1, pp. 16–22, 2003.

[42] D. Koya, K. Hayashi, M. Kitada, A. Kashiwagi, R. Kikkawa, and M. Haneda, "Effects of antioxidants in diabetes-induced oxidative stress in the glomeruli of diabetic rats," *Journal of the American Society of Nephrology*, vol. 14, no. 3, pp. S250–S253, 2003.

[43] S. C. Lee, S. H. Han, J. J. Li et al., "Induction of heme oxygenase-1 protects against podocyte apoptosis under diabetic conditions," *Kidney International*, vol. 76, no. 8, pp. 838–848, 2009.

[44] M. Li, S. Peterson, D. Husney et al., "Long-lasting expression of HO-1 delays progression of type I diabetes in NOD mice," *Cell Cycle*, vol. 6, no. 5, pp. 567–571, 2007.

[45] S. R. Datla, G. J. Dusting, T. A. Mori, C. J. Taylor, K. D. Croft, and F. Jiang, "Induction of heme oxygenase-1 in vivo suppresses NADPH oxidase-derived oxidative stress," *Hypertension*, vol. 50, no. 4, pp. 636–642, 2007.

[46] E. Galkina and K. Ley, "Leukocyte recruitment and vascular injury in diabetic nephropathy," *Journal of the American Society of Nephrology*, vol. 17, no. 2, pp. 368–377, 2006.

[47] J. L. Jin, H. L. Sun, K. K. Dong et al., "Colchicine attenuates inflammatory cell infiltration and extracellular matrix accumulation in diabetic nephropathy," *American Journal of Physiology*, vol. 297, no. 1, pp. F200–F209, 2009.

[48] S. Okada, K. Shikata, M. Matsuda et al., "Intercellular adhesion molecule-1-deficient mice are resistant against renal injury after induction of diabetes," *Diabetes*, vol. 52, no. 10, pp. 2586–2593, 2003.

[49] H. Sugimoto, K. Shikata, K. Hirata et al., "Increased expression of intercellular adhesion molecule-1 (ICAM-1) in diabetic rat glomeruli: glomerular hyperfiltration is a potential mechanism of ICAM-1 upregulation," *Diabetes*, vol. 46, no. 12, pp. 2075–2081, 1997.

[50] T. Wada, K. Furuichi, N. Sakai et al., "Up-regulation of monocyte chemoattractant protein-1 in tubulointerstitial lesions of human diabetic nephropathy," *Kidney International*, vol. 58, no. 4, pp. 1492–1499, 2000.

[51] C. G. Ihm, J. K. Park, S. P. Hong et al., "A high glucose concentration stimulates the expression of monocyte chemotactic peptide 1 in human mesangial cells," *Nephron*, vol. 79, no. 1, pp. 33–37, 1998.

[52] H. Kanamori, T. Matsubara, A. Mima et al., "Inhibition of MCP-1/CCR2 pathway ameliorates the development of diabetic nephropathy," *Biochemical and Biophysical Research Communications*, vol. 360, no. 4, pp. 772–777, 2007.

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Review Article

Oxidative Stress and Heme Oxygenase-1 Regulated Human Mesenchymal Stem Cells Differentiation

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This paper describes the effect of increased expression of HO-1 protein and increased levels of HO activity on differentiation of bone-marrow-derived human MSCs. MSCs are multipotent cells that proliferate and differentiate into many different cell types including adipocytes and osteoblasts. HO, the rate-limiting enzyme in heme catabolism, plays an important role during MSCs differentiation. HO catalyzes the stereospecific degradation of heme to biliverdin, with the concurrent release of iron and carbon monoxide. Upregulation of HO-1 expression and increased HO activity are essential for MSC growth and differentiation to the osteoblast lineage consistent with the role of HO-1 in hematopoietic stem cell differentiation. HO-1 participates in the MSC differentiation process shifting the balance of MSC differentiation in favor of the osteoblast lineage by decreasing PPARγ and increasing osteogenic markers such as alkaline phosphatase and BMP-2. In this paper, we define HO-1 as a target molecule in the modulation of adipogenesis and osteogenesis from MSCs and examine the role of the HO system in diabetes, inflammation, osteoporosis, hypertension, and other pathologies, a burgeoning area of research.

1. Background

1.1. Mesenchymal Stem Cells. Among cells bone-marrow-derived MSCs have attracted a great deal of attention in the past decade because of their high versatility. MSCs have shown promise in the treatment of cardiovascular disease in a series of animal models. Despite being a rare cell population, MSCs can be extensively expanded *in vitro* thus making them of potential use in the clinic [1]. Human MSCs derived from bone marrow are multipotent cells that differentiate and proliferate into many different cell types in various tissues [1–3]. Bone marrow mononuclear cells can be isolated with ease with a Ficoll-Paque PLUS density gradient. Human MSCs give rise to both osteoblastic and adipogenic lineages when cultured with specific differentiation media. The adipogenic media comprise of complete culture medium supplemented with DMEM-high glucose, FBS, insulin, dexamethasone, and indomethacin. The osteogenic media contain ascorbic acid (for appropriate collagen and extracellular matrix production) and β-glycerophosphate (for appropriate mineralization). Several studies demonstrated that an increase in the ROS leads to the elevation of the levels of inflammatory cytokines in adipose tissue. Oxidative stress is a major factor impairing MSCs function resulting in decreased osteogenesis in favor of adipogenesis. Whether MSCs differentiate into osteoblasts or adipocytes is due to multiple signaling pathways including those heavily influenced by HO-1 and -2 [4].

1.2. Role of HO during Bone Formation. Recent studies have shown that several growth factors including OGP enhance differentiation of MSCs to osteoblasts [5] and that EGF and OGP signaling pathways enhance osteoblast cell proliferation. Osteogenic growth peptide is a naturally occurring tetradecapeptide that is both an anabolic agent and a hematopoietic stimulator [6]. For example, OGP increases

osteoblast proliferation, AP activity, and matrix synthesis and mineralization. It prevents glucocorticoid-induced apoptosis and the subsequent bone remodeling alterations that are associated with steroids [7]. Thus, role of HO in the fluctuations of ROS and its effect on osteonectin levels and in MSC-derived osteoblasts will be described. Cytokines and HO activity have a regulatory role in MSCs microenvironment and hematopoiesis [8]. HO attenuates the overall production of ROS through its ability to degrade the prooxidant, heme, resulting in the production of carbon monoxide, biliverdin/bilirubin, and the release of free iron. These three products of heme degradation play an important role in signaling cascades, cell proliferation and differentiation. HO is the enzyme that catalyzes the rate-limiting step in the degradation of heme and exists in two forms: the inducible HO-1 form and the constitutive HO-2 form [4]. During fracture repair, activation of hypoxia-inducible factor (HIF)-1 and its target genes, VEGF and HO-1, regulate bone remodeling. Bone remodeling is a physiological process which includes bone resorption (by osteoclasts) and bone formation (by the osteoblasts) and requires coordination of three cell types, osteocytes, osteoblasts, and osteoclasts. During aging and in several pathologies, the rate of bone turnover increases, but this is characterized by an impaired osteoblastic bone formation compared to osteoclastic bone resorption caused by decreased number and activity of osteoblastic cells (Figure 1) [9–11].

This association suggests a role of HO-1 in bone metabolism. As previously stated, HO has strong implications in bone marrow stem cell differentiation [8, 12]. Most notably, HO-1 expression is increased during osteoblast stem cell development. This increase in HO-1 expression precedes an increase in alkaline phosphatase, bone morphogenic protein, osteonectin, and RUNX-2 mRNA [13]. The function of bone-specific alkaline phosphatase has been shown to be that of a biochemical indicator of bone turnover. Upregulation of HO-1 increased MSC-mediated osteoblasts with an associated decrease in adipocytes. OGP increased HO-1 levels [13]. OGP also lead to an increase in pAKT, an antiapoptotic protein, as well as an increase in eNOS and p-eNOS (Figure 2) [13]. Past research has demonstrated that eNOS is an enzyme expressed in osteoblasts that, when deficient, has been shown to lead to a significant reduction in bone formation in murine models [14]. Both eNOS and NO are stimulators of BMP-2 and increase differentiation of osteoblasts [15, 16]. Increased HO-1 expression has also been shown to increase alkaline phosphatase as well as DNA accumulation and mineralization when compared to osteoblasts not treated with OGP [13]. Osteoblasts cultured in hyperglycemic conditions showed reduced levels of bone BMP-2, osteonectin, pAMPK (a signaling molecule in osteoblasts), and eNOS. The reduction of these osteogenic proteins and enzymes was reversed by OGP which upregulated HO-1 with a subsequent increase in BMP-2, HO-1, eNOS, and pAMPK [13]. HO-1 expression and activity are essential for osteoblast differentiation from MSCs. Although basal levels of HO-1 and HO activity are necessary for osteoblast growth, an increase in HO-1 amplifies osteoblast differentiation. Additionally, HO-1 is required to increase pAKT, pAMPK, peNOS levels, and NO bioavailability [17, 18].

2. HO-1 and Oxidative Stress

An additional important association of HO-1 is this enzyme's influence on oxidative stress and reactive oxygen species (ROS). Hyperglycemia and certain cytokines result in an increase in ROS. HO-1 is inhibited in the presence of high glucose. High glucose suppressed HO-1 expression in both cell lines [19–21] and animal models [22–24].

Intracellularly, reduction-oxidation homeostasis is maintained by the balance between oxidants and antioxidants. Antioxidants including HO, superoxide dismutase, glutathione peroxidase, and catalase are endogenous. Exogenous antioxidants are often derived from food and include vitamins A, C, E, selenium, resveratrol, α-tocopherol, and β carotene [25–27]. When the natural balance between oxidants and antioxidants is altered, ROS can potentially damage cellular structures like DNA, proteins, and phospholipids. This process, called oxidative stress, is implicated in several neurodegenerative and metabolic diseases including obesity and type 2 diabetes. Moderate-to-severe obesity is associated with an increased risk for hypertension and insulin resistance in humans and animals [22, 28, 29]. The antioxidant effects of HO arise from its ability to degrade heme from destabilized heme proteins and from the production of biliverdin and bilirubin, products of HO with potent antioxidant properties. Heme is a prooxidant so, therefore, its breakdown is antioxidative. Although there appears to be a convincingly clear link between HO-1 and apoptosis, the specific mechanism through which HO-1 prevents apoptosis remains unclear [21, 30, 31]. It is likely that part of the anti-apoptotic role of HO-1 is based on its function as an antioxidant enzyme. However, the possibility remains that one or more of its products play additional roles in anti-apoptotic mechanisms. This association between a reduction in ROS with an increase in HO-1 expression was demonstrated by treating MSCs with CoPP, a strong inducer of HO-1. Exposure of the MSCs to CoPP is effective in decreasing ROS while high glucose concentrations increase ROS. A reduction in ROS permits the restoration of osteoblast markers, specifically induction of osteoprotegerin and osteocalcin [4].

The discovery that inhibition of HO-1 expression shifts mesenchymal stem cells to favor adipocytic cells at the expense of osteoblastic cells [4, 13] demonstrates that high glucose has an adipogenic potential and also that a direct link is present between HO-1 suppression and an increase in adipogenesis [13]. The exact mechanisms through which HO-1 affects adipocyte and osteoblast differentiation and protects against oxidative injury remain unclear. However, targeting HO-1 expression is a gateway to increasing osteoblast stem cell differentiation, decreasing oxidative stress, and to the attenuation of osteoporosis through the promotion of bone formation.

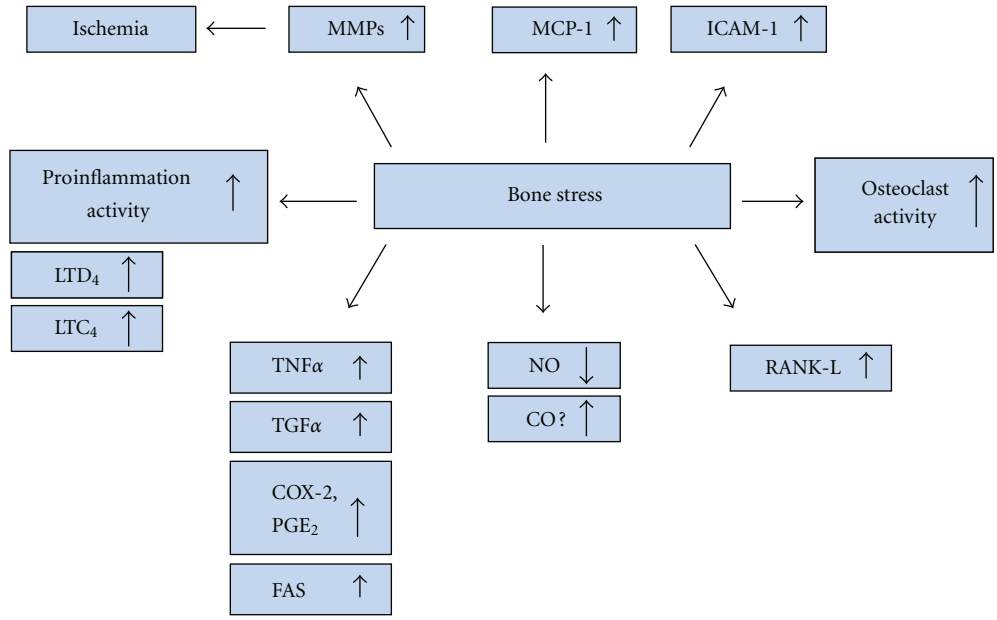

Figure 1: Diagram representing the adverse effects of stress on bone, leading to the releases of inflammatory molecules.

2.1. Introduction of PPARγ and TZD. There appears to be a strong link between HO-1 and PPARγ. PPARγ has been found to increase with the suppression of HO-1 [13]. The increase in PPARγ is associated with a reduction of HO-1 expression and coupled to a significant increase in adipogenesis [13]. PPARγ is a transcription factor that has been implicated in the development of osteoporosis. PPARγ is considered to be the master regulator of adipogenesis [32, 33], and its expression is believed to increase with age and in diabetics. PPARγ is abundantly expressed in mature adipocytes. Its levels are also increased in the livers of animals that have developed steatosis [34, 35]. Loss of the gene that encodes for PPARγ in embryonic fibroblasts results in the complete absence of adipogenesis in murine studies [36]. In addition, adipocyte differentiation can be stimulated by ectopic expression and activation of PPARγ. Conversely, the absence of this gene results in an increase in osteoblasts and bone mass [37]. PPARγ is expressed in human MSCs [38–40]. This evidence coupled with the fact that bone marrow fat increases and bone marrow osteoblasts decrease with age in both animals and humans [41, 42] demonstrates a possible strong link between PPARγ and osteoporosis. Furthermore, PPARγ has been found to decrease the levels of core-binding factor alpha (Cbfa1) and RUNX-2, two factors needed for osteoblast development. By leading to the differentiation of adipocytes over osteoblasts, activation of PPARγ subsequently leads to a decrease in bone mineral density and appears to be strongly correlated to the development and progression of osteoporosis. The stimulation of PPARγ occurs following upregulation of PPARγ expression by TZD [43]. TZDs provide diabetics with many benefits including lower blood glucose levels and reduced rates of atherosclerosis. However, these benefits come with multiple negative effects on bone such as inhibiting osteoblast formation [44–46] and inducing apoptosis of mature osteoblasts. Activation of PPARγ by TZDs also results in the suppression of many vital osteogenic transcription factors in both animal models and humans [47, 48]. The end result of these antiosteogenic effects is a decrease in bone mass. Furthermore, TZD use has been shown to increase the risk of fracture in postmenopausal women [49–51]. A large cohort study showed that the use of TZDs in type 2 diabetics increased the fracture risk in women over 65 years old. This increased fracture risk was seen after only one year of TZD use [51]. The association between TZD and fracture risk is likely due to a discrepancy between bone formation and resorption as rosiglitazone, a thiazolidinedione, has been found to decrease bone mass in the hip and decrease serum osteocalcin levels [52]. Aging leads to a decrease in HO-1 and an increase in bone marrow adiposity with bone loss and an increase in the expression of PPARγ and, ultimately, PPARγ [53]. Extensive research demonstrates that PPARγ plays a pivotal role in regulating MSC specification towards adipogenesis versus osteogenesis and is instrumental in governing both bone mineral density and osteoporosis.

2.2. HO-1 and CYP-450. HO-1 induction increased the levels of cytochrome P450- (P450-) derived epoxyeicosatrienoic acids (EETs), which further decreased PPARγ but increased osteoblasts. We examined if EET levels regulate the MSC-derived adipocytes. Expression of FAS and PPARγ levels was significantly ($P < 0.05$) increased in preadipocyte (14 days of MSC-derived adipocyte differentiation), and conversely pACC and β-catenin were decreased ($P < 0.05$) in preadipocyte. The increase in FAS and PPARγ in preadipocyte was suppressed by the EET. In contrast, the EET treatments significantly increased both pACC and β-catenin compared to cell treated with vehicle solutions.

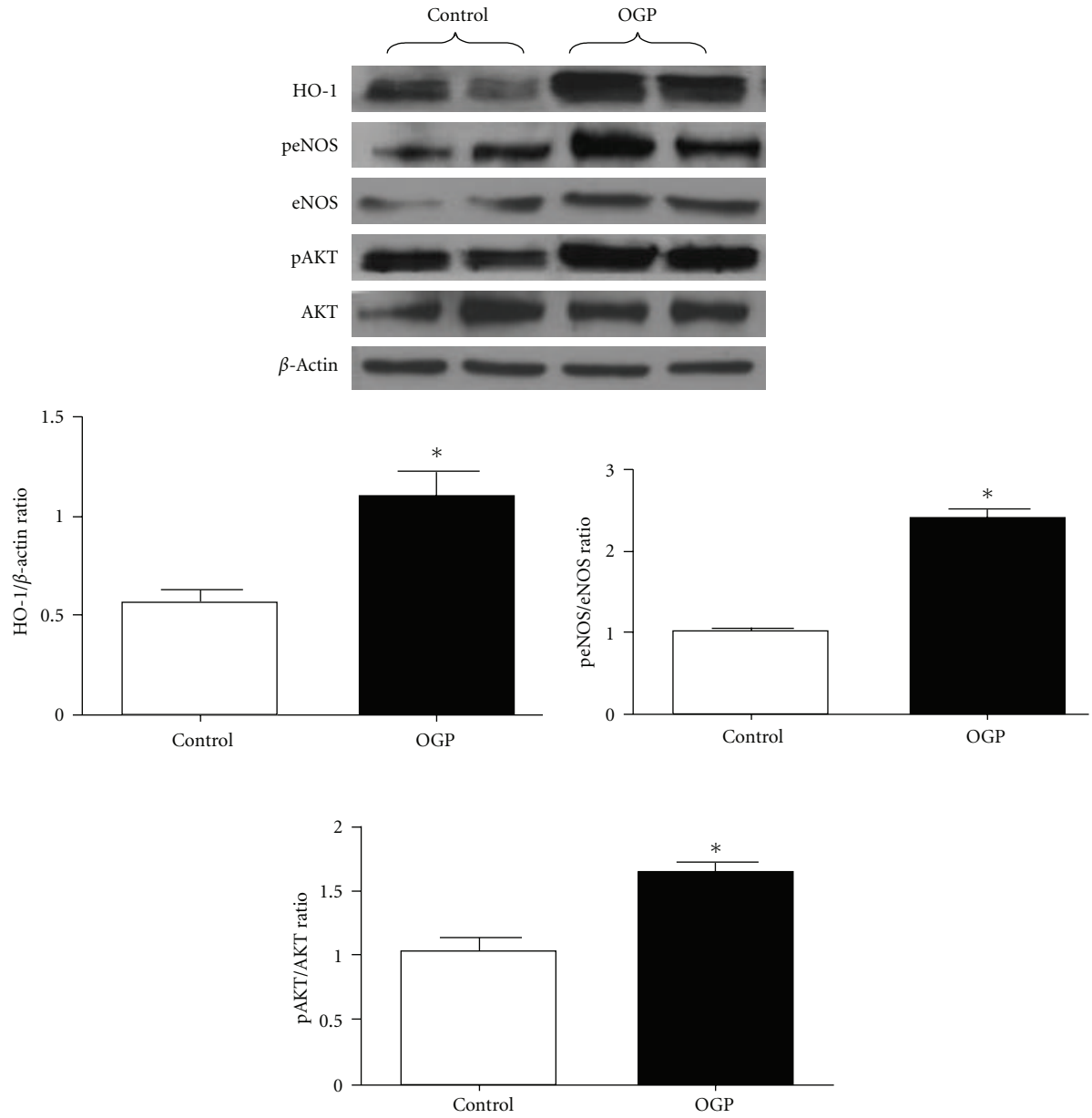

Figure 2: Effect of OGP on HO-1, peNOS/eNOS, and pAKT/AKT proteins expression after 21 days of osteoblast differentiation. Quantitative densitometry evaluation of the proteins ratio was determined. Data are expressed as means ± SEM of three independent experiments; $^*P < 0.05$ control versus OGP.

Further, MSCs-derived adipocyte exhibited a significantly higher expression of PPARγ, SREBP-1, and GLUT4 compared to MSCs-adipocyte grown in the presence of EET [54]. Adipocyte cultured in the absence of the EET agonist showed HO-1 levels that were decreased at day 10 and day 15 compared to adipocyte cultured in the presence of EET. In contrast PPARγ and C/EBPα protein pattern was the reverse by inhibition of HO-1. PPARγ and C/EBPα levels were significantly increased, while Wnt/β-catenin protein levels were decreased when compared with adipocytes cultured in the presence of EET. EET-agonist-treated cells showed an increase in β-catenin. HO-1 expression was greatly diminished during adipogenic differentiation of MSCs, while FAS and PPARγ levels increased. Ectopic expression and activation of PPARγ are sufficient to induce adipocyte differentiation [33]. FAS mRNA levels increased dramatically during 3T3-L1 adipocyte differentiation [55]. In agreement with previous studies, Wnt stimulation facilitates disruption of the axin-based complex [56, 57]. This results in a decrease in the phosphorylation of β-catenin, which enhances β-catenin accumulation and activation leading to an arrest in adipogenesis at the early progenitor stage through the blocking of PPARγ signaling [58, 59]. Furthermore, EETs inhibited MSC-derived stem cell adipogenesis presumably through activation of HO-1/Wnt/β-catenin and the expression of C/EBPα, a marker of adipocyte differentiation [54]. In the

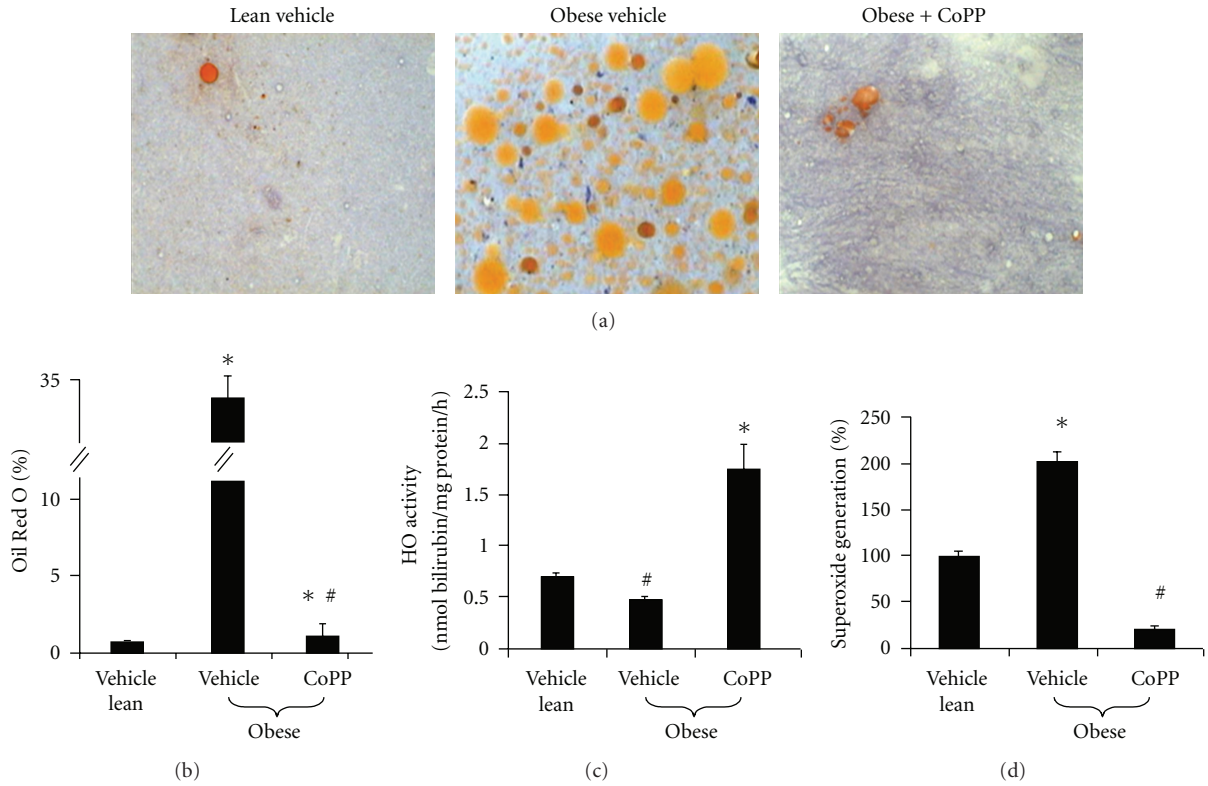

Figure 3: Evaluation of lipid content measured as percentage of Oil Red O staining. (a) Representative photographs demonstrating an increase in lipid droplets in the bone marrow of obese mice compared to lean mice and obese mice treated with CoPP. (b) Quantitative analysis of bone marrow lipid content showing a significant increase in Oil Red O staining in the obese vehicle. Please note bars on the graphs represent the mean ± SEM of three independent experiments; $^{*}P < 0.05$ versus vehicle-treated lean mice; $^{\#}P < 0.05$ versus vehicle-treated ob mice. (c) HO activity of bone marrow cells showing an increase in HO activity in both lean mice and obese mice treated with CoPP when compared to obese mice not receiving treatment. Please note bars on the graphs represent the mean ± SEM of four independent experiments; $^{\#}P < 0.05$ versus vehicle-treated lean mice; $^{*}P < 0.01$ versus vehicle-treated ob mice. (d) Superoxide generation in bone marrow cells is shown to be increased in obese mice and is lowest in the mice treated with CoPP implying an antioxidant effect of HO. Please note bars on the graphs represent the mean ± SEM of four independent experiments; $^{*}P < 0.05$ obese versus vehicle lean; $^{\#}P < 0.001$ versus vehicle-treated obese.

present study, the increase of C/EBPα was prevented by treatment of an EET agonist at day 10 and day 14. Vanella and coworkers provided direct evidence that EET-agonist-induced activation of HO-1 led to the increase in adiponectin and phosphorylation/inactivation of ACC and consequently decreased of FAS levels.

2.3. Diabetes and HO-1. Diabetes directly affects the integrity and functionality of bone in both humans and animals resulting in osteoporosis and an increase in adipogenesis [60–64]. Patients with diabetes frequently have a lower bone mineral density with associated osteopenia or osteoporosis. Because diabetic individuals commonly have decreased bone mass and bone mineral density, they are more susceptible to fractures and impaired bone healing. This correlation between diabetics and an increase in fractures has been well documented in the literature. A large prospective cohort study of over 32,000 postmenopausal women found that those with type 1 diabetes mellitus were 12 times more likely to experience hip fractures than those women without type 1 diabetes. Women with type 2 diabetes had a 1.7-fold increase in hip fractures when compared to women without this disease [65]. Prevention at decreasing osteoporotic fractures in patients with diabetes can involve strict glucose control, prevention and treatment of vascular complications, regular exercise, and fall prevention [66]. Additionally, biochemical markers of bone turnover are negatively affected in diabetics [67]. One such biomechanical marker, osteocalcin, has been found to be decreased in patients with diabetes [68]. However, the exact pathogenesis of the reduction in bone mass seen in diabetics is unknown. Also, the effect of osteoblast stem cell differentiation under high glucose conditions has not been fully elucidated although, as previously stated, strong evidence supports the belief that hyperglycemic environments decrease osteoblast differentiation and increase adipocyte differentiation [4, 13]. Upregulation of HO-1 expression in obesity and type 2 diabetes results in a decrease in visceral and subcutaneous fat content, improved insulin sensitivity, and increased insulin receptor phosphorylation [22, 69–71]. MRI studies showed that upregulation of HO-1 decreased adiposity and adipocyte hypertrophy [17, 70]. The decrease in HO-1 expression was

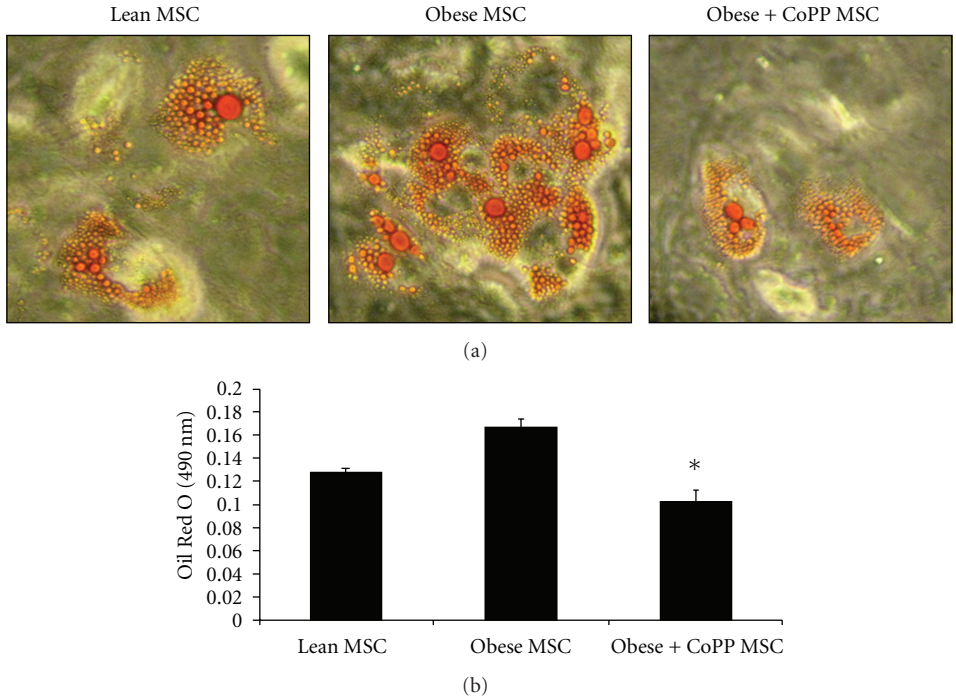

Figure 4: Effect of CoPP on MSC-derived adipogenesis. Adipogenesis was measured as the relative absorbance of Oil Red O at day 10. (a) Representative pictures demonstrating an increase in lipid droplets in the MSC of obese mice compared to lean mice and obese mice treated with CoPP. (b) Quantitative analysis of adipogenesis showing a significant increase in Oil Red O staining in the obese MSC-derived adipocytes. Data are expressed as mean ± SE ($^*P < 0.01$ obese versus CoPP). Note: unpublished data.

associated with an impairment in the mesenchymal stem cells production of adiponectin and increased adipogenesis [13, 22]. In addition, HO-1 gene expression has a differential effect on osteoblasts and adipocyte cell proliferation and differentiation [4, 13]. EETs administration decreased adiposity and insulin resistance in mice and rat models of obesity and diabetes via an increase in HO-1 gene expression and signaling cascade including the activation of AMPK and pAKT [72–74]. Sacerdoti and coworkers [75, 76] studied the interactions between HO-1 gene expression and EETs in vitro and showed that EETs induce HO-1 protein and HO activity. Human stromal-mesenchymal stem cells express CYP450 monooxygenase and form EETs and 20-HETE [8]. Not only 20-HETE but other eicosanoids are correlated with the progression of diabetes [77, 78] as well.

MSCs have the ability to metabolize arachidonic acid to HETE at comparable levels to endothelial cells. Additionally, the EET agonist (NUDSA) inhibited soluble epoxide hydrolase (sEH) and reduced the rate of body weight gain in obese mice which was accompanied by an increase in HO-1 expression [73, 79]. EET crosstalks with HO-1 on the decrease of adipogenesis [54]. The ability of hyperglycemic conditions and diabetes to activate adipocytes to undergo adipogenic differentiation appears to be strongly influenced on the suppression of HO-1. Therefore, it seems that osteoblast differentiation under hyperglycemic conditions decreases secondary to this suppression of HO-1. In addition, high levels of glucose lead to an increase in reactive oxygen species which have been shown to block BMP-2, osteonectin, osteoprotegerin and osteocalcin [4]. A 2010 study used osteoblast-like MG63 cells to demonstrate that hyperglycemic conditions significantly suppressed cell growth, mineralization, and expression of multiple osteoblastic markers (RUNX-2, osteocalcin, osteonectin, type 1 collagen). These high glucose conditions simultaneously stimulated the expression of PPARγ, adipocyte fatty-acid-binding protein (aP2), resistin, and adipsin, all markers of adipogenesis [4].

An additional unpublished study by the authors provides evidence that obese mice have a significant increase in bone marrow adipocytes when compared to both lean mice and obese mice treated with the potent HO-1 inducer CoPP (Figures 3(a), 3(b), and 4). The HO activity in the bone marrow was significantly lower in obese mice compared to lean mice. However, CoPP treatment did increase the HO activity in obese mice (Figure 3(c)). Lastly, superoxide levels were increased in obese mice compared to lean mice. CoPP treatment to obese mice decreased these elevated superoxide levels (Figure 3(d)).

Furthermore, hyperglycemia has been shown to promote cell death in many cell types such as endothelial and mesangial cells [19, 80, 81]. Oxidative stress is a consequence of hyperglycemia that can induce apoptosis via signaling and/or outright molecular damage. In streptozotocin-(STZ-) induced diabetic rats, symptoms of oxidative stress, including increases in cellular heme and apoptosis, were

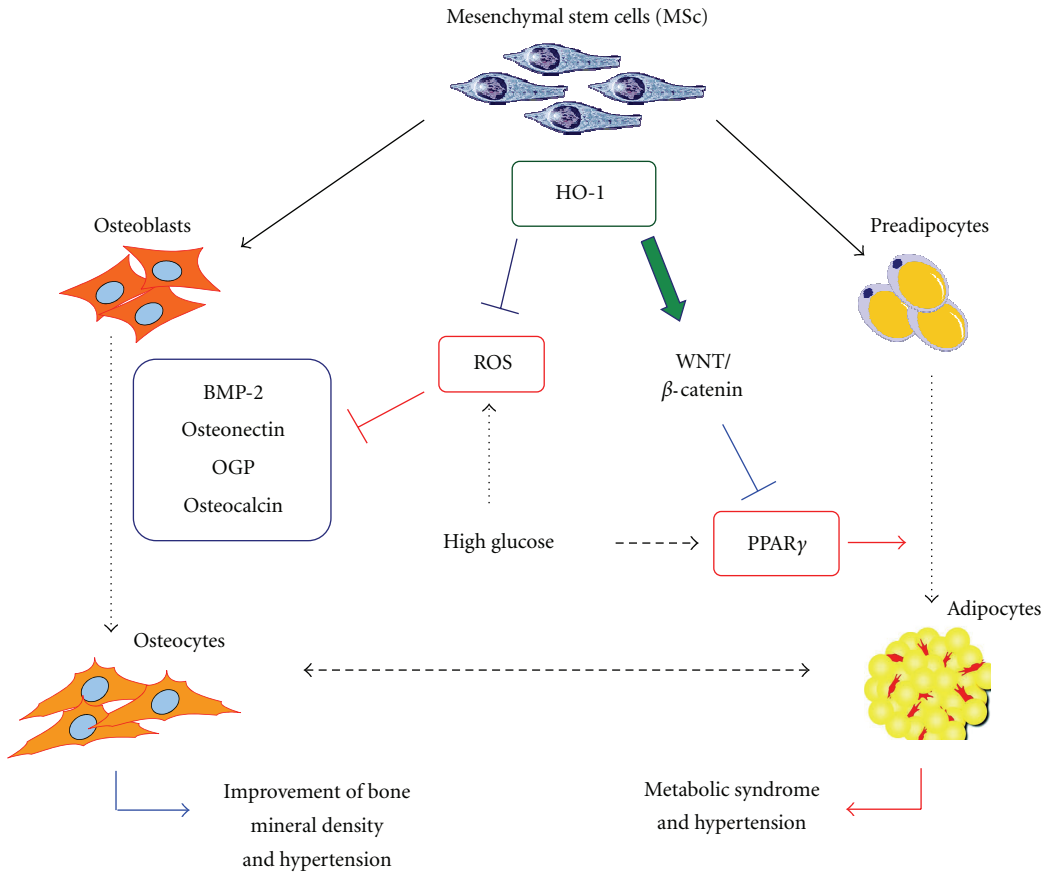

Figure 5: Schematic demonstrating the interplay between HO-1, hyperglycemia, ROS, and PPAR-γ on the regulation of osteoblast and adipocyte differentiation from mesenchymal stem cells.

found to be reversed by upregulation of HO-1 [23]. Elevated glucose levels cause glucose oxidation which leads to an increase of ROS in endothelial cells. This formation of ROS is believed to be the major factor in endothelial dysfunction such as abnormalities in cell cycling and delayed replication. However, these abnormalities are able to be reversed by antioxidant agents and an increased expression of antioxidant enzymes. Overexpression of HO-1 has been shown to make cells resistant to oxidative stress-causing agents while enhancing cell growth and angiogenesis. Such findings further demonstrate the important cytoprotective and antioxidant role of HO-1. Strong evidence exists that diabetic-induced hyperglycemia has a substantial impact on the development and progression of osteoporosis. The stimulation of osteoblast apoptosis as well as the decrease in osteoblast and increase in adipocyte differentiation all contribute to the decreased bone mineral density observed in individuals with diabetes.

2.4. Future Perspectives. Osteoporosis is a complex condition with significant morbidity, characterized by low bone mass, increased fragility, and fracture risk. Although mechanisms are unclear, there is significant evidence showing the interplay between HO-1, PPARγ, oxidative stress, and hyperglycemia. All have a role in the regulation of mesenchymal stem cell differentiation and the development of osteoporosis (Figure 5). Several studies have shown that HO-1 plays a considerable role in reducing oxidative stress, cellular apoptosis, increasing osteoblast differentiation, while simultaneously suppressing adipocyte differentiation from mesenchymal stem cells. HO-1 upregulation causes an increase in alkaline phosphatase and BMP-2 expression and a decrease in PPARγ expression, increasing bone formation and decreasing adipocyte differentiation. Conversely, HO-1 suppression upregulates PPARγ and increases adipogenesis, worse in diabetics, with reduced bone mineral density. Pharmacological and genetic approaches to deliver HO-1 or products of heme degradation remain to be elucidated for the causes, treatment, and prevention of osteoporosis.

Abbreviations

HO-1: Heme oxygenase-1
MSCs: Mesenchymal stem cells
PPARγ: Peroxisome proliferator-activated receptor gamma
GSK3β: Glycogen synthase kinase 3β
aP2: Fatty-acid-binding protein 4 (FABP4)
EGF: Endothelial growth factor
Wnts: Wingless-type

C/EBPα: Adipogenic transcription factors CCAAT/enhancer binding protein α
ROS: Reactive oxygen species
NO: Nitric oxide
BMP-2: Bone morphogenic protein-2
RUNX-2: Runt-related transcription factor 2
OGP: Osteogenic growth peptide
P450: Cytochrome P450
EET: Epoxyeicosatrienoic acids
TZD: Thiazolidinediones
CoPP: Co-protoporphyrin IX
SnMP: Tin-mesoporphyrin IX
AP: Alkaline phosphatase
VEGF: Vascular endothelial growth factor.

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References

[1] M. F. Pittenger, A. M. Mackay, S. C. Beck et al., "Multilineage potential of adult human mesenchymal stem cells," *Science*, vol. 284, no. 5411, pp. 143–147, 1999.

[2] G. Ferrari, G. Cusella-De Angelis, M. Coletta et al., "Muscle regeneration by bone marrow-derived myogenic progenitors," *Science*, vol. 279, no. 5356, pp. 1528–1530, 1998.

[3] I. Barbagallo, D. Tibullo, M. Di Rosa et al., "A cytoprotective role for the heme oxygenase-1/CO pathway during neural differentiation of human mesenchymal stem cells," *Journal of Neuroscience Research*, vol. 86, no. 9, pp. 1927–1935, 2008.

[4] I. Barbagallo, A. Vanella, S. J. Peterson et al., "Overexpression of heme oxygenase-1 increases human osteoblast stem cell differentiation," *Journal of Bone and Mineral Metabolism*, vol. 28, no. 3, pp. 276–288, 2010.

[5] Z. X. Chen, M. Chang, Y. L. Peng et al., "Osteogenic growth peptide C-terminal pentapeptide [OGP(10–14)] acts on rat bone marrow mesenchymal stem cells to promote differentiation to osteoblasts and to inhibit differentiation to adipocytes," *Regulatory Peptides*, vol. 142, no. 1-2, pp. 16–23, 2007.

[6] A. Spreafico, B. Frediani, C. Capperucci et al., "Osteogenic growth peptide effects on primary human osteoblast cultures: potential relevance for the treatment of glucocorticoid-induced osteoporosis," *Journal of Cellular Biochemistry*, vol. 98, no. 4, pp. 1007–1020, 2006.

[7] R. S. Weinstein, J. R. Chen, C. C. Powers et al., "Promotion of osteoclast survival and antagonism of bisphosphonate-induced osteoclast apoptosis by glucocorticoids," *The Journal of Clinical Investigation*, vol. 109, no. 8, pp. 1041–1048, 2002.

[8] N. G. Abraham, "Molecular regulation—biological role of heme in hematopoiesis," *Blood Reviews*, vol. 5, no. 1, pp. 19–28, 1991.

[9] S. Khosla and B. L. Riggs, "Pathophysiology of age-related bone loss and osteoporosis," *Endocrinology and Metabolism Clinics of North America*, vol. 34, no. 4, pp. 1015–1030, 2005.

[10] S. C. Manolagas and A. M. Parfitt, "What old means to bone," *Trends in Endocrinology and Metabolism*, vol. 21, no. 6, pp. 369–374, 2010.

[11] E. S. Strotmeyer, J. A. Cauley, A. V. Schwartz et al., "Nontraumatic fracture risk with diabetes mellitus and impaired fasting glucose in older white and black adults: the health, aging, and body composition study," *Archives of Internal Medicine*, vol. 165, no. 14, pp. 1612–1617, 2005.

[12] N. G. Abraham, J. D. Lutton, and R. D. Levere, "Heme metabolism and erythropoiesis in abnormal iron states: role of δ-aminolevulinic acid synthase and heme oxygenase," *Experimental Hematology*, vol. 13, no. 8, pp. 838–843, 1985.

[13] L. Vanella, D. H. Kim, D. Asprinio et al., "HO-1 expression increases mesenchymal stem cell-derived osteoblasts but decreases adipocyte lineage," *Bone*, vol. 46, no. 1, pp. 236–243, 2010.

[14] K. E. Armour, K. J. Armour, M. E. Gallagher et al., "Defective bone formation and anabolic response to exogenous estrogen in mice with targeted disruption of endothelial nitric oxide synthase," *Endocrinology*, vol. 142, no. 2, pp. 760–766, 2001.

[15] G. Mundy, R. Garrett, S. Harris et al., "Stimulation of bone formation in vitro and in rodents by statins," *Science*, vol. 286, no. 5446, pp. 1946–1949, 1999.

[16] I. R. Garrett, G. Gutierrez, and G. R. Mundy, "Statins and bone formation," *Current Pharmaceutical Design*, vol. 7, no. 8, pp. 715–736, 2001.

[17] S. J. Peterson, D. H. K. Kim, M. Li et al., "The L-4F mimetic peptide prevents insulin resistance through increased levels of HO-1, pAMPK, and pAKT in obese mice," *Journal of Lipid Research*, vol. 50, no. 7, pp. 1293–1304, 2009.

[18] A. L. Kruger, S. J. Peterson, M. L. Schwartzman et al., "Up-regulation of heme oxygenase provides vascular protection in an animal model of diabetes through its antioxidant and anti-apoptotic effects," *Journal of Pharmacology and Experimental Therapeutics*, vol. 319, no. 3, pp. 1144–1152, 2006.

[19] S. Quan, P. M. Kaminski, L. Yang et al., "Heme oxygenase-1 prevents superoxide anion-associated endothelial cell sloughing in diabetic rats," *Biochemical and Biophysical Research Communications*, vol. 315, no. 2, pp. 509–516, 2004.

[20] S. H. Chang, J. Garcia, J. A. Melendez, M. S. Kilberg, and A. Agarwal, "Haem oxygenase 1 gene induction by glucose deprivation is mediated by reactive oxygen species via the mitochondrial electron-transport chain," *Biochemical Journal*, vol. 371, no. 3, pp. 877–885, 2003.

[21] N. G. Abraham, T. Kushida, J. McClung et al., "Heme oxygenase-1 attenuates glucose-mediated cell growth arrest and apoptosis in human microvessel endothelial cells," *Circulation Research*, vol. 93, no. 6, pp. 507–514, 2003.

[22] M. Li, D. H. Kim, P. L. Tsenovoy et al., "Treatment of obese diabetic mice with a heme oxygenase inducer reduces visceral and subcutaneous adiposity, increases adiponectin levels, and improves insulin sensitivity and glucose tolerance," *Diabetes*, vol. 57, no. 6, pp. 1526–1535, 2008.

[23] S. Turkseven, A. Kruger, C. J. Mingone et al., "Antioxidant mechanism of heme oxygenase-1 involves an increase in superoxide dismutase and catalase in experimental diabetes," *American Journal of Physiology*, vol. 289, no. 2, pp. H701–H707, 2005.

[24] M. A. Di Noia, S. Van Driesche, F. Palmieri et al., "Heme oxygenase-1 enhances renal mitochondrial transport carriers and cytochrome c oxidase activity in experimental diabetes," *The Journal of Biological Chemistry*, vol. 281, no. 23, pp. 15687–15693, 2006.

[25] M. A. Puertollano, M. A. de Pablo, and G. Álvarez de Cienfuegos, "Anti-oxidant properties of N-acetyl-L-cysteine do not improve the immune resistance of mice fed dietary lipids to Listeria monocytogenes infection," *Clinical Nutrition*, vol. 22, no. 3, pp. 313–319, 2003.

[26] L. Mueller and V. Boehm, "Antioxidant activity of β-carotene compounds in different in vitro assays," *Molecules*, vol. 16, no. 2, pp. 1055–1069, 2011.

[27] M. Tiedge, S. Lortz, J. Drinkgern, and S. Lenzen, "Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells," *Diabetes*, vol. 46, no. 11, pp. 1733–1742, 1997.

[28] B. Larsson, "Obesity, fat distribution and cardiovascular disease," *International Journal of Obesity*, vol. 15, supplement 2, pp. 53–57, 1991.

[29] J. E. Hall, "The kidney, hypertension, and obesity," *Hypertension*, vol. 41, no. 3, pp. 625–633, 2003.

[30] A. Pileggi, R. Damaris Molano, T. Berney et al., "Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation," *Diabetes*, vol. 50, no. 9, pp. 1983–1991, 2001.

[31] S. Brouard, L. E. Otterbein, J. Anrather et al., "Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis," *Journal of Experimental Medicine*, vol. 192, no. 7, pp. 1015–1025, 2000.

[32] S. E. Schadinger, N. L. R. Bucher, B. M. Schreiber, and S. R. Farmer, "PPARγ2 regulates lipogenesis and lipid accumulation in steatotic hepatocytes," *American Journal of Physiology*, vol. 288, no. 6, pp. E1195–E1205, 2005.

[33] J. Liu and S. R. Farmer, "Regulating the balance between peroxisome proliferator-activated receptor γ and β-catenin signaling during adipogenesis: a glycogen synthase kinase 3β phosphorylation-defective mutant of β-catenin inhibits expression of a subset of adipogenic genes," *The Journal of Biological Chemistry*, vol. 279, no. 43, pp. 45020–45027, 2004.

[34] O. Gavrilova, M. Haluzik, K. Matsusue et al., "Liver peroxisome proliferator-activated receptor gamma contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass," *The Journal of Biological Chemistry*, vol. 278, no. 36, pp. 34268–34276, 2003.

[35] K. Matsusue, M. Haluzik, G. Lambert et al., "Liver-specific disruption of PPARγ in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes," *The Journal of Clinical Investigation*, vol. 111, no. 5, pp. 737–747, 2003.

[36] N. Kubota, Y. Terauchi, H. Miki et al., "PPARγ mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance," *Molecular Cell*, vol. 4, no. 4, pp. 597–609, 1999.

[37] T. Akune, S. Ohba, S. Kamekura et al., "PPARγ insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors," *The Journal of Clinical Investigation*, vol. 113, no. 6, pp. 846–855, 2004.

[38] B. Lecka-Czernik, I. Gubrij, E. J. Moerman et al., "Inhibition of Osf2/Cbfa1 expression and terminal osteoblast differentiation by PPARγ2," *Journal of Cellular Biochemistry*, vol. 74, no. 3, pp. 357–371, 1999.

[39] E. D. Rosen and B. M. Spiegelman, "PPARgamma: a nuclear regulator of metabolism, differentiation, and cell growth," *The Journal of Biological Chemistry*, vol. 276, no. 41, pp. 37731–37734, 2001.

[40] O. Braissant, F. Foufelle, C. Scotto, M. Dauça, and W. Wahli, "Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-α, -β, and -γ in the adult rat," *Endocrinology*, vol. 137, no. 1, pp. 354–366, 1996.

[41] S. G. Moore and K. L. Dawson, "Red and yellow marrow in the femur: age-related changes in appearance at MR imaging," *Radiology*, vol. 175, no. 1, pp. 219–223, 1990.

[42] J. M. Gimble, C. E. Robinson, X. Wu, and K. A. Kelly, "The function of adipocytes in the bone marrow stroma: an update," *Bone*, vol. 19, no. 5, pp. 421–428, 1996.

[43] M. Kawai, K. M. Sousa, O. A. MacDougald, and C. J. Rosen, "The many facets of PPARγ: novel insights for the skeleton," *American Journal of Physiology*, vol. 299, no. 1, pp. E3–E9, 2010.

[44] M. A. Sorocéanu, D. Miao, X. Y. Bai, H. Su, D. Goltzman, and A. C. Karaplis, "Rosiglitazone impacts negatively on bone by promoting osteoblast/osteocyte apoptosis," *Journal of Endocrinology*, vol. 183, no. 1, pp. 203–216, 2004.

[45] G. Mabilleau, A. Mieczkowska, and M. E. Edmonds, "Thiazolidinediones induce osteocyte apoptosis and increase sclerostin expression," *Diabetic Medicine*, vol. 27, no. 8, pp. 925–932, 2010.

[46] A. K. McDonough, R. S. Rosenthal, X. Cao, and K. G. Saag, "The effect of thiazolidinediones on BMD and osteoporosis," *Nature Clinical Practice Endocrinology and Metabolism*, vol. 4, no. 9, pp. 507–513, 2008.

[47] K. R. Shockley, O. P. Lazarenko, P. J. Czernik, C. J. Rosen, G. A. Churchill, and B. Lecka-Czernik, "PPARγ2 nuclear receptor controls multiple regulatory pathways of osteoblast differentiation from marrow mesenchymal stem cells," *Journal of Cellular Biochemistry*, vol. 106, no. 2, pp. 232–246, 2009.

[48] B. Lecka-Czernik, C. Ackert-Bicknell, M. L. Adamo et al., "Activation of peroxisome proliferator-activated receptor γ (PPARγ) by rosiglitazone suppresses components of the insulin-like growth factor regulatory system in vitro and in vivo," *Endocrinology*, vol. 148, no. 2, pp. 903–911, 2007.

[49] A. V. Schwartz, D. E. Sellmeyer, E. Vittinghoff et al., "Thiazolidinedione use and bone loss in older diabetic adults," *Journal of Clinical Endocrinology and Metabolism*, vol. 91, no. 9, pp. 3349–3354, 2006.

[50] B. Lecka-Czernik, "Bone as a target of type 2 diabetes treatment," *Current Opinion in Investigational Drugs*, vol. 10, no. 10, pp. 1085–1090, 2009.

[51] Z. A. Habib, S. L. Havstad, K. Wells, G. Divine, M. Pladevall, and L. K. Williams, "Thiazolidinedione use and the longitudinal risk of fractures in patients with type 2 diabetes mellitus," *Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 2, pp. 592–600, 2010.

[52] A. Grey, M. Bolland, G. Gamble et al., "The peroxisome proliferator-activated receptor-γ agonist rosiglitazone decreases bone formation and bone mineral density in healthy postmenopausal women: a randomized, controlled trial," *Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 4, pp. 1305–1310, 2007.

[53] M. Kawai and C. J. Rosen, "PPARγ: a circadian transcription factor in adipogenesis and osteogenesis," *Nature Reviews Endocrinology*, vol. 6, no. 11, pp. 629–636, 2010.

[54] L. Vanella, D. H. Kim, K. Sodhi et al., "Crosstalk between EET and HO-1 downregulates Bach1 and adipogenic marker expression in mesenchymal stem cell derived adipocytes," *Prostaglandins and Other Lipid Mediators*, vol. 96, no. 1–4, pp. 54–62, 2011.

[55] N. Moustaid and H. S. Sul, "Regulation of expression of the fatty acid synthase gene in 3T3-L1 cells by differentiation and triiodothyronine," *The Journal of Biological Chemistry*, vol. 266, no. 28, pp. 18550–18554, 1991.

[56] R. S. Jope and G. V. W. Johnson, "The glamour and gloom of glycogen synthase kinase-3," *Trends in Biochemical Sciences*,

vol. 29, no. 2, pp. 95–102, 2004.

[57] S. L. Etheridge, G. J. Spencer, D. J. Heath, and P. G. Genever, "Expression profiling and functional analysis of Wnt signaling mechanisms in mesenchymal stem cells," *Stem Cells*, vol. 22, no. 5, pp. 849–860, 2004.

[58] S. E. Ross, N. Hemati, K. A. Longo et al., "Inhibition of adipogenesis by Wnt signaling," *Science*, vol. 289, no. 5481, pp. 950–953, 2000.

[59] M. Okamura, H. Kudo, K. I. Wakabayashi et al., "COUP-TFII acts downstream of Wnt/β-catenin signal to silence PPARγ gene expression and repress adipogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 14, pp. 5819–5824, 2009.

[60] P. Tontonoz, E. Hu, R. A. Graves, A. I. Budavari, and B. M. Spiegelman, "mPPARγ2: tissue-specific regulator of an adipocyte enhancer," *Genes and Development*, vol. 8, no. 10, pp. 1224–1234, 1994.

[61] E. J. Hamilton, V. Rakic, W. A. Davis et al., "Prevalence and predictors of osteopenia and osteoporosis in adults with Type 1 diabetes," *Diabetic Medicine*, vol. 26, no. 1, pp. 45–52, 2009.

[62] J. L. Fowlkes, R. C. Bunn, L. Liu et al., "Runt-related transcription factor 2 (RUNX2) and RUNX2-related osteogenic genes are down-regulated throughout osteogenesis in type 1 diabetes mellitus," *Endocrinology*, vol. 149, no. 4, pp. 1697–1704, 2008.

[63] A. V. Schwartz, "Diabetes mellitus: does it affect bone?" *Calcified Tissue International*, vol. 73, no. 6, pp. 515–519, 2003.

[64] A. V. Schwartz, D. E. Sellmeyer, K. E. Ensrud et al., "Older women with diabetes have an increased risk of fracture: a prospective study," *Journal of Clinical Endocrinology and Metabolism*, vol. 86, no. 1, pp. 32–38, 2001.

[65] K. K. Nicodemus and A. R. Folsom, "Type 1 and type 2 diabetes and incident hip fractures in postmenopausal women," *Diabetes Care*, vol. 24, no. 7, pp. 1192–1197, 2001.

[66] N. K. Lee, H. Sowa, E. Hinoi et al., "Endocrine regulation of energy metabolism by the skeleton," *Cell*, vol. 130, no. 3, pp. 456–469, 2007.

[67] S. Yaturu, S. Humphrey, C. Landry, and S. K. Jain, "Decreased bone mineral density in men with metabolic syndrome alone and with type 2 diabetes," *Medical Science Monitor*, vol. 15, no. 1, pp. CR5–CR9, 2009.

[68] H. He, R. Liu, T. Desta, C. Leone, L. C. Gerstenfeld, and D. T. Graves, "Diabetes causes decreased osteoclastogenesis, reduced bone formation, and enhanced apoptosis of osteoblastic cells in bacteria stimulated bone loss," *Endocrinology*, vol. 145, no. 1, pp. 447–452, 2004.

[69] H. K. Dong, A. P. Burgess, M. Li et al., "Heme oxygenase-mediated increases in adiponectin decrease fat content and inflammatory cytokines tumor necrosis factor-α and interleukin-6 in Zucker rats and reduce adipogenesis in human mesenchymal stem cells," *Journal of Pharmacology and Experimental Therapeutics*, vol. 325, no. 3, pp. 833–840, 2008.

[70] A. Nicolai, M. Li, D. H. Kim et al., "Heme oxygenase-1 induction remodels adipose tissue and improves insulin sensitivity in obesity-induced diabetic rats," *Hypertension*, vol. 53, no. 3, pp. 508–515, 2009.

[71] S. J. Peterson, G. Drummond, D. H. Kim et al., "L-4F treatment reduces adiposity, increases adiponectin levels, and improves insulin sensitivity in obese mice," *Journal of Lipid Research*, vol. 49, no. 8, pp. 1658–1669, 2008.

[72] A. Burgess, M. Li, L. Vanella et al., "Adipocyte heme oxygenase-1 induction attenuates metabolic syndrome in both male and female obese mice," *Hypertension*, vol. 56, no. 6, pp. 1124–1130, 2010.

[73] K. Sodhi, K. Inoue, K. H. Gotlinger et al., "Epoxyeicosatrienoic acid agonist rescues the metabolic syndrome phenotype of HO-2-null mice," *Journal of Pharmacology and Experimental Therapeutics*, vol. 331, no. 3, pp. 906–916, 2009.

[74] J. Cao, K. Sodhi, K. Inoue et al., "Lentiviral-human heme oxygenase targeting endothelium improved vascular function in angiotensin II animal model of hypertension," *Human Gene Therapy*, vol. 22, no. 3, pp. 271–282, 2011.

[75] D. Sacerdoti, M. Bolognesi, M. Di Pascoli et al., "Rat mesenteric arterial dilator response to 11,12-epoxyeicosatrienoic acid is mediated by activating heme oxygenase," *American Journal of Physiology*, vol. 291, no. 4, pp. H1999–H2002, 2006.

[76] D. Sacerdoti, C. Colombrita, M. Di Pascoli et al., "11,12-Epoxyeicosatrienoic acid stimulates heme-oxygenase-1 in endothelial cells," *Prostaglandins and Other Lipid Mediators*, vol. 82, no. 1–4, pp. 155–161, 2007.

[77] M. L. Schwartzman, P. Iserovich, K. Gotlinger et al., "Profile of lipid and protein autacoids in diabetic vitreous correlates with the progression of diabetic retinopathy," *Diabetes*, vol. 59, no. 7, pp. 1780–1788, 2010.

[78] P. Luo and M.-H. Wang, "Eicosanoids, β-cell function, and diabetes," *Prostaglandins and Other Lipid Mediators*, vol. 95, no. 1–4, pp. 1–10, 2011.

[79] D. H. Kim, L. Vanella, K. Inoue et al., "Epoxyeicosatrienoic acid agonist regulates human mesenchymal stem cell-derived adipocytes through activation of HO-1-pAKT signaling and a decrease in PPARγ," *Stem Cells and Development*, vol. 19, no. 12, pp. 1863–1873, 2010.

[80] A. L. Kruger, S. Peterson, S. Turkseven et al., "D-4F induces heme oxygenase-1 and extracellular superoxide dismutase, decreases endothelial cell sloughing, and improves vascular reactivity in rat model of diabetes," *Circulation*, vol. 111, no. 23, pp. 3126–3134, 2005.

[81] R. Mishra, S. N. Emancipator, T. Kern, and M. S. Simonson, "High glucose evokes an intrinsic proapoptotic signaling pathway in mesangial cells," *Kidney International*, vol. 67, no. 1, pp. 82–93, 2005.

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Review Article

Novel Insights into the Vasoprotective Role of Heme Oxygenase-1

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Cardiovascular risk factors contribute to enhanced oxidative stress which leads to endothelial dysfunction. These events trigger platelet activation and their interaction with leukocytes and endothelial cells, thus contributing to the induction of chronic inflammatory processes at the vascular wall and to the development of atherosclerotic lesions and atherothrombosis. In this scenario, endogenous antioxidant pathways are induced to restrain the development of vascular disease. In the present paper, we will discuss the role of heme oxygenase (HO)-1 which is an enzyme of the heme catabolism and cleaves heme to form biliverdin and carbon monoxide (CO). Biliverdin is reduced enzymatically to the potent antioxidant bilirubin. Recent evidence supports the involvement of HO-1 in the antioxidant and antiinflammatory effect of cyclooxygenase(COX)-2-dependent prostacyclin in the vasculature. Moreover, the role of HO-1 in estrogen vasoprotection is emerging. Finally, possible strategies to develop novel therapeutics against cardiovascular disease by targeting the induction of HO-1 will be discussed.

1. Introduction

For many years, atherosclerosis was considered an age-related process characterized by the passive accumulation of lipids in the vessel wall. However, the most recent lines of evidence have clearly shown that it is a complex process in which multiple pathogenic factors contribute to trigger and sustain vessel wall damage, leading to myocardial infarction, stroke, and sudden death [1]. In particular, there is an increasing appreciation of atherosclerosis as a dynamic and progressive disease starting with endothelial dysfunction which may trigger platelet activation and their interaction with leukocytes and endothelial cells. This process may contribute to the induction of chronic inflammation at the vascular wall [2].

Several lines of evidence suggest that oxidative stress may promote endothelial dysfunction through increased production of reactive oxygen species (ROS). Increased levels of diverse ROS are produced in the vessel wall and they individually or in combination may contribute to the pathogenesis of vascular disease. Thus, increased lipid peroxidation has been identified as a key mechanism for the development of atherosclerosis and inflammatory vascular damage. In fact, intracellular oxidative signals may induce the expression of a selective set of vascular inflammatory genes thus linking oxidative stress and inflammation in atherogenesis [3, 4].

Endothelial cells generate several protective mediators to regulate the functions of underlying vascular smooth muscle cells and circulating cells [5]. Among them, cyclooxygenase (COX)-2-dependent prostacyclin (PGI_2) plays a central role [5]. COX-2 is among endothelial genes upregulated by steady laminar shear stress (LSS) [6], which characterizes "atherosclerotic lesion-protected areas" [7]. COX activity of the enzyme catalyzes the conversion of free arachidonic acid to prostaglandin $(PG)G_2$, which is then converted to PGH_2 through its peroxidase activity [8]. Endothelial cells may transform PGH_2 to a different array of the prostanoids (i.e., PGD_2, PGE_2, and PGI_2) along the vascular beds; however, robust evidence sustains that PGI_2 is the dominant

prostanoid produced in the macrocirculation [4, 9]. PGI_2 exhibits properties of relevance to atheroprotection. In fact, it acts as a general restraint on endogenous stimuli to platelet activation, vascular proliferation and contraction, and cell adhesion [4]. It has been reported that PGI_2 has antioxidant function before and in the early stage of atherogenesis through the induction of the antioxidant enzyme heme oxygenase (HO)-1 [10].

Recently, we provide evidence that COX-2-dependent PGI_2 (induced by steady LSS) upregulates HO-1, which halts the proatherogenic cytokine, tumor necrosis factor (TNF)-α, in human endothelial cells [11]. Altogether, these data strongly support the key role of HO-1 pathway in the vasoprotective phenotype induced by PGI_2.

In this paper we aim (i) to summarize the major features of the biology of HO-1 system by relating them to the role of this antioxidant enzyme in normal and pathological states, such as vascular inflammation and angiogenesis; (ii) to shed some light on the molecular mechanisms involved in the interplay between HO-1 system and the vasoprotective PGI_2.

2. Biology of HO

HO plays a central role in regulating the levels of intracellular heme by catalyzing the oxidative degradation of heme to liberate free iron, carbon monoxide (CO), and biliverdin in mammalian cells [20]. Biliverdin is metabolized to bilirubin by biliverdin reductase. Excess free heme catalyzes the formation of ROS, which leads to endothelial dysfunction as seen in numerous pathologic vascular conditions including systemic hypertension and diabetes, as well as in ischemia/reperfusion injury. The HO system, through its products, may cause different effects on the vascular system: (i) prevention of endothelial cell apoptosis; (ii) attenuation of the inflammatory response in the vessel wall; (iii) regulation of the vascular tone; (iv) participation in angiogenesis and vasculogenesis. Among all products of HO-1, bilirubin and biliverdin are the most potent endogenous scavengers of ROS [21] and CO exerts antiapoptotic and anti-inflammatory effects through the induction of soluble guanylyl cyclase. It suppresses the production of TNF-α, interleukin (IL)-1β and CCL4 chemokine (macrophage inflammatory protein-1β) and induces the synthesis of anti-inflammatory IL-10 [22]. Finally, free iron, despite participation in Fenton reaction that leads to formation of highly reactive hydroxyl radicals, activates Fe-ATPase. It is a transporter that removes intracellular iron as well as induces expression of ferritin heavy chains which sequester free iron and exert specific cytoprotective roles [23].

Three isoforms of HO have been described: an inducible isoform, HO-1, and two constitutively expressed isoforms, HO-2 and HO-3. HO-1 is a 32 kDa microsomal protein considered to be a protective, early stress-response agent that may have additional nonenzymatic activities related to its mitochondrial localization and nuclear translocation. The expression of HO-1 is generally very low in normal tissues, apart from liver and spleen, where it participates in the processing of senescent or damaged erythrocytes and in protection against oxidative damage caused by free porphyrins [24]. In all tissues, low basal expression of HO-1 can be upregulated by a wide variety of stimuli that cause oxidative stress, including its substrate heme, heavy metals, cytokines, ultraviolet rays, lipopolysaccharide, hydrogen peroxide, growth factors, nitric oxide (NO), and also CO [25]. HO-2, a 36-kDa protein which is constitutively expressed, is localized primarily in the brain, testis, and vascular endothelium [26, 27]. Recently it has been postulated a novel role for HO-2 in the regulation of the inflammatory and reparative response to injury, which is a cytoprotective mechanism typically associated with HO-1 induction. HO-2 may constitute an essential protective circuit responsible of a basal tone of anti-inflammatory signals critical to the execution of self-resolving inflammatory-reparative processes [28]. HO-3, a lastly cloned 33-kDa protein, which is a pseudogene derived from HO-2 transcript, has been found only in rats [29].

3. Regulation of HO-1 Gene Expression

There are different mechanisms involved in the modulation of HO-1 expression.

It has been reported that mitogen-activated protein kinases (MAPKs), phosphatidyl inositol 3-kinase/Akt, protein kinase (PK)A, PKC, and PKG [30], nuclear factor E2-related factor 2 (Nrf2), Bach1 (bric-a-brac, tramtrack, and broad complex and cap "n" collar homology 1), activator protein-1 (AP-1), nuclear factor-κB (NF-κB), cyclic adenosine monophosphate-responsive element-binding protein, and activating transcription factor 2 (ATF-2) [31] participate in HO-1 gene regulation. The transcription factor Nrf2 plays a central role in the transcriptional activation of HO-1 and many other genes encoding phase II drug-metabolizing enzymes in response to oxidative stress. Activation of Nrf2 is regulated by the cytosolic protein Keap1 that negatively modulates the nuclear translocation of Nrf2 and facilitates degradation of Nrf2 via the proteasome. Upon activation, Nrf2 enters the nucleus where it binds to the AU-rich elements (AREs) in the HO-1 promoter to trigger gene expression [17]. Nrf2 has been recently reported to regulate the induction of HO-1 in response to various forms of cellular stress, including hemodynamic, oxidative, and endoplasmic reticulum stress [32–34]. Moreover, fibroblasts and lung tissue from Nrf2-deficient animals express reduced levels of HO-1 [35, 36], further implicating Nrf2 in the induction of HO-1 [37].

Other transcription factors have been identified, such as the transcription factor Yin Yang (YY)1 that is a downstream effector of CO produced by HO-1 [38] and hypoxia-inducible factor (HIF)-1. It has been found that the increase in the transcription factor YY1 is involved in the inhibition of neointimal hyperplasia in vivo by HO-1 [38] and that the HIF-1 stabilization induces cardioprotection via HO-1 expression [39]. The results of Dawn and Bolli [39] show that HIF-1-mediated upregulation of HO-1 is beneficial to the ischemic myocardium.

In addition to the transcriptional regulation, it has been reported that a posttranslational mechanism may exist to

attenuate HO-1 expression. It has been demonstrated that two miRNAs, miR-217 and miR-377, combine to attenuate HO-1 protein expression, resulting in a significant reduction in HO-1 enzyme activity. The knockdown of both miR-217 and miR-377 increases HO-1 protein expression, while the overexpression of the same miRNAs leads to attenuation of protein expression [40]. Recently, Lin et al. show that HO-1 is subjected to posttranslational regulation by the ubiquitin-proteasome system through an endoplasmic reticulum-associated degradation pathway [41]. Proteasome inhibition significantly decreased HO-1 protein degradation. Increased HO-1 expression by MG-132, a proteasome inhibitor, has been shown to protect astrocytes from heme-mediated oxidative injury [42].

4. Polymorphisms in HO-1 Gene

Three polymorphisms in the 5′ flanking region of the HO-1 gene have been described: a (GT)n dinucleotide length polymorphism [43] and two single-nucleotide polymorphisms (SNPs), G(-1135)A and T(-413)A [44]. Only two, the (GT)n repeat polymorphism and the T(-413)A SNP, have been reported to exert functional importance by influencing the level of HO-1 expression in different organ systems. Thus, they may enhance or suppress the susceptibility to various disease conditions, including the maintenance of pregnancy [44, 45] and various cardiovascular (CV) disease [44].

In view of the apparently beneficial effects of placental HO-1 expression for the pregnancy outcome, the relationship between idiopathic recurrent miscarriage and a (GT)n repeat microsatellite polymorphism of HO-1 gene has been investigated [45]. The results from this study firstly showed the association between the HO-1 (GT)n microsatellite polymorphism in the human HO-1 promoter regulatory region and women with idiopathic recurrent miscarriage in a relatively large Caucasian population, supporting the hypothesis that HO-1 polymorphisms among human population might contribute to some unexplained cases of pregnancy disorders, such as fetal growth retardation and preeclampsia [46].

HO-1 plays a critical role in protecting the CV system from the damaging effects of oxidative stress. The two functional polymorphisms of HO-1 gene have been associated with CV disease and have different frequency distributions based upon ethnicity [44]. In particular, a significant association between the AA genotype of a T(-413)A polymorphism and arterial hypertension in Japanese women, but not in men, was observed [47]. This polymorphism was suggested to be associated with a higher expression of HO-1 and the authors suggest that an interaction between estrogen-induced expression of NO synthase and HO-1-derived CO, which attenuates NO-induced vasodilation, may explain their findings [47]. However, the inconsistency between men and women raises some doubts on the reproducibility of these data. Moreover, the same authors demonstrated that the AA genotype of the T(-413)A polymorphism may reduce the incidence of ischemic heart disease, even if it may potentially increase the risk of hypertension [48].

(GT)n dinucleotide repeat in the HO-1 gene promoter shows a length polymorphism that modulates the level of gene transcription [43]. Compared with long (GT)n repeats, short (GT)n repeats in the human HO-1 gene promoter were shown to have higher transcriptional activity in response to oxidative stress [49]. It has been shown that length polymorphism in the HO-1 gene promoter is related to coronary artery disease susceptibility in Japanese people, but this association was found only in patients with hypercholesterolemia or diabetes mellitus or in smokers [50], thus suggesting that HO-1 may play an antiatherogenic role in Japanese patients with these coronary risk factors.

Moreover, (GT)n microsatellite polymorphism was reported to be associated with emphysema, restenosis after percutaneous transluminal angioplasty, and coronary artery disease [49, 51, 52]. However, in some studies the association between HO-1 polymorphisms and CV disease was not confirmed. A study based on a large number of 1807 patients showed that the (GT)n dinucleotide repeats length polymorphism located in the promoter region of the human HO-1 gene is not associated with the development of restenosis and major adverse clinical events following coronary stenting [53]. Similarly, Turpeinen et al. [54] showed that HO-1 gene polymorphisms have no significant role in outcome of kidney transplantation in the Finnish population. A recent prospective case-control study of more than 3000 participants showed that neither the (GT)n dinucleotide repeat nor the T(-413)A polymorphism in the HO-1 promoter is associated with angiographic coronary artery disease, myocardial infarction, or survival rate in Caucasians undergoing coronary angiography [55]. Thus, altogether these studies leave still open the debate about the functional relevance of both variants of polymorphisms in HO-1 promoter. It is not unusual that studies of genetic polymorphisms produce divergent results, especially if small numbers of cases and controls are examined; often positive associations seen in small studies have been disproven in subsequent larger studies. In conclusion, even if the regulation of HO-1 gene may be determined, at least in part, by genetics, neither the (GT)n dinucleotide repeat nor the T(-413)A polymorphism of the HO-1 gene can be considered reliable genetic markers for CV disease.

5. Role of HO-1 in Vascular Inflammation

HO-1 represses inflammation by removing the proinflammatory molecule heme and by generating CO and the bile pigments, biliverdin, and bilirubin. These HO-1 reaction products are capable of blocking innate and adaptive immune responses by modifying the activation, differentiation, maturation, and/or polarization of numerous cell types, including endothelial cells, monocytes/macrophages, dendritic cells, T lymphocytes, mast cells, and platelets. These cellular actions by CO and bile pigments result in diminished leukocyte recruitment and infiltration, and proinflammatory mediator production within atherosclerotic lesions [56].

The role of HO-1 in inflammation is demonstrated in HO-1 knockout mice, in which HO-1 deficiency leads to increased production of proinflammatory cytokines [57].

In patients subjected to bypass surgery, a higher activity of HO-1 resulted in a lower concentration of IL-6 [58]. HO-1 has been reported to reduce inflammatory cell rolling, adhesion, and migration from the vascular compartment, by downregulating the function and expression of adhesion molecules on the vessel wall [59, 60]. In contrast, inhibition of HO-1 increases adhesion molecule expression [61–63].

Preclinical and clinical evidence clearly suggests that the progression of atherosclerosis is associated with inflammation [64]. Different studies have been performed to understand whether HO-1 can be protective in the pathogenesis of this disease. Experimental evidence demonstrates that the induction of HO-1 in vascular cells suppresses oxidized low-density-lipoprotein (LDL)-induced monocyte transmigration and inhibits atherosclerotic lesion formation in LDL receptor (LDLR) knockout mice [65, 66]. Interestingly, the levels of bilirubin in the normal human population correlate inversely with the incidence of atherosclerotic events [67] and it has been shown that bilirubin attenuates vascular endothelial activation and dysfunction in vitro [12].

Recent interest has also focused on peroxisome proliferator-activated receptor δ (PPARδ) ligands and induction of HO-1 expression. Ali et al. showed for the first time in vivo that PPARδ ligands induce vascular endothelial HO-1 expression, thus supporting the hypothesis that PPARδ represents an important potential target for the treatment of endothelial dysfunction and atherogenesis [13]. Finally, it has been shown that, in human monocytes, HO-1 activity is involved in attenuation of TNF-α production [68].

6. Cross-Talk between HO-1 and PGI_2

PGI_2 is considered a major prostanoid generated in the macrocirculation (both in endothelial cells and vascular smooth muscle cells) [5, 9], where it inhibits platelet activation, vascular smooth muscle cell contraction and proliferation, leukocyte-endothelial cell interactions [69], and cholesteryl ester hydrolase and induces thrombomodulin, an important inhibitor of blood coagulation [70, 71]. PGI_2 acts mostly through I prostanoid receptor (IP), a rhodopsin-like class A, 7-transmembrane-spanning G-protein-coupled receptor (GPCR), which activates membrane-bound adenylyl cyclase and the subsequent formation of the second messenger cyclic adenosine monophosphate (cAMP) [14]. Recently, studies in animal experimental models have shown that COX-2-derived PGI_2 confers atheroprotection in female mice lacking the LDLR (an animal model of atherosclerosis), through the induction of HO-1 [10]. However, the possible contribution of endothelial COX-1 to PGI_2 biosynthesis and of endothelial COX-2 to the generation of other prostanoids, in particular PGE_2 [72], has not been completely clarified. In fact, recent results suggest a cardioprotective role of PGE_2 via E prostanoid receptors (EP)2 and EP4 [73, 74]; on the other hand, it is important to underline that PGE_2, due its important role in inflammation, may enhance plaque burden and plaque destabilization in humans [75].

Thus, recently, we performed a study in human umbilical vein endothelial cells (HUVECs) exposed a physiological fluid mechanical stimulus in vitro [11] (Figure 1(a)) with the aims to (i) distinguish between the vasoprotective function of COX-2 and COX-1 and (ii) evaluate the contribution of different prostanoids to endothelial vasoprotection. In this study, we showed that in HUVECs exposed to uniform LSS of 10 dyn/cm^2 (characteristically associated with lesion-protected areas), COX-2, but not COX-1 and downstream synthases, was significantly induced, and this translated into enhanced biosynthesis not only of PGI_2, but also of other prostanoids, such as PGE_2 and PGD_2 (Figure 1(b)). Pharmacological studies, using a selective COX-2 inhibitor (NS-398) and a nonselective COX inhibitor (aspirin), showed that both COX-2 and COX-1 contributed to PGI_2 generation while only COX-1 contributed to PGE_2 and PGD_2. In the same study, we found that steady LSS reduces the synthesis and release of TNF-α (a known mediator of endothelial dysfunction and atherogenesis) [76, 77] from endothelial cells. Interestingly, we found that LSS-dependent reduction of TNF-α generation was completely countered by NS-398, aspirin, or the specific PGI_2 receptor (IP) antagonist RO3244794 [78] (Figure 1(b)). Altogether, these results support the role of COX-2-dependent PGI_2 in LSS-dependent reduction of endothelial TNF-α generation. Since LSS induced the expression of HO-1 and this effect was inhibited by NS-398, aspirin, or the IP antagonist, we hypothesized that the induction of HO-1, as a consequence of COX-2-dependent PGI_2 generation, is involved in LSS-dependent reduction of endothelial TNF-α biosynthesis. This hypothesis was confirmed by the use of the novel imidazole-based HO-1 inhibitor QC15 [79]. In fact, we showed that the inhibition of HO-1 activity was associated with a complete abrogation of LSS-dependent inhibition of TNF-α biosynthesis (Figure 1(b)). Altogether these results support the contribution of LSS-induced PGI_2 in the anti-inflammatory effect of HO-1 in endothelial cells. This seems to be a novel protective action of endothelial PGI_2 which may work in physiological conditions [11].

Further specific studies have to be performed to clarify the molecular pathways involved in the regulation of the vasoprotective gene HO-1 by COX-2-dependent PGI_2 in endothelial cells. We proposed that IP receptor signalling, through the activation of PKA, may induce the phosphorylation of glycogen synthase kinase (GSK)-3 [15], thus causing its inactivation and the loss of the capacity to phosphorylate Nrf2 [16]. This might translate into the stabilization of Nrf2 and its translocation into the nucleus, where it promotes the transcription of antioxidant and phase II genes, including HO-1 [11] (Figure 2). Furthermore, it has been shown that the Kruppel-like factor(KLF)-2 is increased in endothelial cells exposed to LSS [18]. This transcription factor may enhance antioxidant activity of Nrf2 by increasing its nuclear localization and activation [19]. The synergistic activity of the 2 transcription factors (Nrf2 and KLF-2) represents the major contribution to the shear-stress-elicited transcriptome in endothelial cells (Figure 2). Altogether our study provides evidence that COX-2-dependent PGI_2 (induced by steady LSS) upregulates HO-1 which halts TNF-α generation in human endothelial cells [11]. This vasoprotective effect is abrogated by COX inhibitors, thus suggesting that inhibition of COX-2-dependent PGI_2 might contribute to acceleration

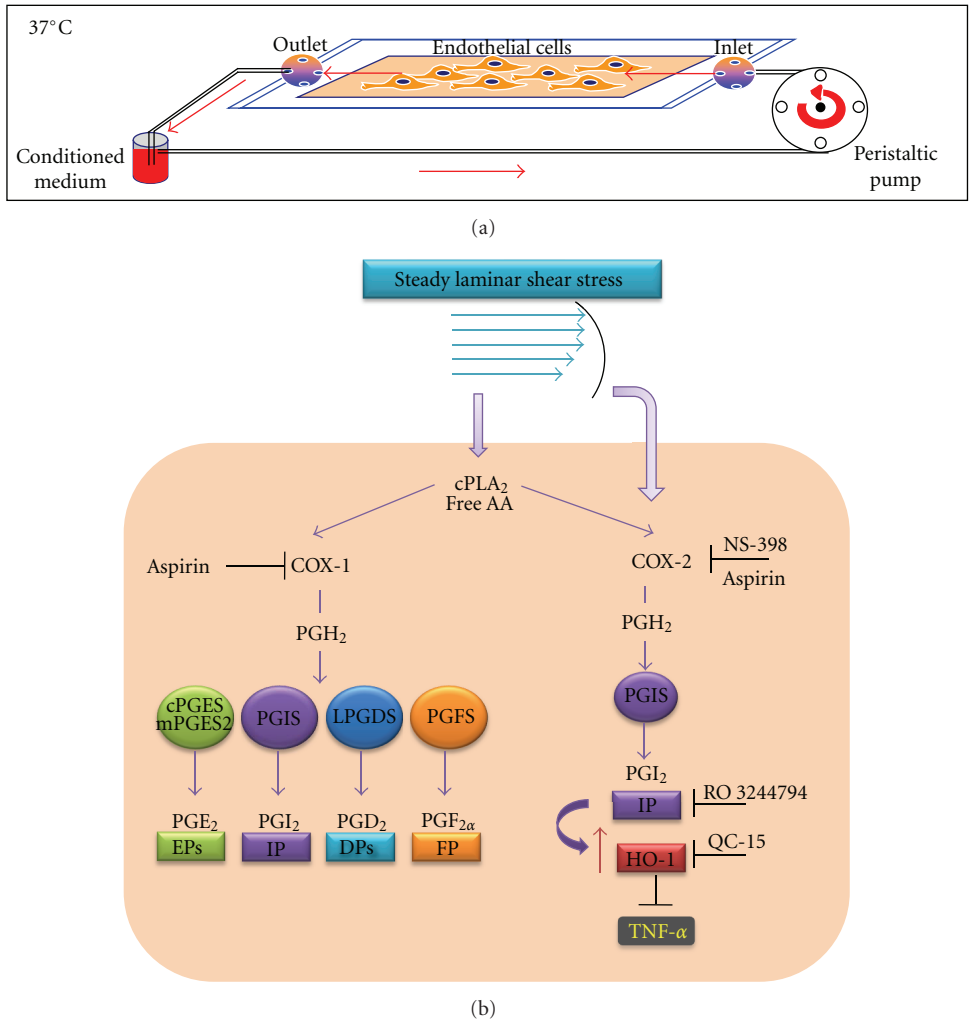

Figure 1: Exposure of endothelial cells to steady laminar shear stress (LSS). (a) HUVECs (0.8 to 1×10^6 cells per glass slide) are shear stressed using a parallel plate flow chamber connected to a constant pressure drop flow loop, maintained at 37°C and gassed continuously with a humidified mixture of 5% CO_2 in air. Endothelial monolayers are continuously perfused in a closed circuit at an estimated shear stress of 10 dyn/cm^2 (flow rate of 2.53 mL/min; shear rate of 1400 sec^{-1}) with 7 mL of perfusion DMEM-medium199 (50% vol/vol), supplemented with 5% fetal calf serum, 1% glutamine, and antibiotics for 6 hours [11]. (b) In HUVEC, steady LSS activates $cPLA_2$, thus releasing free arachidonic acid (AA) from cell membrane phospholipids, the substrate of cyclooxygenase isoenzymes (COX-1 and COX-2). In addition, LSS upregulates COX-2 expression in HUVEC, without affecting the expression of COX-1 and downstream synthases (such as cPGES, mPGES2, PGIS, LPGDS, PGFS) [11]. Both COX-1 and COX-2 participate in the biosynthesis of PGE_2, PGI_2, PGD_2, and $PGF_{2\alpha}$ as suggested by the finding that aspirin (a nonselective COX inhibitor) affects the levels of all these prostanoids. Differently, the selective COX-2 inhibitor (NS-398) affected only PGI_2 in HUVECs exposed to LSS which overexpressed COX-2. COX-2-dependent PGI_2, induced by LSS, through the interaction with a specific receptor (IP), causes the induction of HO-1. It constrains TNF-α biosynthesis in HUVECs under this experimental condition. In fact, LSS-dependent reduction of TNF-α generation is completely countered by the selective COX-2 inhibitor NS-398, the nonselective COX inhibitor aspirin, or the specific PGI_2 receptor (IP) antagonist RO3244794 [11, 12]. Finally, by the use of the novel imidazole-based HO-1 inhibitor QC15 [13], it has been shown that HO-1 induction in response to COX-dependent PGI_2 plays a role in LSS-dependent reduction of TNF-α biosynthesis [11].

of atherogenesis in patients taking traditional (t) nonsteroidal anti-inflammatory drugs (NSAIDs) and NSAIDs selective for COX-2 (coxibs).

7. Role of HO-1 in Angiogenesis

Angiogenesis involves the formation of new blood vessels and is critical for fundamental events such as development and repair after injury [80]. Recently, it has been shown that HO-1 and its gaseous product CO have potent proangiogenic properties in addition to well-recognized anti-inflammatory, antioxidant, and antiapoptotic effects [80]. Angiogenic factors, such as vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1), mediate their proangiogenic effects through induction of HO-1, making it an attractive target for therapeutic intervention [80]. It has been reported that the role of HO-1 in angiogenesis regulation could be "good" or "bad." The role of HO-1 in

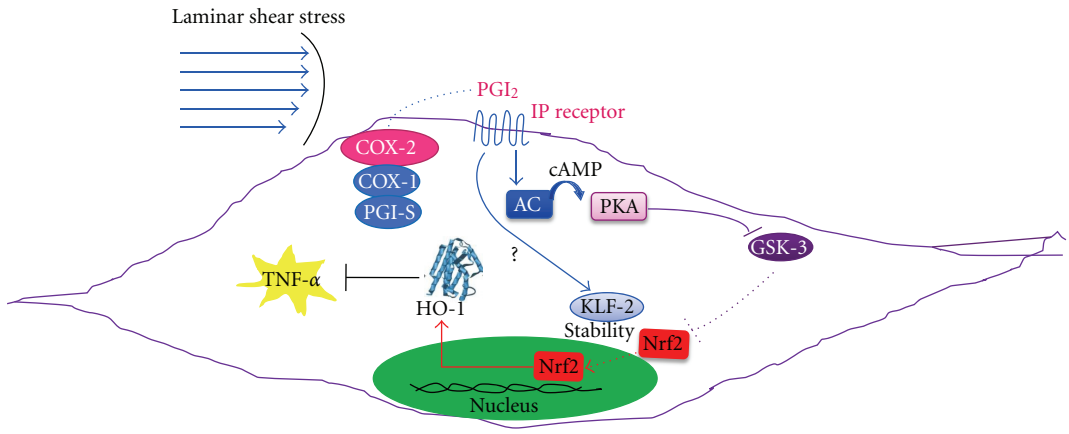

Figure 2: Postulated molecular mechanism involved in the induction of HO-1 by COX-2-dependent PGI_2, in endothelial cells exposed to steady laminar shear stress (LSS). In endothelial cells exposed to uniform LSS (characteristically associated with atherosclerotic lesion-protected areas), COX-2 is overexpressed [11]. PGI_2, mainly produced by the combined activity of COX-2 and PGI_2-synthase (PGIS), interacts with its specific receptor, IP [14]. This interaction may lead to the activation of adenylate cyclase (AC), causing an increase of intracellular levels of cyclic AMP (cAMP) and subsequently to the activation of protein kinase A (PKA) [14]. PKA may phosphorylate glycogen synthase kinase(GSK)-3 [15], causing its inactivation and the loss of the capacity to phosphorylate nuclear factor E2-related factor 2 (Nrf2) [16]. Reduced phosphorylation of Nrf2 causes its stabilization and translocation into the nucleus, where it promotes the transcription of antioxidant and phase II genes, including HO-1 [11, 17]. In addition to Nrf2, Kruppel-like factor(KLF)-2 is increased in endothelial cells exposed to LSS [18]. KLF2 enhances antioxidant activity of Nrf2 by increasing its nuclear localization and activation [19]. The synergistic activity of these two transcription factors forms a major contribution to the shear-stress-elicited transcriptome in endothelial cells. The overexpression of HO-1 in endothelial cells by LSS exerts an anti-inflammatory action through its capacity to inhibit the biosynthesis and release of TNF-α [11].

favoring angiogenesis responses is crucial for proper placental vascularization, wound healing, and neovascularization of ischemic heart. However, it may have detrimental outcomes in diseases where new blood vessel formation is undesirable, such as in tumor neovascularization [80].

Zhao and collaborators recently showed that a partial deficiency of maternal HO-1 resulted in the malformation of fetomaternal interface, alteration of the placental vasculature, insufficiency of spiral artery remodeling, and alteration of uterine natural killer cell differentiation and maturation [46]. These changes were independent of the fetal genotype, but relied on the maternal HO-1 level, which determined the balance of expression levels of pro- and antiangiogenic factors in the deciduas region [46]. According to these results a reduction in HO-1 placental expression was associated with recurrent miscarriages, spontaneous abortions, and preeclampsia [81]. These findings are in agreement with the results that HO-1 polymorphisms (as described above) are associated with idiopathic recurrent miscarriage in a relatively large Caucasian population of women [45].

The replacement of damaged capillaries and reestablishment of the normal oxygen amounts to a wound are accomplished by neovascularization. Wound-healing process includes a coagulation phase (characterized by endothelial dysfunction and platelet activation), an early extracellular matrix deposition, the release of factors by platelets, an inflammatory phase, and the resulting granulation, which are all events that rely on angiogenesis [82]. Growth factors including VEGF, chemokines like SDF-1, and hypoxia-inducible factors (HIFs) also coordinate the multifaceted events involved in wound healing [83, 84]. Interestingly, compared with wild-type littermate mice, HO-1-deficient mice exhibit impaired wound healing due, in part, to reduced recruitment of endothelial progenitor cells (EPCs) and capillary formation at the site of injury [85]. In addition, the induction of HO-1 in wounded skin was relatively weak and delayed in diabetic mice, in which also angiogenesis and wound closure were impaired. In such animals, local delivery of HO-1 transgene, using adenoviral vectors, accelerated the wound healing and increased the vascularization [86].

It has been recognized that HO-1 has a protective effect in ischemic myocardium by the increasing of expression of angiogenic growth factors in the infarcted tissue [87]. VEGF is a strong therapeutic reagent by inducing angiogenesis in ischemic myocardium [88], and it can mediate the ischemia-induced mobilization of EPCs from bone marrow [89]. Lin et al. showed that HO-1 gene transfer after myocardial infarction provides protection at least in part by promoting angiogenesis through inducing angiogenic growth factors [90]. In addition, preclinical and clinical studies have demonstrated that mesenchymal stem cells (MSCs) transplantation can attenuate ventricular remodeling and augment cardiac function when implanted into the infarcted myocardium. In HO-1-transfected MSCs-treated hearts, the myocardial apoptosis was marked with significantly reduced fibrotic area and the cardiac function and remodeling were also significantly improved [87].

It is important to point out that in addition to the numerous lines of evidence supporting the positive role of HO-1 in angiogenesis regulation, several authors reported

of the negative effects of this enzyme in tumor angiogenesis. In particular, it has been shown that several human tumors, including renal cell and prostate cancer, express high levels of HO-1 [91, 92]. HO-1 may promote tumor cell survival [93], hindering the effectiveness of anticancer therapies [94]. In contrast, inhibition of HO-1 has been shown to enhance tumor regression in animal models [95], suggesting that the HO-1 pathway may be a therapeutic target in carcinogenesis [80].

However, in prostatic cancer cells (PC3), HO-1 seems to be antiangiogenic. In fact, Ferrando et al. [96] identified a set of inflammatory and proangiogenic genes downregulated in response to HO-1 overexpression, in particular VEGFA, VEGFC, HIF1α, and $\alpha 5\beta 1$ integrin. An in vivo angiogenic assay showed that intradermal inoculation of PC3 cells stably transfected with HO-1 (PC3HO-1) generated tumors less vascularized than controls, with decreased microvessel density and reduced CD34 and MMP9 positive staining. Interestingly, longer-term grown PC3HO-1 xenografts displayed reduced neovascularization with the subsequent downregulation of VEGFR2 expression. Additionally, HO-1 repressed NF-κB-mediated transcription, which strongly suggests that HO-1 may regulate angiogenesis through this pathway. Taken together, these data support a key role of HO-1 as a modulator of the angiogenic switch in prostate carcinogenesis ascertaining it as a logical target for intervention therapy [96].

8. Interplay between HO-1 and Estrogen

Estrogen has both rapid and longer-term direct effects on CV tissues mediated by the two estrogen receptors, ER-α and ER-β [97]. Estradiol promotes endothelial cell growth, protects endothelial cells against damage by oxidants and cholesterol, and induces the generation of endothelial-derived vasodilators, such as NO and prostanoids [98]. In fact, premenopausal women are less susceptible to myocardial infarction and stroke than are males of the same age group, an advantage that is lost after menopause [99]. Several animal studies and some small clinical trials support a cardioprotective action of estrogens [100, 101]. E_2 retards atherogenesis in animal models [102] and improves endothelial disfunction in hyperlipidemic women [103].

Recently, Egan et al. [10] found that deletion of PGI_2 receptor (IP) removes the atheroprotective effect of estrogen in ovariectomized female mice. The atheroprotective role of estrogen, in this setting, seems to be mediated by the induction of PGI_2 biosynthesis. PGI_2 activates its plasma membrane receptor IP which causes the induction of the antioxidant HO-1 in the vasculature. In fact, in vitro experiments, in mouse aortic smooth muscle cells (MASMCs), showed that estrogen acts on ER-α to upregulate the production of atheroprotective PGI_2 through the induction of COX-2 [10]. MASMCs lacking the PGI_2 receptor (IP/KO) showed an increased oxidative stress suggesting that IP modulates oxidant stress under basal conditions. In addition, cicaprost, an IP agonist, increased HO-1 protein expression in wild-type MASMCs but not in IP/KO MASMCs. The involvement of IP signalling in the induction of vascular HO-1 was shown also in vivo. Thus, IP deletion decreased aortic HO-1 protein expression in female mice lacking both the IP and the LDL scavenger receptor (LDLR) (IP/LDLR DKO) [10]. These data showed that the atheroprotective role of estrogen is mediated by enhanced generation of COX-2-dependent PGI_2 and suggest that chronic treatment of patients with NSAIDs selective for COX-2 (coxibs) or tNSAIDs could undermine the estrogen-mediated protection from CV disease in pre-menopausal females. A study was performed to estimate the interaction, in a general population setting (using information from the UK's General Practice Research Database), between tNSAIDs and hormone therapy on the occurrence of acute myocardial infarction and death from coronary heart disease [101]. The researchers found that current use of hormone replacement therapy was associated with a lower risk of heart attack than nonuse. However, when looking at women who used tNSAIDs at the same time as hormone replacement therapy, the researchers found no suggestion of a reduction in risk of heart attack. These findings suggest that hormone therapy and NSAIDs might interact, with NSAIDs acting against a role for hormone replacement therapy in preventing heart attacks. This pharmacodynamic interaction might play a role, at least in part, in the uncertain results regarding the effect of postmenopausal hormone therapy on heart disease in women [104].

9. Cross-Talk between HO-1 and Cytochrome P-450-Derived Epoxyeicosatrienoic Acids

A molecular crosstalk between the cytochrome P-450-derived epoxyeicosatrienoic acids (EETs) and HO-1 gene expression was studied by Sacerdoti and coworkers [105, 106]. EETs induce HO-1 expression and signalling cascade [107], including activation of AMP-activated kinase (AMPK) and pAKT, thus reducing adiposity and insulin resistance in animal model of obesity and diabetes. In addition, EETs decrease MSC-derived adipocyte stem cell differentiation by the upregulation of HO-1-adiponectin-AKT signalling, suggesting that EET agonist may have potential therapeutic role in the treatment of dyslipidemia, diabetes, and the metabolic syndrome [108]. The potential action of EETs as intracellular lipid signalling modulators of adipogenesis was further supported by the recent finding that the treatment with EET agonists inhibits adipogenesis and decreases the levels of inflammatory cytokines. Interestingly, these effects are associated with the increase of HO-1 expression, which occurs through the inhibition of a negative regulator of HO-1 expression, Bach-1 [109].

10. Conclusions and Perspectives

Vascular health depends on a delicate balance in the vascular wall of prooxidative and antioxidant cellular mechanisms [10, 11]. Several lines of evidence have shown that HO-1 plays a central role in the vasoprotection effects of PGI_2. COX-2-dependent PGI_2 (induced by steady LSS) upregulates HO-1 which halts TNF-α generation in human endothelial cells [11]. Thus, clinical conditions associated with reduced

generation of vascular PGI_2 or the inhibition of COX-2-dependent PGI_2 by coxibs and tNSAIDs may cause CV hazard [5], at least in part, through downregulation of HO-1 expression. In fact, HO system could attenuate/block the progression of vascular diseases via its antioxidant, anti-inflammatory, and antiproliferative effects.

Due to several beneficial effects of HO-1 for the CV system, it has emerged as a promising therapeutic target in the treatment of vascular disease. Pharmacological induction or gene transfer of HO-1 ameliorates vascular dysfunction in animal models of atherosclerosis, postangioplasty restenosis, vein graft stenosis, thrombosis, myocardial infarction, and hypertension, while inhibition of HO-1 activity or gene deletion exacerbates these disorders [110].

Gene therapy and gene transfer, including site- and organ-specific targeted gene transfer have become powerful tools for studying the potential role of HO-1 in the treatment of CV diseases. HO-1 induction by pharmacological agents or the in vitro gene transfer of human HO-1 into endothelial cells increases cell cycle progression and attenuates angiotensin II, TNF-α, and heme-mediated DNA damage. In addition, administration of human HO-1 to rats in advance of schemia/reperfusion injury considerably reduces tissue damage [111]. On the other hand, it should be point out that overexpression of human HO-1 may lead to some possible side effects. In particular, it may accelerate tumor growth, stimulates early stages of angiogenesis [80], increases the occurrence of metastasis and resistance to chemotherapy and photodynamic therapy [112].

Currently, gene therapy with the use of antioxidant genes, such as HO-1, is emerging as a promising approach for selecting CV pathologies, in particular for patient groups not suitable for conventional therapies [113]. However, in this area a further improvement of gene transfer vectors and transfer protocols to more efficiently transduce different cell types of the CV system is still required and diagnostic means for better identification of patients most likely to benefit from gene therapy interventions are lacking.

Authors' Contribtion

E. Marcantoni and L. di Francesco contributed equally to this paper.

References

[1] E. Bartoloni, Y. Shoenfeld, and R. Gerli, "Inflammatory and autoimmune mechanisms in the induction of atherosclerotic damage in systemic rheumatic diseases: two faces of the same coin," *Arthritis Care and Research*, vol. 63, no. 2, pp. 178–183, 2011.

[2] M. Gawaz, H. Langer, and A. E. May, "Platelets in inflammation and atherogenesis," *Journal of Clinical Investigation*, vol. 115, no. 12, pp. 3378–3384, 2005.

[3] K. K. Griendling and G. A. FitzGerald, "Oxidative stress and cardiovascular injury. Part I: basic mechanisms and in vivo monitoring of ROS," *Circulation*, vol. 108, no. 16, pp. 1912–1916, 2003.

[4] P. Minuz, C. Fava, and A. Lechi, "Lipid peroxidation, isoprostanes and vascular damage," *Pharmacological Reports*, vol. 58, pp. 57–68, 2006.

[5] T. Grosser, S. Fries, and G. A. FitzGerald, "Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities," *Journal of Clinical Investigation*, vol. 116, no. 1, pp. 4–15, 2006.

[6] J. N. Topper, J. Cai, D. Falb, and M. A. Gimbrone, "Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 19, pp. 10417–10422, 1996.

[7] C. K. Zarins, D. P. Giddens, and B. K. Bharadvaj, "Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress," *Circulation Research*, vol. 53, no. 4, pp. 502–514, 1983.

[8] C. D. Funk, "Prostaglandins and leukotrienes: advances in eicosanoid biology," *Science*, vol. 294, no. 5548, pp. 1871–1875, 2001.

[9] S. Moncada, E. A. Higgs, and J. R. Vane, "Human arterial and venous tissues generate prostacyclin (prostaglandin X), a potent inhibitor of platelet aggregation," *Lancet*, vol. 1, no. 8001, pp. 18–21, 1977.

[10] K. M. Egan, J. A. Lawson, S. Fries et al., "COX-2-derived prostacyclin confers atheroprotection on female mice," *Science*, vol. 306, no. 5703, pp. 1954–1957, 2004.

[11] L. Di Francesco, L. Totani, M. Dovizio et al., "Induction of prostacyclin by steady laminar shear stress suppresses tumor necrosis factor-α biosynthesis via heme oxygenase-1 in human endothelial cells," *Circulation Research*, vol. 104, no. 4, pp. 506–513, 2009.

[12] K. Kawamura, K. Ishikawa, Y. Wada et al., "Bilirubin from heme oxygenase-1 attenuates vascular endothelial activation and dysfunction," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 1, pp. 155–160, 2005.

[13] F. Ali, N. S. Ali, A. Bauer et al., "PPARσ and PGC1α act cooperatively to induce haem oxygenase-1 and enhance vascular endothelial cell resistance to stress," *Cardiovascular Research*, vol. 85, no. 4, pp. 701–710, 2010.

[14] S. Narumiya, Y. Sugimoto, and F. Ushikubi, "Prostanoid receptors: structures, properties, and functions," *Physiological Reviews*, vol. 79, no. 4, pp. 1193–1226, 1999.

[15] X. Fang, S. X. Yu, Y. Lu, R. C. Bast, J. R. Woodgett, and G. B. Mills, "Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 22, pp. 11960–11965, 2000.

[16] M. Salazar, A. I. Rojo, D. Velasco, R. M. De Sagarra, and A. Cuadrado, "Glycogen synthase kinase-3β inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2," *Journal of Biological Chemistry*, vol. 281, no. 21, pp. 14841–14851, 2006.

[17] X. M. Liu, K. J. Peyton, D. Ensenat et al., "Nitric oxide stimulates heme oxygenase-1 gene transcription via the Nrf2/ARE complex to promote vascular smooth muscle cell survival," *Cardiovascular Research*, vol. 75, no. 2, pp. 381–389, 2007.

[18] S. SenBanerjee, Z. Lin, G. B. Atkins et al., "KLF2 is a novel transcriptional regulator of endothelial proinflammatory

activation," *Journal of Experimental Medicine*, vol. 199, no. 10, pp. 1305–1315, 2004.

[19] J. O. Fledderus, R. A. Boon, O. L. Volger et al., "KLF2 primes the antioxidant transcription factor Nrf2 for activation in endothelial cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 7, pp. 1339–1346, 2008.

[20] N. G. Abraham and A. Kappas, "Pharmacological and clinical aspects of heme oxygenase," *Pharmacological Reviews*, vol. 60, no. 1, pp. 79–127, 2008.

[21] R. Stocker, Y. Yamamoto, and A. F. McDonagh, "Bilirubin is an antioxidant of possible physiological importance," *Science*, vol. 235, no. 4792, pp. 1043–1046, 1987.

[22] L. E. Otterbein, F. H. Bach, J. Alam et al., "Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway," *Nature Medicine*, vol. 6, no. 4, pp. 422–428, 2000.

[23] R. R. Crichton, S. Wilmet, R. Legssyer, and R. J. Ward, "Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells," *Journal of Inorganic Biochemistry*, vol. 91, no. 1, pp. 9–18, 2002.

[24] R. Tenhunen, H. S. Marver, and R. Schmid, "The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 61, no. 2, pp. 748–755, 1968.

[25] S. W. Ryter, J. Alam, and A. M. K. Choi, "Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications," *Physiological Reviews*, vol. 86, no. 2, pp. 583–650, 2006.

[26] N. G. Abraham, G. S. Drummond, J. D. Lutton, and A. Kappas, "The biological significance and physiological role of heme oxygenase," *Cellular Physiology and Biochemistry*, vol. 6, no. 3, pp. 129–168, 1996.

[27] W. K. McCoubrey Jr., J. F. Ewing, and M. D. Maines, "Human heme oxygenase-2: Characterization and expression of a full-length cDNA and evidence suggesting that the two HO-2 transcripts may differ by choice of polyadenylation signal," *Archives of Biochemistry and Biophysics*, vol. 295, no. 1, pp. 13–20, 1992.

[28] F. Seta, L. Bellner, R. Rezzani et al., "Heme oxygenase-2 is a critical determinant for execution of an acute inflammatory and reparative response," *American Journal of Pathology*, vol. 169, no. 5, pp. 1612–1623, 2006.

[29] W. K. Mccoubrey Jr., T. J. Huang, and M. D. Maines, "Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3," *European Journal of Biochemistry*, vol. 247, no. 2, pp. 725–732, 1997.

[30] R. Stocker and M. A. Perrella, "Heme oxygenase-1: a novel drug target for atherosclerotic diseases?" *Circulation*, vol. 114, no. 20, pp. 2178–2189, 2006.

[31] Y. M. Kim, H. O. Pae, J. E. Park et al., "Heme oxygenase in the regulation of vascular biology: from molecular mechanisms to therapeutic opportunities," *Antioxidants and Redox Signaling*, vol. 14, no. 1, pp. 137–167, 2011.

[32] X. L. Chen, S. E. Varner, A. S. Rao et al., "Laminar flow induction of antioxidant response element-mediated genes in endothelial cells: a novel anti-inflammatory mechanism," *Journal of Biological Chemistry*, vol. 278, no. 2, pp. 703–711, 2003.

[33] T. Ishii, K. Itoh, E. Ruiz et al., "Role of Nrf2 in the regulation of CD36 and stress protein expression in murine macrophages: activation by oxidatively modified LDL and 4-hydroxynonenal," *Circulation Research*, vol. 94, no. 5, pp. 609–616, 2004.

[34] X. M. Liu, K. J. Peyton, D. Ensenat et al., "Endoplasmic reticulum stress stimulates heme oxygenase-1 gene expression in vascular smooth muscle: role in cell survival," *Journal of Biological Chemistry*, vol. 280, no. 2, pp. 872–877, 2005.

[35] L. Leung, M. Kwong, S. Hou, C. Lee, and J. Y. Chan, "Deficiency of the Nrf1 and Nrf2 transcription factors results in early embryonic lethality and severe oxidative stress," *Journal of Biological Chemistry*, vol. 278, no. 48, pp. 48021–48029, 2003.

[36] H. Y. Cho, A. E. Jedlicka, S. P. M. Reddy et al., "Role of NRF2 in protection against hyperoxic lung injury in mice," *American Journal of Respiratory Cell and Molecular Biology*, vol. 26, no. 2, pp. 175–182, 2002.

[37] X. M. Liu, K. J. Peyton, D. Ensenat et al., "Nitric oxide stimulates heme oxygenase-1 gene transcription via the Nrf2/ARE complex to promote vascular smooth muscle cell survival," *Cardiovascular Research*, vol. 75, no. 2, pp. 381–389, 2007.

[38] K. Beck, B. J. Wu, J. Ni et al., "Interplay between heme oxygenase-1 and the multifunctional transcription factor Yin Yang 1 in the inhibition of intimal hyperplasia," *Circulation Research*, vol. 107, no. 12, pp. 1490–1497, 2010.

[39] B. Dawn and R. Bolli, "HO-1 induction by HIF-1: a new mechanism for delayed cardioprotection?" *American Journal of Physiology*, vol. 289, no. 2, pp. H522–H524, 2005.

[40] J. D. Beckman, C. Chen, J. Nguyen et al., "Regulation of heme oxygenase-1 protein expression by miR-377 in Combination with miR-217," *Journal of Biological Chemistry*, vol. 286, no. 5, pp. 3194–3202, 2011.

[41] P. H. Lin, M. T. Chiang, and L. Y. Chau, "Ubiquitin-proteasome system mediates heme oxygenase-1 degradation through endoplasmic reticulum-associated degradation pathway," *Biochimica et Biophysica Acta*, vol. 1783, no. 10, pp. 1826–1834, 2008.

[42] J. Chen and R. F. Regan, "Increasing expression of heme oxygenase-1 by proteasome inhibition protects astrocytes from heme-mediated oxidative injury," *Current Neurovascular Research*, vol. 2, no. 3, pp. 189–196, 2005.

[43] T. Kimpara, A. Takeda, K. Watanabe et al., "Microsatellite polymorphism in the human heme oxygenase-1 gene promoter and its application in association studies with Alzheimer and Parkinson disease," *Human Genetics*, vol. 100, no. 1, pp. 145–147, 1997.

[44] M. Exner, E. Minar, O. Wagner, and M. Schillinger, "The role of heme oxygenase-1 promoter polymorphisms in human disease," *Free Radical Biology and Medicine*, vol. 37, no. 8, pp. 1097–1104, 2004.

[45] D. Denschlag, R. Marculescu, G. Unfried et al., "The size of a microsatellite polymorphism of the haem oxygenase 1 gene is associated with idiopathic recurrent miscarriage," *Molecular Human Reproduction*, vol. 10, no. 3, pp. 211–214, 2004.

[46] H. Zhao, J. Azuma, F. Kalish, R. J. Wong, and D. K. Stevenson, "Maternal heme oxygenase 1 regulates placental vasculature development via angiogenic factors in mice," *Biology of Reproduction*, vol. 85, no. 5, pp. 1005–1012, 2011.

[47] K. Ono, T. Mannami, and N. Iwai, "Association of a promoter variant of the haeme oxygenase-1 gene with hypertension in women," *Journal of Hypertension*, vol. 21, no. 8, pp. 1497–1503, 2003.

[48] K. Ono, Y. Goto, S. Takagi et al., "A promoter variant of the heme oxygenase-1 gene may reduce the incidence of ischemic heart disease in Japanese," *Atherosclerosis*, vol. 173, no. 2, pp. 315–319, 2004.

[49] N. Yamada, M. Yamaya, S. Okinaga et al., "Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema," *American Journal of Human Genetics*, vol. 66, no. 1, pp. 187–195, 2000.

[50] H. Kaneda, M. Ohno, J. Taguchi et al., "Heme oxygenase-1 gene promoter polymorphism is associated with coronary artery disease in Japanese patients with coronary risk factors," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 22, no. 10, pp. 1680–1685, 2002.

[51] M. Exner, M. Schillinger, E. Minar et al., "Heme oxygenase-1 gene promoter microsatellite polymorphism is associated with restenosis after percutaneous transluminal angioplasty," *Journal of Endovascular Therapy*, vol. 8, no. 5, pp. 433–440, 2001.

[52] M. Schillinger, M. Exner, E. Minar et al., "Heme oxygenase-1 genotype and restenosis after balloon angioplasty: a novel vascular protective factor," *Journal of the American College of Cardiology*, vol. 43, no. 6, pp. 950–957, 2004.

[53] K. Tiroch, W. Koch, N. Von Beckerath, A. Kastrati, and A. Schömig, "Heme oxygenase-1 gene promoter polymorphism and restenosis following coronary stenting," *European Heart Journal*, vol. 28, no. 8, pp. 968–973, 2007.

[54] H. Turpeinen, L. E. Kyllönen, J. Parkkinen, J. Laine, K. T. Salmela, and J. Partanen, "Heme oxygenase 1 gene polymorphisms and outcome of renal transplantation," *International Journal of Immunogenetics*, vol. 34, no. 4, pp. 253–257, 2007.

[55] N. Lüblinghoff, K. Winkler, B. R. Winkelmann et al., "Genetic variants of the promoter of the heme oxygenase-1 gene and their influence on cardiovascular disease (The Ludwigshafen Risk and Cardiovascular Health Study)," *BMC Medical Genetics*, vol. 10, article no. 36, 2009.

[56] W. Durante, "Protective role of heme oxygenase-1 against inflammation in atherosclerosis," *Frontiers in Bioscience*, vol. 16, no. 6, pp. 2372–2388, 2011.

[57] M. H. Kapturczak, C. Wasserfall, T. Brusko et al., "Heme oxygenase-1 modulates early inflammatory responses: evidence from the heme oxygenase-1-deficient mouse," *American Journal of Pathology*, vol. 165, no. 3, pp. 1045–1053, 2004.

[58] H. Taha, K. Skrzypek, I. Guevara et al., "Role of heme oxygenase-1 in human endothelial cells: lesson from the promoter allelic variants," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 8, pp. 1634–1641, 2010.

[59] F. A. D. T. G. Wagener, J. L. Da Silva, T. Farley, T. De Witte, A. Kappas, and N. G. Abraham, "Differential effects of heme oxygenase isoforms on heme mediation of endothelial intracellular adhesion molecule expression," *Journal of Pharmacology and Experimental Therapeutics*, vol. 291, no. 1, pp. 416–423, 1999.

[60] F. A. D. T. G. Wagener, A. Eggert, O. C. Boerman et al., "Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase," *Blood*, vol. 98, no. 6, pp. 1802–1811, 2001.

[61] S. Hayashi, R. Takamiya, T. Yamaguchi et al., "Induction of heme oxygenase-1 suppresses venular leukocyte adhesion elicited by oxidative stress: role of bilirubin generated by the enzyme," *Circulation Research*, vol. 85, no. 8, pp. 663–671, 1999.

[62] T. J. Vachharajani, J. Work, A. C. Issekutz, and D. N. Granger, "Heme oxygenase modulates selectin expression in different regional vascular beds," *American Journal of Physiology*, vol. 278, no. 5, pp. H1613–H1617, 2000.

[63] J. D. Belcher, H. Mahaseth, T. E. Welch, L. E. Otterbein, R. P. Hebbel, and G. M. Vercellotti, "Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice," *Journal of Clinical Investigation*, vol. 116, no. 3, pp. 808–816, 2006.

[64] P. Libby, Y. Okamoto, V. Z. Rocha, and E. Folco, "Inflammation in atherosclerosis: transition from theory to practice," *Circulation Journal*, vol. 74, no. 2, pp. 213–220, 2010.

[65] K. Ishikawa, M. Navab, N. Leitinger, A. M. Fogelman, and A. J. Lusis, "Induction of heme oxygenase-1 inhibits the monocyte transmigration induced by mildly oxidized LDL," *Journal of Clinical Investigation*, vol. 100, no. 5, pp. 1209–1216, 1997.

[66] K. Ishikawa, D. Sugawara, X. P. Wang et al., "Heme oxygenase-1 inhibits atherosclerotic lesion formation in LDL-receptor knockout mice," *Circulation Research*, vol. 88, no. 5, pp. 506–512, 2001.

[67] M. Mayer, "Association of serum bilirubin concentration with risk of coronary artery disease," *Clinical Chemistry*, vol. 46, no. 11, pp. 1723–1727, 2000.

[68] Y. Drechsler, A. Dolganiuc, O. Norkina et al., "Heme oxygena-1 mediates the anti-inflammatory effects of acute alcohol on IL-10 induction involving p38 MAPK activation in monocytes," *Journal of Immunology*, vol. 177, no. 4, pp. 2592–2600, 2006.

[69] T. Kobayashi, Y. Tahara, M. Matsumoto et al., "Roles of thromboxane A_2 and prostacyclin in the development of atherosclerosis in apoE-deficient mice," *Journal of Clinical Investigation*, vol. 114, no. 6, pp. 784–794, 2004.

[70] S. Moncada, R. Gryglewski, S. Bunting, and J. R. Vane, "An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation," *Nature*, vol. 263, no. 5579, pp. 663–665, 1976.

[71] K. Rabausch, E. Bretschneider, M. Sarbia et al., "Regulation of thrombomodulin expression in human vascular smooth muscle cells by COX-2-derived prostaglandins," *Circulation research*, vol. 96, no. 1, pp. e1–e6, 2005.

[72] D. W. Kawka, M. Ouellet, P. O. Hétu, I. I. Singer, and D. Riendeau, "Double-label expression studies of prostacyclin synthase, thromboxane synthase and COX isoforms in normal aortic endothelium," *Biochimica et Biophysica Acta*, vol. 1771, no. 1, pp. 45–54, 2007.

[73] C. R. J. Kennedy, Y. Zhang, S. Brandon et al., "Salt-sensitive hypertension and reduced fertility in mice lacking the prostaglandin EP2 receptor," *Nature Medicine*, vol. 5, no. 2, pp. 217–220, 1999.

[74] A. M. Hristovska, L. E. Rasmussen, P. B. L. Hansen et al., "Prostaglandin E_2 induces vascular relaxation by E-prostanoid 4 receptor-mediated activation of endothelial nitric oxide synthase," *Hypertension*, vol. 50, no. 3, pp. 525–530, 2007.

[75] U. T. Shankavaram, W. C. Lai, S. Netzel-Arnett et al., "Monocyte membrane type 1-matrix metalloproteinase: prostaglandin-dependent regulation and role in metalloproteinase-2 activation," *Journal of Biological Chemistry*, vol. 276, no. 22, pp. 19027–19032, 2001.

[76] P. Barath, M. C. Fishbein, J. Cao, J. Berenson, R. H. Helfant, and J. S. Forrester, "Detection and localization of tumor necrosis factor in human atheroma," *American Journal of Cardiology*, vol. 65, no. 5, pp. 297–302, 1990.

[77] C. Cardillo, F. Schinzari, N. Mores et al., "Intravascular tumor necrosis factor α blockade reverses endothelial dysfunction in rheumatoid arthritis," *Clinical Pharmacology and Therapeutics*, vol. 80, no. 3, pp. 275–281, 2006.

[78] K. R. Bley, A. Bhattacharya, D. V. Daniels et al., "RO1138452 and RO3244794: characterization of structurally distinct,

potent and selective IP (prostacyclin) receptor antagonists," *British Journal of Pharmacology*, vol. 147, no. 3, pp. 335–345, 2006.

[79] R. T. Kinobe, Y. Ji, J. Z. Vlahakis et al., "Effectiveness of novel imidazole-dioxolane heme oxygenase inhibitors in renal proximal tubule epithelial cells," *Journal of Pharmacology and Experimental Therapeutics*, vol. 323, no. 3, pp. 763–770, 2007.

[80] J. Dulak, J. Deshane, A. Jozkowicz, and A. Agarwal, "Heme oxygenase-1 and carbon monoxide in vascular pathobiology: focus on angiogenesis," *Circulation*, vol. 117, no. 2, pp. 231–241, 2008.

[81] F. Lyall and L. Myatt, "The role of the placenta in pre-eclampsia—a workshop report," *Placenta*, vol. 23, no. 1, pp. S142–S145, 2002.

[82] S. M. Bauer, R. J. Bauer, and O. C. Velazquez, "Angiogenesis, vasculogenesis, and induction of healing in chronic wounds," *Vascular and Endovascular Surgery*, vol. 39, no. 4, pp. 293–306, 2005.

[83] D. J. Ceradini, A. R. Kulkarni, M. J. Callaghan et al., "Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1," *Nature Medicine*, vol. 10, no. 8, pp. 858–864, 2004.

[84] O. M. Tepper, J. M. Capla, R. D. Galiano et al., "Adult vasculogenesis occurs through in situ recruitment, proliferation, and tubulization of circulating bone marrow-derived cells," *Blood*, vol. 105, no. 3, pp. 1068–1077, 2005.

[85] J. Deshane, S. Chen, S. Caballero et al., "Stromal cell-derived factor 1 promotes angiogenesis via a heme oxygenase 1-dependent mechanism," *Journal of Experimental Medicine*, vol. 204, no. 3, pp. 605–618, 2007.

[86] A. Grochot-Przeczek, R. Lach, J. Mis et al., "Heme oxygenase-1 accelerates cutaneous wound healing in mice," *PLoS One*, vol. 4, no. 6, Article ID e5803, 2009.

[87] B. Zeng, G. Lin, X. Ren, Y. Zhang, and H. Chen, "Overexpression of HO-1 on mesenchymal stem cells promotes angiogenesis and improves myocardial function in infarcted myocardium," *Journal of Biomedical Science*, vol. 17, no. 1, article no. 80, 2010.

[88] H. Huwer, C. Welter, C. Özbek et al., "Simultaneous surgical revascularization and angiogenic gene therapy in diffuse coronary artery disease," *European Journal of Cardio-thoracic Surgery*, vol. 20, no. 6, pp. 1128–1134, 2001.

[89] O. Jeon, S. J. Song, S. H. Bhang, C. Y. Choi, M. J. Kim, and B. S. Kim, "Additive effect of endothelial progenitor cell mobilization and bone marrow mononuclear cell transplantation on angiogenesis in mouse ischemic limbs," *Journal of Biomedical Science*, vol. 14, no. 3, pp. 323–330, 2007.

[90] H. H. Lin, Y. H. Chen, P. F. Chang, Y. T. Lee, S. F. Yet, and L. Y. Chau, "Heme oxygenase-1 promotes neovascularization in ischemic heart by coinduction of VEGF and SDF-1," *Journal of Molecular and Cellular Cardiology*, vol. 45, no. 1, pp. 44–55, 2008.

[91] A. I. Goodman, M. Choudhury, J. L. Da Silva, M. L. Schwartzman, and N. G. Abraham, "Overexpression of the heme oxygenase gene in renal cell carcinoma," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 214, no. 1, pp. 54–61, 1997.

[92] M. D. Maines and P. A. Abrahamsson, "Expression of heme oxygenase-1 (HSP32) in human prostate: normal, hyperplastic, and tumor tissue distribution," *Urology*, vol. 47, no. 5, pp. 727–733, 1996.

[93] H. Was, T. Cichon, R. Smolarczyk et al., "Overexpression of heme oxygenase-1 in murine melanoma: increased proliferation and viability of tumor cells, decreased survival of mice," *American Journal of Pathology*, vol. 169, no. 6, pp. 2181–2198, 2006.

[94] D. Nowis, M. Legat, T. Grzela et al., "Heme oxygenase-1 protects tumor cells against photodynamic therapy-mediated cytotoxicity," *Oncogene*, vol. 25, no. 24, pp. 3365–3374, 2006.

[95] J. Fang, T. Sawa, T. Akaike et al., "In vivo antitumor activity of pegylated zinc protoporphyrin: targeted inhibition of heme oxygenase in solid tumor," *Cancer Research*, vol. 63, no. 13, pp. 3567–3574, 2003.

[96] M. Ferrando, G. Gueron, B. Elguero et al., "Heme oxygenase 1 (HO-1) challenges the angiogenic switch in prostate cancer," *Angiogenesis*, vol. 14, no. 4, pp. 467–479, 2011.

[97] M. E. Mendelsohn and R. H. Karas, "The protective effects of estrogen on the cardiovascular system," *New England Journal of Medicine*, vol. 340, no. 23, pp. 1801–1811, 1999.

[98] M. Rosselli, B. Imthurn, P. J. Keller, E. K. Jackson, and R. K. Dubey, "Circulating nitric oxide (nitrite/nitrate) levels in postmenopausal women substituted with 17β-estradiol and norethisterone acetate: a two-year follow-up study," *Hypertension*, vol. 25, no. 4, pp. 848–853, 1995.

[99] M. E. Mendelsohn, "Protective effects of estrogen on the cardiovascular system," *American Journal of Cardiology*, vol. 89, no. 12, pp. 12E–18E, 2002.

[100] R. K. Dubey, B. Imthurn, M. Barton, and E. K. Jackson, "Vascular consequences of menopause and hormone therapy: importance of timing of treatment and type of estrogen," *Cardiovascular Research*, vol. 66, no. 2, pp. 295–306, 2005.

[101] L. A. García Rodríguez, K. Egan, and G. A. FitzGerald, "Traditional nonsteroidal anti-inflammatory drugs and postmenopausal hormone therapy: a drug-drug interaction?" *PLoS Medicine*, vol. 4, no. 5, article e157, 2007.

[102] P. A. K. Bourassa, P. M. Milos, B. J. Gaynor, J. L. Breslow, and R. J. Aiello, "Estrogen reduces atherosclerotic lesion development in apolipoprotein E-deficient mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 19, pp. 10022–10027, 1996.

[103] P. Collins, G. M. C. Rosano, P. M. Sarrel et al., "17β-estradiol attenuates acetylcholine-induced coronary arterial constriction in women but not men with coronary heart disease," *Circulation*, vol. 92, no. 1, pp. 24–30, 1995.

[104] G. M. C. Rosano, C. Vitale, A. Silvestri, and M. Fini, "Hormone replacement therapy and cardioprotection: the end of the tale?" *Annals of the New York Academy of Sciences*, vol. 997, pp. 351–357, 2003.

[105] D. Sacerdoti, M. Bolognesi, M. Di Pascoli et al., "Rat mesenteric arterial dilator response to 11,12-epoxyeicosatrienoic acid is mediated by activating heme oxygenase," *American Journal of Physiology*, vol. 291, no. 4, pp. H1999–H2002, 2006.

[106] D. Sacerdoti, C. Colombrita, M. Di Pascoli et al., "11,12-Epoxyeicosatrienoic acid stimulates heme-oxygenase-1 in endothelial cells," *Prostaglandins and Other Lipid Mediators*, vol. 82, no. 1–4, pp. 155–161, 2007.

[107] K. Sodhi, K. Inoue, K. H. Gotlinger et al., "Epoxyeicosatrienoic acid agonist rescues the metabolic syndrome phenotype of HO-2-null mice," *Journal of Pharmacology and Experimental Therapeutics*, vol. 331, no. 3, pp. 906–916, 2009.

[108] D. H. Kim, L. Vanella, K. Inoue et al., "Epoxyeicosatrienoic acid agonist regulates human mesenchymal stem cell-derived adipocytes through activation of HO-1-pAKT signaling and a decrease in PPARγ," *Stem Cells and Development*, vol. 19, no. 12, pp. 1863–1873, 2010.

[109] L. Vanella, D. H. Kim, K. Sodhi et al., "Crosstalk between EET and HO-1 downregulates Bach1 and adipogenic marker

expression in mesenchymal stem cell derived adipocytes," *Prostaglandins and Other Lipid Mediators*, vol. 96, no. 1–4, pp. 54–62, 2011.

[110] W. Durante, "Targeting heme oxygenase-1 in vascular disease," *Current drug targets*, vol. 11, no. 12, pp. 1504–1516, 2010.

[111] S. J. Peterson and W. H. Frishman, "Targeting heme oxygenase: therapeutic implications for diseases of the cardiovascular system," *Cardiology in Review*, vol. 17, no. 3, pp. 99–111, 2009.

[112] S. Tanaka, T. Akaike, J. Fang et al., "Antiapoptotic effect of haem oxygenase-1 induced by nitric oxide in experimental solid tumour," *British Journal of Cancer*, vol. 88, no. 6, pp. 902–909, 2003.

[113] A. L. Levonen, E. Vähäkangas, J. K. Koponen, and S. Ylä-Herttuala, "Antioxidant gene therapy for cardiovascular disease: current status and future perspectives," *Circulation*, vol. 117, no. 16, pp. 2142–2150, 2008.

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Research Article

Vasculitis, Atherosclerosis, and Altered HDL Composition in Heme-Oxygenase-1-Knockout Mice

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To elucidate roles of heme oxygenase-1 (HO-1) in cardiovascular system, we have analyzed one-year-old HO-1-knockout mice. Homozygous HO-1-knockout mice had severe aortitis and coronary arteritis with mononuclear cellular infiltration and fatty streak formation even on a standard chow diet. Levels of plasma total cholesterol and HDL were similar among the three genotypes. However, homozygous HO-1-knockout mice had lower body weight and plasma triglyceride. HO-1-deficiency resulted in alteration of the composition of HDL. The ratio of apolipoprotein AI to AII in HO-1-knockout mice was reduced about 10-fold as compared to wild-type mice. In addition, paraoxonase, an enzyme against oxidative stress, was reduced less than 50% in HO-1-knockout mice. The knockout mice also exhibited significant elevation of plasma lipid hydroperoxides. This study using aged HO-1-knockout mice strengthened the idea that HO-1 functions to suppress systemic inflammation in artery wall and prevents plasma lipid peroxidation.

1. Introduction

Heme oxygenase (HO) oxidatively catalyzes heme to biliverdin, carbon monoxide, and free iron using NADPH-cytochrome P450 reductase as an electron donor [1–3]. A number of studies suggest a potential protective function of this enzyme against oxidative stress under various conditions with transcriptional activation of heme oxygenase-1 (HO-1) [4, 5]. The antioxidant activity of HO derives from both the elimination of prooxidant heme and the biological activities of its products. Biliverdin and its metabolite by biliverdin reductase, bilirubin, effectively inhibit LDL oxidation [6, 7]. Free iron regulates ferritin synthesis through an iron-responsive element [8]. Ferritin has been shown to have cytoprotective effects against oxidative injuries [9]. Carbon monoxide (CO) modulates the activity of soluble guanylate synthetase like nitric oxide [10] and p38 mitogen-activated kinase activity [11].

Accumulating evidence suggests that oxidized LDL (oxLDL) plays an important role during the early phases of atherogenesis via its proinflammatory properties [5]. We recently reported that HO-1 is remarkably induced by mildly oxLDL in both endothelial cells and smooth muscle cells [12]. HO-1 expression was also highly responsive to oxidized bioactive oxidized phospholipids existing in LDL [12]. In addition, using artery wall cocultures we found that HO-1 inhibits oxLDL-dependent monocyte chemotaxis through its products bilirubin and biliverdin [12]. We then performed in vivo studies to examine the role of HO-1 on the development of atherosclerosis. HO-1 was highly expressed in atherosclerotic lesion in C57BL/6J, apoE-knockout, and LDL-receptor-knockout mice [13]. When we modulate HO expression in high-fat-fed LDL-receptor knockout mice, HO inhibition significantly resulted in the progression of atherosclerotic lesion formation compared to the mice in which HO was induced [13]. These results lead us to hypothesize the protective roles of HO-1 for atherogenesis.

High-density lipoproteins (HDLs) are considered to work for antiatherogenesis. These antiatherogenic properties have been explained by reverse cholesterol transport from

cells to liver [14]. However, previous studies revealed that HDL itself works as an antioxidant for minimally oxidized low-density lipoproteins [15] and that paraoxonase and apolipoprotein AI in HDL play an important role as antioxidants [16, 17]. However, it is reported that oxidative modification of HDL loses the effect to stimulate efflux of cholesterol from foam cells [18] and that oxidized HDL activates platelets similar to oxidized LDL [19].

To further strengthen the idea that HO-1 functions as an anti-inflammatory enzyme in artery wall and that HO-1 exhibits antioxidative effects on plasma lipoproteins, we analyzed one-year-old HO-1-knockout mice bred on a standard chow diet. Studies using HO-1 knockout mice [4, 20, 21] revealed that (i) HO-1-homozygous-knockout ($HO\text{-}1^{-/-}$) mice develop an anemia with accumulation of iron in liver and kidney, (ii) cultured $HO\text{-}1^{-/-}$ embryonic fibroblasts produce high free radicals when exposed to hydrogen peroxides, paraquat, or cadmium chloride, (iii) $HO\text{-}1^{-/-}$ mice are vulnerable to mortality when challenged with endotoxin, and (ix) HO-1 expression ensures to survive cardiac xenograft.

In this study, we examined artery walls and plasma lipoproteins of male HO-1-knockout mice between 1- and 1.5-year old which were bred on a standard chow diet. HO-1-knockout mice had severe infiltration of mononuclear cells at their ascending aortic wall and coronary arteries and small atherosclerotic lesion in aortic sinus. In HO-1 knockout mice, HDL appears to be oxidized. Furthermore, the composition of apolipoprotein AI and AII in HDL altered. These results show HDL oxidation in HO-1-knockout mice. In addition, HO-1-knockout mice showed lower plasma paraoxonase level and higher lipid peroxide level, suggesting that the absence of HO-1 resulted in the oxidation of plasma lipoproteins and activate inflammatory responses in arterial wall.

2. Materials and Methods

2.1. Reagents. Reagents utilized were obtained from Sigma unless otherwise specified.

2.2. Animal Handling and Procedures. All animal experiments were conducted in accordance with the guidelines of the UCLA and Fukushima Medical University Animal Research Committee. The generation of mice containing targeted disruption of the HO-1 gene was done as previously described [4]. HO-1-deficient mice were generated with C57BL/6J and 129/sv mixed genetic background. C57BL/6J mice for backcross were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were fed a standard rodent chow diet containing 4% (wt/wt) fat and <0.04% (wt/wt) cholesterol (Oriental Bio, Tsukuba, Japan). Animals were housed four to five per cage and maintained in a temperature-controlled room with a 12-hour light/dark cycle, and animals were strictly monitored for microorganisms.

2.3. Atherosclerotic Lesion Analysis. Following sacrifice, the heart and proximal aorta were excised and washed in phosphate-buffered saline to remove blood. The basal portion of the heart and proximal aorta were embedded in OCT compound (Tissue Tek, Elkhart, IN), frozen on dry ice, and stored at -70°C until they were sectioned. Serial 10-μm cryosections were collected on poly-D-lysine-coated slides, stained with oil red O and hematoxylin, and examined by light microscopy. Atherosclerotic lesion area was calculated using serial sections of the first 400 μm of the ascending aorta as previously described [22]. Hematoxylin-eosin and Elastica Van Gieson staining were done with the paraformaldehyde fixed sections.

2.4. Hematocrit, Plasma Lipoprotein Analyses, and Lipid Peroxidation Assay. Blood was collected from the retro-orbital plexus of mice fasted overnight using heparin-coated capillaries (Fisher Scientific) into a heparin-treated Microtainer tube (Becton Dickinson) and centrifuged at 4°C. The hematocrit was determined by the use of capillary microhematocrit technique in blood obtained. Plasma cholesterol and triglyceride concentrations were determined enzymatically as described previously [23].

2.5. Plasma Lipoproteins and Lipid Analyses. Lipoprotein fractions from pooled mice plasma were isolated by fast performance liquid chromatography [23]. Lipoprotein concentrations are expressed according to their protein content. The characterization of isolated HDL fractions from mouse plasma has previously reported [24]. The protein content of lipoproteins was measured using the method of Lowry et al. [25]. HDL fraction was electrophoresed by 1% agarose gel and stained with Nile Red. SDS-PAGE with 4–20% gradient gel was performed according to the procedure of Laemmli [26].

2.6. Paraoxonase Assay. Paraoxonase activities were assayed using paraoxon as substrate [27]. The cuvette contained 1.0 mM paraoxon in 20 mM Tris/HCl (pH 8.0). The reaction was initiated by the addition of the plasma, and the increase in the absorbance at 405 nm was recorded. Blanks were included to correct for spontaneous hydrolysis of paraoxon. Enzymatic activity was calculated from the molar extinction coefficient $1310\,M^{-1}\,cm^{-1}$. 1 unit of paraoxonase activity is defined as 1 nmol of 4-nitrophenol formed per min under the above assay conditions [27]. For purified paraoxonase standard solutions, paraoxonase isoforms were isolated as described previously [27].

2.7. Other Procedures. Plasma lipid peroxidation as a malondialdehyde was measured with a kit from Oxis (Portland, OR). All values are expressed as means ± SD. Significant difference was determined by one-way ANOVA analysis with Fisher's post hoc test. $P < 0.05$ was considered significant.

3. Results and Discussion

3.1. Aortitis, Coronary Arteritis, and Atherosclerosis in HO-1-Knockout Mice. To elucidate the effect of HO-1 on vascular system, we examined male HO-1-knockout mice aged over 1 year which were bred on a standard rodent chow diet.

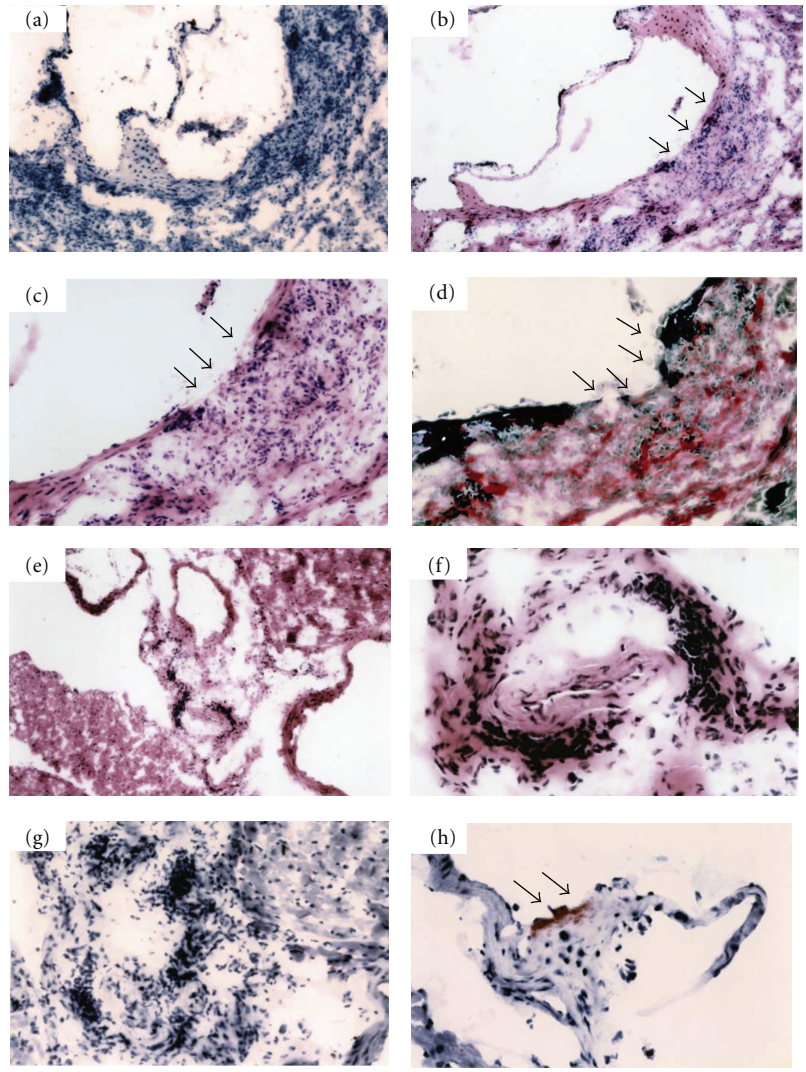

Figure 1: Severe aortitis, coronary arteritis and small atherosclerotic lesion on a standard rodent chow diet after 1 year old of HO-$1^{-/-}$ mice. (a, g, h) Oil red O staining. (b, c, e, f) Hematoxylin- eosin staining. (d) Elastica Van Gieson staining. (d) Elastic fibers in the vessel walls are torn at the site of severe mononuclear cell infiltration. Magnifications at (a, e) ×40, (b) ×100, (c, d) ×200, and (f, g, h) ×400. Predominant sites of aortitis (b, c, d) and initial fatty streak formation (h) are indicated by arrows. These photomicrographs are representative of numerous sections examined.

Table 1: Body weight and plasma lipid levels of HO-1-knockout mice.

HO-1 genotype	+/+($n = 7$)	+/−($n = 6$)	−/−($n = 5$)
Body weight (g)	45.6 ± 4.3	43.8 ± 3.8	30.6 ± 3.9*
Hematocrit (%)	46 ± 4	44 ± 2	41 ± 2
Total cholesterol (mg/dL)	92 ± 18	87 ± 11	86 ± 15
Triglyceride (mg/dL)	106 ± 38	26 ± 4	14 ± 3*
HDL-cholesterol (mg/dL)	73 ± 8	72 ± 11	65 ± 9
Free Fatty Acid (mg/dL)	43 ± 7	41 ± 8	36 ± 3

Lipid levels are given in mg/dL ± S.D. Values for lipid levels on a standard chow diet were from mice of 50% C57BL/6 and 50% 129/Sv genetic background. *$P < 0.05$.

These mice had a mixed genetic background of 129Sv and C57BL/6J. HO-$1^{-/-}$ mice had severe infiltration of mononuclear cells at their ascending aortic wall (100%) (Figures 1(a) and 1(b)). This infiltration was transmural, and the intimal elastic laminal structure was severely destroyed (Figures 1(c), and 1(d)). This mononuclear cellular infiltration was not only observed at aorta but also observed at coronary arteries (Figures 1(e), 1(f), and 1(g)). Oil red O staining revealed small atherosclerotic lesion in aortic sinus of HO-$1^{-/-}$ mice even under a standard chow diet though wild-type mice did not develop such lesions (Figure 1(h)). These inflammatory changes of arteries were also observed in heterozygous mice; however, this was less frequently. It is unclear whether these arterial inflammations in HO-1-deficient mice are the response against microorganisms or autoimmune response [28].

3.2. Altered HDL Properties in HO-1-Knockout Mice. Table 1 shows body weights and plasma lipid levels of

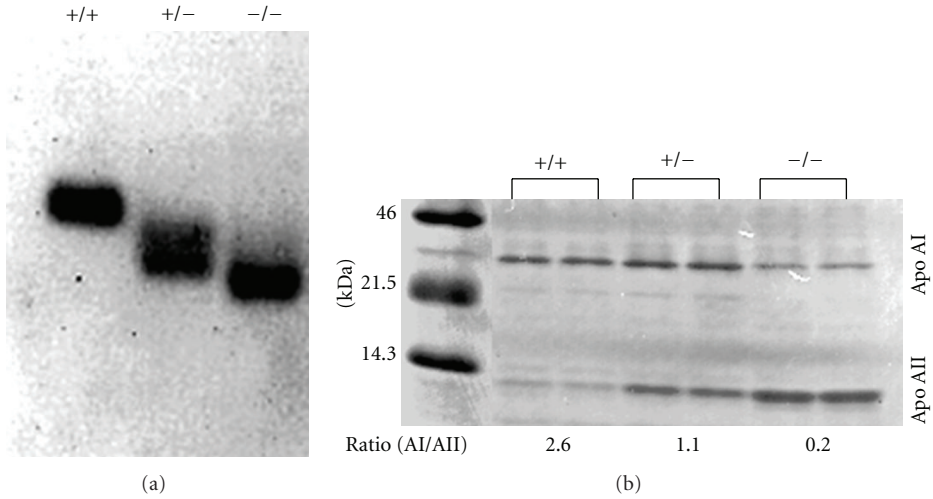

Figure 2: (a) Agarose electrophoresis of HDL in HO-1-knockout mice. 11 μg protein of HDL fraction was electrophoresed in 1% agarose gel and stained with Nile Red. (b) Change of Apolipoprotein AI/AII ratio in HO-1-knockout mice. 1 μg protein HDL was subjected to SDS-PAGE and stained with Nile Red.

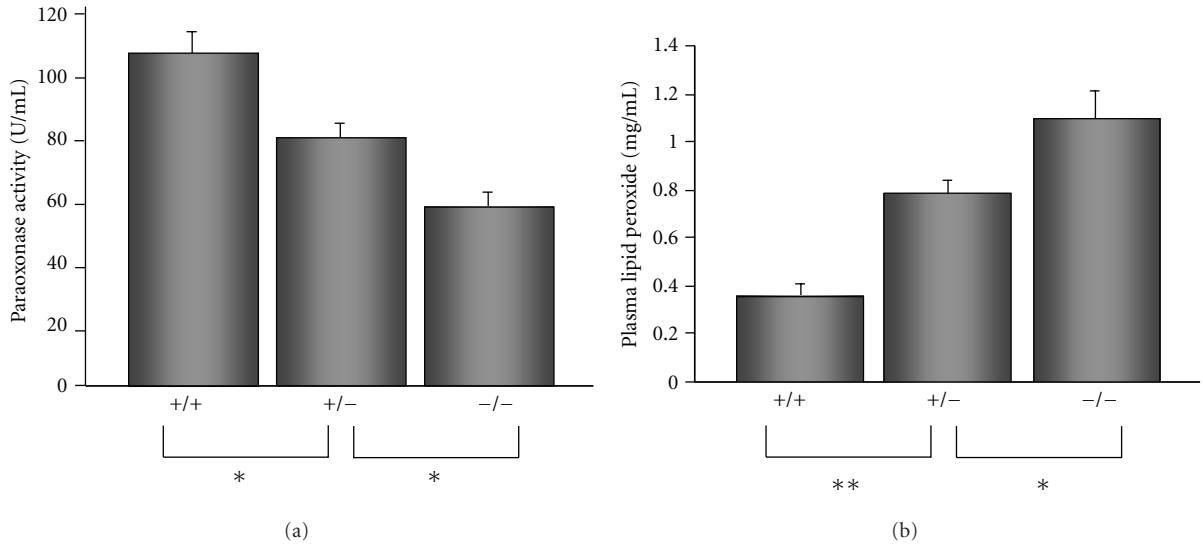

Figure 3: (a) Plasma paraoxonase activities in HO-1-knockout mice. PON activities were determined using arylesterase as substrate and are given as a percentage ± S.D. (b) Plasma lipid hydroperoxide levels in HO-1-knockout mice. Estimates of lipid peroxidation products were obtained by malondialdehyde (MDA) measurements of plasma. Data represents mean ± S.D. from duplicate. Asterisks represent statistically significant differences ($^*P < 0.05$, $^{**}P < 0.01$).

HO-1-knockout mice analyzed. HO-$1^{-/-}$ mice were significantly lighter than HO-1 wild-type (HO-$1^{+/+}$) and HO-1-heterozygous-knockout (HO-$1^{+/-}$) mice. There were no differences in total cholesterol, HDL, and free fatty acid levels among three genotypes; however, triglyceride levels of HO-$1^{-/-}$ and HO-$1^{+/-}$ were much lower than those of HO-$1^{+/+}$ mice. HDLs were prepared by discontinuous density gradient ultracentrifugation from pooled fresh plasma of each genotype, electrophoresed on 1% agarose gel and stained with Nile Red. 11 μg HDL of HO-$1^{-/-}$ and $^{+/-}$ mice runs faster than that of HO-$1^{+/+}$ mice, suggesting HDLs of HO-$1^{-/-}$ and $^{+/-}$ mice are more negatively charged or smaller in size (Figure 2(a)). To examine the composition of apolipoproteins in HDL, 1 μg HDL was subjected to 4–20% SDS-PAGE gel. The ratios of apolipoprotein AI and apolipoprotein AII were different among three genotypes (Figure 2(b)). Apolipoprotein AI was major protein in HO-$1^{+/+}$, whereas apolipoprotein AII was a major in HO-$1^{-/-}$ mice. The ratios of apolipoprotein AI to AII were 2.6, 1.1, and 0.2 in HO-$1^{+/+}$, $^{+/-}$, and $^{-/-}$ mice, respectively, by densitometric analyzes. There are more than 10-fold changes of the ratio of apolipoprotein AI to AII between HO-$1^{+/+}$ and HO-$1^{-/-}$ mice. Studies using transgenic mice models suggested that apolipoprotein AI is antiatherogenic [29] and apolipoprotein AII is atherogenic [23]. Though we cannot explain the mechanism why the absence of HO-1

resulted in the compositional change of HDL and produced oxidized HDL, it may be possible to understand HO-1 functions to prevent HDL particle and apolipoproteins from oxidative stress. It is reported that apolipoprotein AI starts denaturation and is easy to make oligomers in oxidized HDL [30]. However, in this study, we did not find oligomeric bands. It will be also interesting to examine whether HO-$1^{-/-}$ mice have more susceptibilities to atherogenesis by high-fat diet challenges.

Paraoxonase is a calcium-dependent esterase that is known to catalyze hydrolysis of organophosphates and widely expressed in the liver, kidney, intestine, and plasma [31]. Paraoxonase has been suggested to contribute to antioxidant protection of HDL to LDL oxidation in vitro [32] and in vivo [33]. HO-$1^{-/-}$ mice had decreased plasma paraoxonase activity less than 50% compared to HO-$1^{+/+}$ mice (Figure 3(a)). This reduced paraoxonase activity may imply that HDL in HO-$1^{-/-}$ mice was suffered by stronger oxidative stress. There may be unknown relationship between reduced paraoxonase activity and the compositional changes of apolipoprotein AI to AII. Plasma lipid peroxides levels of HO-$1^{-/-}$ mice were higher than those of HO-$1^{+/+}$ mice (Figure 3(b)), suggesting that HO-1 functions to suppress lipoprotein oxidation presumably by production of antioxidants, biliverdin and bilirubin.

Over 95% of HO-$1^{-/-}$ mice die in utero with unknown reason [4]. It may be not easy for HO-$1^{-/-}$ mice to survive without intrinsic antioxidant systems although the reason only a part of those survives is not still clear. In addition, changes in HO-$1^{-/-}$ mice such as lower body weight and decrease of plasma triglyceride need to be further examined by analyzing food consumption and metabolic rate. However, our data using HO-$1^{-/-}$ mice directly suggests a significant function of HO-1 as an anti-inflammatory molecule in artery wall and for native HDL.

Abbreviations

HO: Heme oxygenase
HDL: High-density lipoprotein
LDL: Low-density lipoprotein
oxLDL: Oxidized LDL
−/−: Homozygous knockout
+/−: Heterozygous knockout
+/+: Wild type.

References

[1] M. D. Maines and A. Kappas, "Cobalt induction of hepatic heme oxygenase; with evidence that cytochrome P-450 is not essential for this enzyme activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 71, no. 11, pp. 4293–4297, 1974.

[2] M. D. Maines, N. G. Ibrahim, and A. Kappas, "Solubilization and partial purification of heme oxygenase from rat liver," *Journal of Biological Chemistry*, vol. 252, no. 16, pp. 5900–5903, 1977.

[3] T. Yoshida and G. Kikuchi, "Purification and properties of heme oxygenase from pig spleen microsomes," *Journal of Biological Chemistry*, vol. 253, no. 12, pp. 4224–4229, 1978.

[4] K. D. Poss and S. Tonegawa, "Reduced stress defense in heme oxygenase 1-deficient cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 20, pp. 10925–10930, 1997.

[5] R. Stocker and M. A. Perrella, "Heme oxygenase-1: a novel drug target for atherosclerotic diseases?" *Circulation*, vol. 114, no. 20, pp. 2178–2189, 2006.

[6] R. Stocker, Y. Yamamoto, A. F. McDonagh, A. N. Glazer, and B. N. Ames, "Bilirubin is an antioxidant of possible physiological importance," *Science*, vol. 235, no. 4792, pp. 1043–1046, 1987.

[7] K. Kawamura, K. Ishikawa, Y. Wada et al., "Bilirubin from heme oxygenase-1 attenuates vascular endothelial activation and dysfunction," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 1, pp. 155–160, 2005.

[8] E. C. Theil, "Regulation of ferritin and transferrin receptor mRNAs," *Journal of Biological Chemistry*, vol. 265, no. 9, pp. 4771–4774, 1990.

[9] G. Balla, H. S. Jacob, J. Balla et al., "Ferritin: a cytoprotective antioxidant strategem of endothelium," *Journal of Biological Chemistry*, vol. 267, no. 25, pp. 18148–18153, 1992.

[10] J. R. Stone and M. A. Marletta, "Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states," *Biochemistry*, vol. 33, no. 18, pp. 5636–5640, 1994.

[11] S. W. Ryter, J. Alam, and A. M. K. Choi, "Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications," *Physiological Reviews*, vol. 86, no. 2, pp. 583–650, 2006.

[12] K. Ishikawa, M. Navab, N. Leitinger, A. M. Fogelman, and A. J. Lusis, "Induction of heme oxygenase-1 inhibits the monocyte transmigration induced by mildly oxidized LDL," *Journal of Clinical Investigation*, vol. 100, no. 5, pp. 1209–1216, 1997.

[13] K. Ishikawa, D. Sugawara, X. P. Wang et al., "Heme oxygenase-1 inhibits atherosclerotic lesion formation in LDL-receptor knockout mice," *Circulation Research*, vol. 88, no. 5, pp. 506–512, 2001.

[14] M. S. Brown, Y. K. Ho, and J. L. Goldstein, "The cholesteryl ester cycle in macrophage foam cells. Continual hydrolysis and re-esterification of cytoplasmic cholesteryl esters," *Journal of Biological Chemistry*, vol. 255, no. 19, pp. 9344–9352, 1980.

[15] A. D. Watson, J. A. Berliner, S. Y. Hama et al., "Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein," *Journal of Clinical Investigation*, vol. 96, no. 6, pp. 2882–2891, 1995.

[16] M. Aviram, M. Rosenblat, C. L. Bisgaier, R. S. Newton, S. L. Primo-Parmo, and B. N. La Du, "Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions: a possible peroxidative role for paraoxonase," *Journal of Clinical Investigation*, vol. 101, no. 8, pp. 1581–1590, 1998.

[17] W. A. Boisvert, A. S. Black, and L. K. Curtiss, "ApoA1 reduces free cholesterol accumulation in atherosclerotic lesions of apoE-deficient mice transplanted with apoE-expressing macrophages," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 19, no. 3, pp. 525–530, 1999.

[18] M. Navab, S. T. Reddy, B. J. Van Lenten, G. M. Anantharamaiah, and A. M. Fogelman, "The role of dysfunctional HDL in atherosclerosis," *Journal of Lipid Research*, vol. 50, pp. S145–S149, 2009.

[19] Y. Takahashi, H. Chiba, K. Matsuno et al., "Native lipoproteins inhibit platelet activation induced by oxidized lipoproteins," *Biochemical and Biophysical Research Communications*, vol. 222, no. 2, pp. 453–459, 1996.

[20] K. D. Poss and S. Tonegawa, "Heme oxygenase 1 is required for mammalian iron reutilization," *Proceedings of the National*

Academy of Sciences of the United States of America, vol. 94, no. 20, pp. 10919–10924, 1997.

[21] M. P. Soares, Y. Lin, J. Anrather et al., "Expression of heme oxygenase-1 can determine cardiac xenograft survival," *Nature Medicine*, vol. 4, no. 9, pp. 1073–1077, 1998.

[22] J. H. Qiao, P. Z. Xie, M. C. Fishbein et al., "Pathology of atheromatous lesions in inbred and genetically engineered mice: genetic determination of arterial calcification," *Arteriosclerosis and Thrombosis*, vol. 14, no. 9, pp. 1480–1497, 1994.

[23] C. H. Warden, C. C. Hedrick, J. H. Qiao, L. W. Castellani, and A. J. Lusis, "Atherosclerosis in transgenic mice overexpressing apolipoprotein A-II," *Science*, vol. 261, no. 5120, pp. 469–472, 1993.

[24] R. J. Havel, H. A. Eder, and J. H. Bragdon, "The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum," *The Journal of Clinical Investigation*, vol. 34, no. 9, pp. 1345–1353, 1955.

[25] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein measurement with the Folin phenol reagent," *The Journal of Biological Chemistry*, vol. 193, no. 1, pp. 265–275, 1951.

[26] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680–685, 1970.

[27] K. N. Gan, A. Smolen, H. W. Eckerson, and B. N. La Du, "Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities," *Drug Metabolism and Disposition*, vol. 19, no. 1, pp. 100–106, 1991.

[28] D. Willis, A. R. Moore, R. Frederick, and D. A. Willoughby, "Heme oxygenase: a novel target for the modulation of the inflammatory response," *Nature Medicine*, vol. 2, no. 1, pp. 87–90, 1996.

[29] E. M. Rubin, R. M. Krauss, E. A. Spangler, J. G. Verstuyft, and S. M. Clift, "Inhibition of early atherogenesis in transgenic mice by human apolipoprotein A1," *Nature*, vol. 353, no. 6341, pp. 265–267, 1991.

[30] R. L. Artola, C. B. Conde, L. Bagatolli, R. P. Pécora, G. D. Fidelio, and S. C. Kivatinitz, "High-density lipoprotein from hypercholesterolemic animals has peroxidized lipids and oligomeric apolipoprotein A-I. Its putative role in atherogenesis," *Biochemical and Biophysical Research Communications*, vol. 239, no. 2, pp. 570–574, 1997.

[31] G. S. Getz and C. A. Reardon, "Paraoxonase, a cardioprotective enzyme: continuing issues," *Current Opinion in Lipidology*, vol. 15, no. 3, pp. 261–267, 2004.

[32] M. I. Mackness, S. Arrol, and P. N. Durrington, "Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein," *FEBS Letters*, vol. 286, no. 1-2, pp. 152–154, 1991.

[33] D. M. Shih, L. Gu, Y. R. Xia et al., "Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis," *Nature*, vol. 394, no. 6690, pp. 284–287, 1998.

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Research Article

Renal Heme Oxygenase-1 Induction with Hemin Augments Renal Hemodynamics, Renal Autoregulation, and Excretory Function

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Heme oxygenases (HO-1; HO-2) catalyze conversion of heme to free iron, carbon monoxide, and biliverdin/bilirubin. To determine the effects of renal HO-1 induction on blood pressure and renal function, normal control rats ($n = 7$) and hemin-treated rats ($n = 6$) were studied. Renal clearance studies were performed on anesthetized rats to assess renal function; renal blood flow (RBF) was measured using a transonic flow probe placed around the left renal artery. Hemin treatment significantly induced renal HO-1. Mean arterial pressure and heart rate were not different (115 ± 5 mmHg versus 112 ± 4 mmHg and 331 ± 16 versus 346 ± 10 bpm). However, RBF was significantly higher (9.1 ± 0.8 versus 7.0 ± 0.5 mL/min/g, $P < 0.05$), and renal vascular resistance was significantly lower (13.0 ± 0.9 versus 16.6 ± 1.4 [mmHg/(mL/min/g)], $P < 0.05$). Likewise, glomerular filtration rate was significantly elevated (1.4 ± 0.2 versus 1.0 ± 0.1 mL/min/g, $P < 0.05$), and urine flow and sodium excretion were also higher (18.9 ± 3.9 versus $8.2 \pm 1.0\,\mu$L/min/g, $P < 0.05$ and 1.9 ± 0.6 versus $0.2 \pm 0.1\,\mu$mol/min/g, $P < 0.05$, resp.). The plateau of the autoregulation relationship was elevated, and renal vascular responses to acute angiotensin II infusion were attenuated in hemin-treated rats reflecting the vasodilatory effect of HO-1 induction. We conclude that renal HO-1 induction augments renal function which may contribute to the antihypertensive effects of HO-1 induction observed in hypertension models.

1. Introduction

Heme metabolism is catalyzed by heme oxygenases (HO), which convert heme to carbon monoxide (CO), biliverdin, and free iron [1]. Two isoforms of HO are expressed in the kidney, HO-1 and HO-2 [2, 3]. The kidney has relatively low basal level of HO activity [2, 4] that is mainly derived from the constitutive HO-2 [5–7]. Renal HO-2 is localized to epithelial cells of the proximal tubule, thick ascending limb and distal tubule, connecting tubule, and principal cells of the collecting ducts [8]. Renal HO-1 is induced under certain pathological conditions and in response to several agents [3, 9–11]. The pattern of HO-1 expression in the kidney varies with different inducers utilized [2, 12]. Acute treatment with hemin increases renal cortical dialysate CO concentration and causes diuresis and natriuresis [13]. Increases in renal perfusion pressure (RPP) induce renal CO production, and HO inhibition prevents the pressure-dependent increase in CO and attenuates pressure natriuresis [14]. This suggests that induction of HO-1 could modulate renal hemodynamics and renal excretory function.

HO inhibition during nitric oxide synthesis inhibition constricts afferent arterioles (Aff-Art) [15], and exogenous CO administration dilates renal Aff-Art from normal rats [15, 16]. Furthermore, endogenously produced CO exerts a vasodilatory influence on the renal circulation, and inhibition of HO decreases renal blood flow (RBF) [17–21]. Bilirubin is also produced from heme metabolism by HO and biliverdin reductase and is an abundant endogenous antioxidant [22]. Bilirubin scavenges reactive oxygen species [23–25] and inhibits angiotensin II-mediated activation of NADPH oxidase [26, 27], effects that also potentially cause dilation of the renal microvasculature. Chronic treatment of angiotensin-II-infused hypertensive rats with bilirubin

attenuates urinary protein excretion [28], and inhibition of bilirubin metabolism attenuates angiotensin II-dependent hypertension in mice [29]. Upregulation of renal HO-1 increases CO and biliverdin/bilirubin production, reduces NADPH oxidase-mediated oxidative stress [26], inhibits cortical 20-HETE synthesis [30], and inhibits thromboxane synthase [31]. Overexpression of HO-1 reduces the pressor responsiveness to angiotensin II [32], and inhibition of HO activity magnifies the renal vasoconstrictor effect of angiotensin II and enhances pressure-induced constriction of isolated pressurized renal interlobular arteries [20]. These results implicate HO-derived metabolites as important modulators of renal microcirculatory function. However, recent data indicate that blood pressure and RBF responses to increased angiotensin II or inhibition of nitric oxide are not significantly enhanced in HO-2 knockout mice [33].

This study was designed to examine the hypothesis that renal HO-1 induction augments renal hemodynamics and renal excretory function. To test this hypothesis, we performed renal clearance and renal hemodynamic studies on control and hemin-treated rats. Accordingly, the aims of this study were (1) to determine the effects of HO-1 induction on renal blood flow (RBF), glomerular filtration rate (GFR), and renal excretory function and (2) to determine the effects of HO-1 induction on RBF autoregulatory responses to changes in RPP and on the renal vasoconstrictor responses to angiotensin II.

2. Methods

2.1. Animal Treatment. All experimental protocols were approved by Tulane Institutional Animal Care and Use Committee. Male Sprague Dawley rats weighing 300–400 g were fed a normal rat diet (TD 90229, Harlan-Teklad) with free access to water. Two groups of rats were studied: (1) control rats and (2) Hemin-treated rats which received 4 i.p. injections of hemin for 4 consecutive days (3 mg/100 g Bwt./day) prior to the acute experiment.

2.2. Renal Function Studies. As described by Patterson et al. [34] and Wang et al. [35], on the day of acute experiment, rats were anesthetized with inactin (thiobutabarbital sodium, Sigma, Saint Louis, USA, 100 mg/kg, i.p.) and placed on thermostatically controlled heated surgical table to maintain rectal temperature at 37°C. A polyethylene tube was placed in the trachea and the animals were allowed to breathe air enriched with oxygen (95% O_2/5% CO_2). The left carotid artery was cannulated to allow continuous monitoring of systemic arterial blood pressure and heart rate. The left femoral vein was cannulated to allow infusion of solutions. The left kidney was exposed from a flank incision, gently freed, and placed in a plastic cup. For timed urine collections, a catheter was introduced into the ureter and passed to the pelvis. The renal artery was separated carefully from the renal vein which enabled placement of a noncannulating flow probe, 1 mm in the diameter, connected with a Transonic flowmeter (Transonic System Inc., Ithaca, NY, USA) for measurement of the total RBF. During surgery, an isotonic saline solution containing albumin (6 g/dL) was infused at 1.2 mL/h (for 300 g body weight). Following the surgical procedures, an isotonic saline solution containing 1% albumin and 7.5% polyfructosan (Inutest, Fresenius Kabi, Austria) was infused; initially a priming dose was infused at the rate of 1.6 mL/kg for 5 min followed by a continuous infusion at 1.2 mL/h and a stabilization period of about 1 h was allowed.

2.2.1. Experimental Protocol

Protocol 1. experiments were performed to determine the effects of HO-1 induction with hemin on renal hemodynamics and renal excretory function. After the stabilization period, at least six 15-minute collection periods were initiated during which measurements of mean arterial pressure (MAP), heart rate (HR), RBF, renal vascular resistance (RVR), GFR, urine flow (UV), and urinary sodium excretion ($U_{Na}V$) were averaged. Measurements for all collection periods were then averaged. The data from control and hemin-treated rats were compared.

Protocol 2. these experiments were done to determine the effects of HO-1 induction with hemin on RBF autoregulatory responses. An aortic clamp was placed above the junction of the left renal artery to regulate RPP to the left kidney. The left femoral artery was cannulated, and the catheter was advanced up the aorta to measure RPP at the left kidney. A Transonic flow probe placed around the left renal artery was used to measure changes in RBF in response to decreases in RPP. After the stabilization period, RPP was decreased to 110, 100, 90, and 80 mmHg and was maintained for 5 minutes at each level. MAP, HR, RBF, and RPP were averaged for each time period at different RPP.

Protocol 3. these experiments were done to determine the effects of HO-1 induction with hemin on renal vascular responses to angiotensin II. After the stabilization period, MAP and RBF were measured during i.v. infusion of vehicle followed by angiotensin II (Ang II 50, 100, and 200 ng/kg/min). Each infusion period lasted 30 minutes, and RBF responses were averaged during the last 20 minutes.

2.3. Analytical Procedures and Hemin Preparation. Urine volumes were determined gravimetrically. Blood samples were collected in heparinized tubes and centrifuged at 2500 g for 10 min at 4°C to separate the plasma, which was stored at −20°C. Plasma and urine sodium concentrations were measured by flame photometry. Polyfructosan concentrations in urine and plasma samples were measured by standard spectrophotometry. GFR was determined from the clearance of polyfructosan.

Hemin (Sigma-Aldrich, St. Louis, MO): 50 mg hemin was dissolved in 1 mL of 0.1 M NaOH; this solution was diluted using deionized water and pH was adjusted to 7.8; the final volume was adjusted to 5 mL.

2.3.1. Tissue Preparation. Left kidneys were immediately collected and frozen at −80°C until used for Western blot.

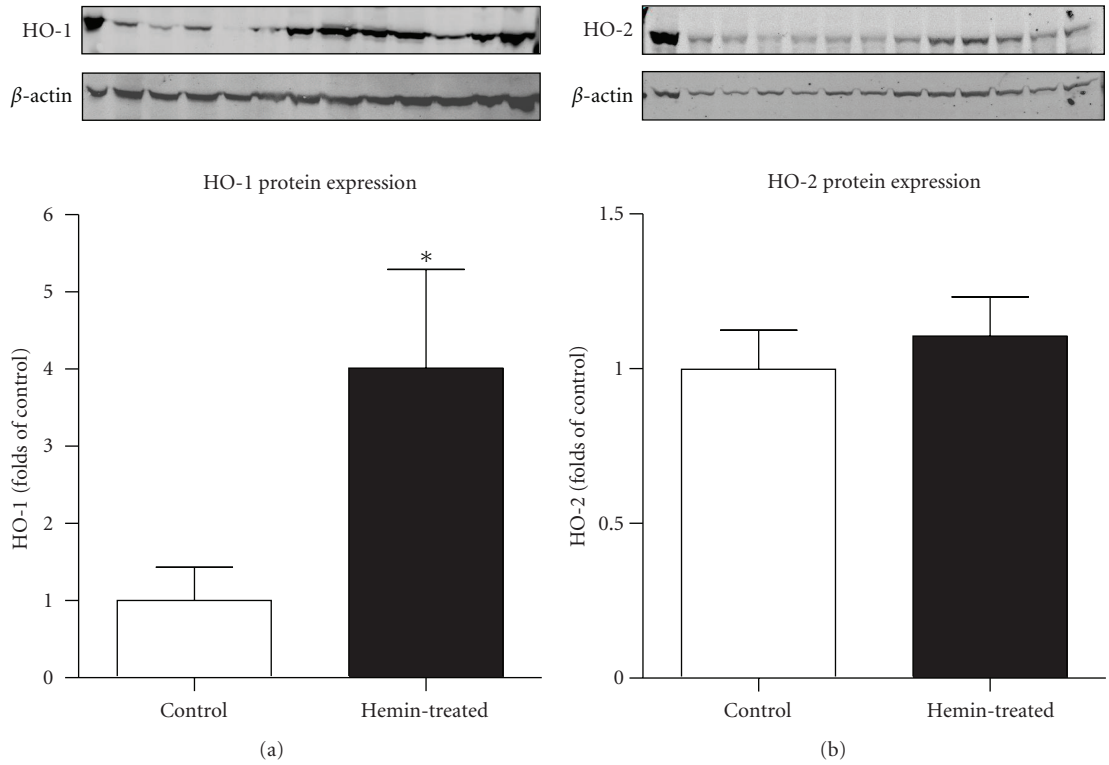

Figure 1: Western blot and densitometry analysis showing the effect of treatment with Hemin (3 mg/100 g body wt/day for 4 days) on renal HO-1 and HO-2 protein expression ($n = 6$) compared to control rats ($n = 6$). Results are normalized by β-actin and expressed as folds of control and presented as mean ± SE for each group, $^*P < 0.05$ versus Control.

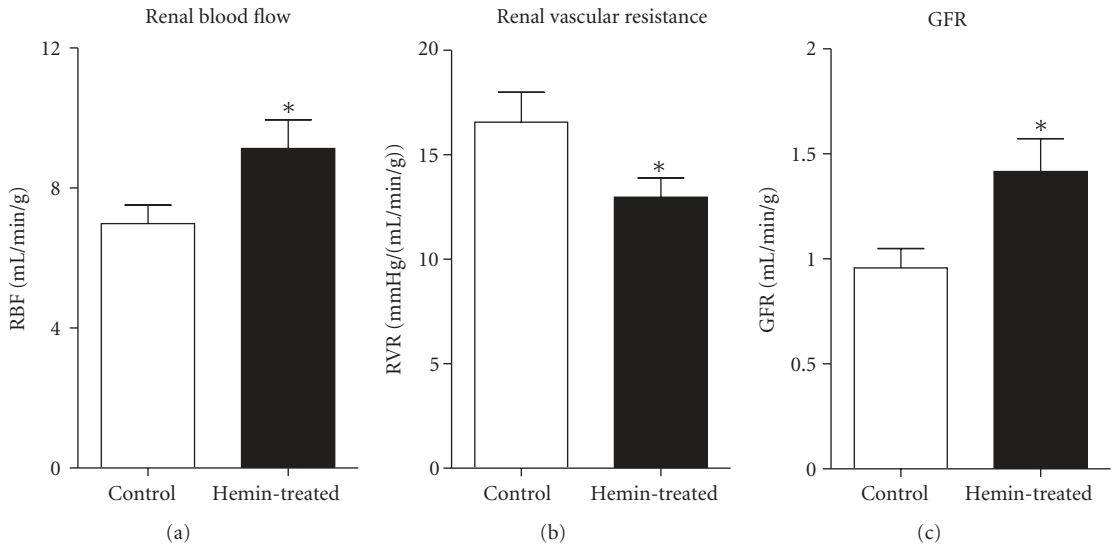

Figure 2: Renal blood flow (RBF), renal vascular resistance (RVR), and glomerular filtration rate (GFR) in hemin-treated rats ($n = 6$) compared to control rats ($n = 7$). Results are expressed as mean ± SE for each group, $^*P < 0.05$ versus Control.

Tissues were homogenized in buffer at pH 7.4, containing 0.25 M sucrose. The homogenates were centrifuged in an Eppendorf centrifuge at 10,000 g for 10 min at 4°C to remove unbroken cells and the supernatant was stored at −80°C. Protein concentration was determined according to the method of Bradford (BioRad, Hercules, CA) [36].

2.3.2. Western Blot Analysis. As previously described [30], cell-free homogenates (10,000 ×g supernatant) of kidney preparations were separated by SDS/polyacrylamide gel electrophoresis and transferred to a hydrophobic polyvinylidene difluoride (PVDF) transfer membrane (Amersham-GE Biosciences, Piscataway, NJ). The membranes were incubated

Table 1: Hemodynamics and renal excretory measurements in hemin-treated rats compared to control untreated rats.

	Control ($n = 7$)	Hemin ($n = 6$)
Mean arterial pressure, MAP (mmHg)	112 ± 4	115 ± 5
Heart rate, HR (bpm)	346 ± 10	331 ± 16
Glomerular filtration rate, GFR (mL/min/g)	1.0 ± 0.1	1.4 ± 0.2*
Renal blood flow, RBF (mL/min/gm)	7.0 ± 0.5	9.1 ± 0.8*
Renal vascular resistance, RVR [mmHg/(mL/min/g)]	16.6 ± 1.4	13.0 ± 0.9*
Urine flow, UV (μL/min/g)	8.2 ± 1.0	18.9 ± 3.9*
Sodium excretion, $U_{Na}V$ (μmol/min/g)	0.2 ± 0.1	1.9 ± 0.6*

* $P < 0.05$ versus control.

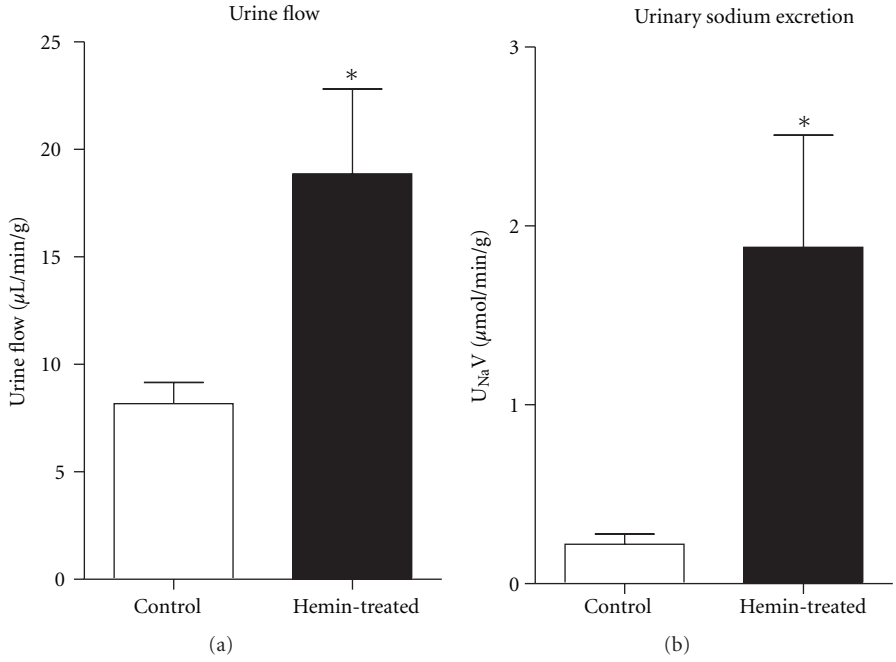

Figure 3: Urine flow (UV) and urinary sodium excretion ($U_{Na}V$) in hemin-treated rats ($n = 6$) compared to control rats ($n = 7$). Results are expressed as mean ± SE for each group, *$P < 0.05$ versus Control.

with Odyssey blocking reagent (LI-COR Biosciences, Lincoln, Nebraska) at 4°C overnight. The membranes were incubated for 1 hr with one of the following antibodies: rabbit anti-rat HO-1 and HO-2 polyclonal antibodies (1 : 1000, Stressgen Biotechnologies Corp, Victoria, BC, Canada) or mouse anti-β-actin monoclonal antibody. The membranes were washed with phosphate buffered saline tween-20 (PBST) and subsequently probed with fluorescent tagged secondary antibodies at a dilution of 1 : 15000. The signal was detected using Odyssey fluorescent scanner.

2.4. Statistical Analysis. Results are presented as mean ± SE for a number (n) of experiments. Unpaired t-test was used to analyze differences in basal measurements, and HO-1 and HO-2 protein expression between the control and hemin-treated groups. Repeated measures one-way ANOVA followed by Bonferroni's multiple comparison test was used to analyze changes within the same group. Repeated measures two-way ANOVA followed by Bonferroni's multiple comparison test was used to analyze differences between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. HO-1 Protein Expression Is Induced in Kidneys from Hemin-Treated Rats. HO-1 protein expression significantly increased by 4.0 ± 1.3-fold in kidneys from hemin-treated rats compared to kidneys from normal control rats ($n = 6$, $P < 0.05$) (Figure 1). No differences in renal HO-2 expression between hemin-treated rats and normal control rats were detected (Figure 1).

3.2. Renal HO-1 Induction with Hemin Is Associated with Augmented Renal Hemodynamics and Excretory Function without

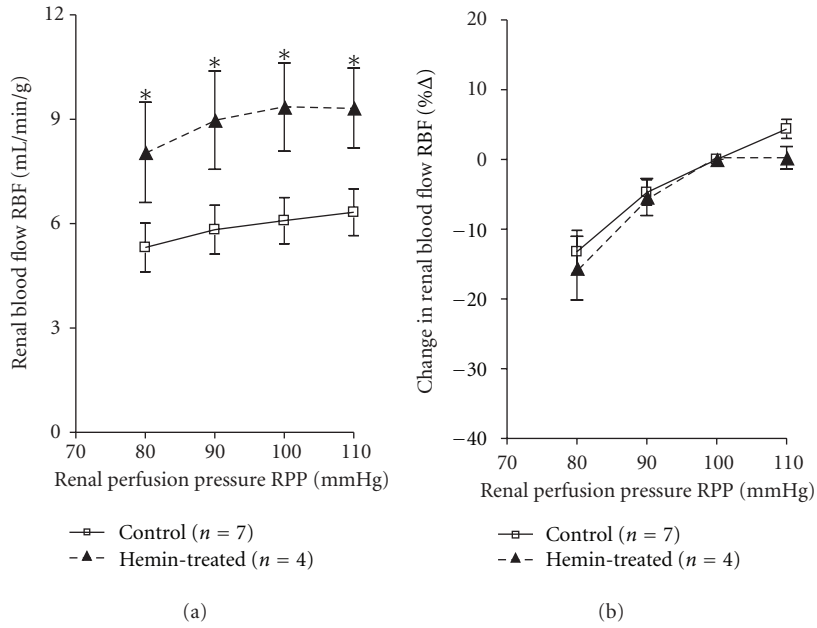

Figure 4: Renal blood flow (RBF) autoregulatory responses to changes in renal perfusion pressure (RPP) in hemin-treated rats ($n = 4$) compared to control rats ($n = 7$). Results are expressed as mean ± SE for each group, $^{*}P < 0.05$ versus Control.

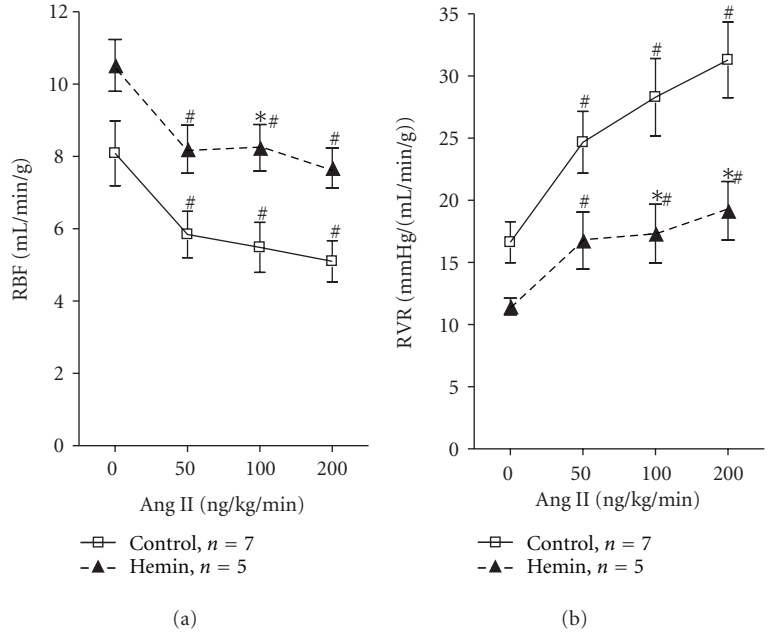

Figure 5: Changes in RBF and RVR in response to infusion of angiotensin II in hemin-treated rats ($n = 5$) compared to control rats ($n = 7$). Results are expressed as mean ± SE for each group, $^{*}P < 0.05$ versus Control, $^{\#}P < 0.05$ versus baseline before angiotensin II infusion.

Alteration in Mean Arterial Pressure or Heart Rate. HR and MAP were not significantly different in hemin-treated rats compared to control rats (Table 1). While RBF and GFR were significantly higher, RVR was significantly lower in hemin-treated rats (Table 1 and Figure 2). These data indicate that renal HO-1induction is associated with renal vasodilation in hemin-treated rats.

Hemin-treated rats also had increased urine flow and urinary sodium excretion ($U_{Na}V$) indicating that renal HO-1 induction is associated with augmented renal excretory function (Table 1 and Figure 3).

3.3. Hemin-Treatment Increases the RBF Autoregulatory Plateau in Response to Decreases in RPP. Figure 4(a) shows that RBF was significantly higher in hemin-treated rats compared to control rats at all measured RPP points indicating that the vasodilatory effect is maintained at different renal

perfusion pressures. Figure 4(b) demonstrates that RBF autoregulatory responses to changes in RPP were not altered in hemin-treated rats indicating that renal autoregulatory responses are maintained with a tendency to show improved autoregulatory capability.

3.4. Renal HO-1 Induction with Hemin Is Associated with Attenuated Renal Vasoconstrictor Responses to Ang II and Maintained Pressor Responses. Basal MAP was not significantly different between control and hemin treated rats (126.0 ± 2.1 mmHg, $n = 7$ versus 118.9 ± 3.9 mmHg, $n = 5$). RBF was significantly higher in hemin treated rats compared to control rats (10.5 ± 0.7 mL/min/g, $n = 5$ versus 8.1 ± 0.9 mL/min/g, $n = 7$, $P < 0.05$).

In response to angiotensin II infusion, MAP significantly increased to the same extent in both groups. In response to infusion with Ang II 50, 100, and 200 ng/kg/min, MAP increased from 126.0 ± 2.1 mmHg to 134.9 ± 1.7, 142.7 ± 1.6, and 150.7 ± 2.2 mmHg in the control group ($n = 7$) and from 118.9 ± 3.9 to 131.4 ± 7.2, 136.9 ± 9.3, and 142.7 ± 9.7 mmHg in the hemin-treated rats ($n = 5$). RBF significantly decreased in response to infusion with Ang II 50, 100, and 200 ng/min/kg in both groups; however, RBF was significantly higher in the hemin-treated group compared to the control group ($P < 0.0001$). RBF during infusion with 200 ng/min/kg Ang II was 7.7 ± 0.6 mL/min/g in hemin-treated group ($n = 5$) compared to 5.1 ± 0.6 mL/min/g in control group ($n = 7$) (Figure 5). The reduced responsiveness to Ang II infusion in the hemin treated group is shown by the attenuated increases in RVR with the Ang II infusions.

4. Discussion

HO-1 plays a major renoprotective role when induced under certain pathophysiological conditions [4, 9, 17, 19]. Although several reports examined effects of acute activation or inhibition of HO via acute administration of the substrate hemin or HO inhibitors, the effects of chronic induction of HO-1 on renal hemodynamics and renal excretory function have not been examined before. To determine the effects of HO-1 on renal function, hemin was used to induce HO-1 expression [1]. Hemin increases HO activity via increasing substrate availability and also by transcriptional activation of hmox-1 gene [37]. Treating with HO-1 inducers causes differential induction of HO-1 in the kidney; increased expression of HO-1 in different renal structures may exert different effects on renal function [38]. As expected, in our study chronic hemin administration increased HO-1 expression whereas it did not affect HO-2 expression.

In rats treated with hemin, MAP was not different compared to untreated rats; however, hemin-treated rats had significantly higher RBF and lower RVR. These results indicate that renal HO-1 induction exerts a renal vasodilatory effect. This is consistent with previously reported data that acute heme administration increases RBF, urine flow, and sodium excretion [13]. In addition, hemin-treated rats had significantly higher GFR compared to control rats indicating that the vasodilatory response is mediated through dilation of renal arterioles. These data are consistent with previous reports showing that acute inhibition of HO decreases RBF [18, 20] and infusion of CO increased RBF and GFR. In addition, CO causes vasodilation of afferent arterioles [15]. Interestingly, this renal vasodilatory response in hemin-treated rats was associated with significantly higher urine flow (2-fold) and sodium excretion (10-fold) (Figure 3), indicating that HO-1 induction exerts diuretic and natriuretic responses. This is consistent with previous data showing that acute heme administration causes natriuresis [13], while acute HO inhibition decreases urine flow and sodium excretion [39]. With chronic treatment, induction of HO-1 may be in different segments of the nephron and may be inhibiting other ion transporters; however, the effects of HO products on tubular transporters have not been well characterized. In addition, previous research shows that acute inhibition of HO with CrMP attenuates pressure-natriuresis, indicating that HO participates in the natriuretic response to increases in arterial pressure [14]. We speculate that the effects of chronic HO-1 induction on renal function are mediated directly via increases in CO and/or bilirubin production. However, indirect effects via regulation of other heme enzymes and/or renal transporters cannot be excluded.

To determine the effect of this vasodilatory response on renal autoregulatory capability, renal perfusion pressure was decreased using an aortic clamp and the corresponding RBF was recorded. Data presented in Figure 4 indicate that renal autoregulatory responses are maintained in hemin-treated rats albeit at higher RBF and changes in RBF in response to decreases in RPP were comparable in hemin-treated and control rats. This indicates that the renal vascular responses to changes in RPP are maintained; however, the plateau of the autoregulation relationship is elevated reflecting the vasodilatory effect of HO-1 induction. This may protect the kidney from excessive vasoconstriction during hypertension while limiting the deleterious effects of the increases in renal perfusion pressure, which could explain the renoprotective role of HO induction in different models of hypertension [38]. To examine this hypothesis, changes in RBF in response to acute infusion of angiotensin II were compared between hemin-treated and control untreated rat.

Angiotensin II infusion increased MAP to the same level in hemin-treated rats and control rats; however, renal vasoconstrictor responses were significantly attenuated in hemin-treated rats (Figure 5). This observation is consistent with previous data showing that the inhibition of HO with SnMP magnifies the renal vasoconstrictor effect of angiotensin II [20]. Although RBF significantly decreased in both control and hemin-treated rats, the increase in RVR in response to angiotensin II was significantly greater in control rats than in hemin-treated rats (Figure 5). This finding is of significant importance because induction of HO-1 by heme administration to angiotensin-II-infused hypertensive rats increases creatinine clearance and decreases proteinuria [17], indicating a renoprotective effect of HO-1 during angiotensin II hypertension. In addition, chronic hemin infusions normalize blood pressure in spontaneously

hypertensive rats [40] and induction of HO-1 prevents the blood pressure increase in angiotensin-II-infused mice [41].

In summary, the present study demonstrates that pharmacological renal HO-1 induction increases RBF, GFR, urine flow, and sodium excretion; these effects are conducive to renoprotection. Renal autoregulatory responses are maintained but operate at a higher RBF during HO-1 induction. Finally, renal HO-1 induction with hemin significantly attenuates renal vasoconstrictor responses to angiotensin II. Under several pathophysiological conditions, HO-1 is induced in the kidney [9]; renal HO-1 induction under these circumstances might play a renoprotective role by attenuating excessive renal vasoconstrictor responses.

Disclosure

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References

[1] R. Tenhunen, H. S. Marver, and R. Schmid, "The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 61, no. 2, pp. 748–755, 1968.

[2] J. L. Da Silva, B. A. Zand, L. M. Yang, H. E. Sabaawy, E. Lianos, and N. G. Abraham, "Heme oxygenase isoform-specific expression and distribution in the rat kidney," *Kidney International*, vol. 59, no. 4, pp. 1448–1457, 2001.

[3] N. Hill-Kapturczak, S. H. Chang, and A. Agarwal, "Heme oxygenase and the kidney," *DNA and Cell Biology*, vol. 21, no. 4, pp. 307–321, 2002.

[4] F. T. Botros, M. L. Schwartzman, C. T. Stier Jr., A. I. Goodman, and N. G. Abraham, "Increase in heme oxygenase-1 levels ameliorates renovascular hypertension," *Kidney International*, vol. 68, no. 6, pp. 2745–2755, 2005.

[5] N. G. Abraham, G. S. Drummond, J. D. Lutton, and A. Kappas, "The biological significance and physiological role of heme oxygenase," *Cellular Physiology and Biochemistry*, vol. 6, no. 3, pp. 129–168, 1996.

[6] M. D. Maines, "The heme oxygenase system: a regulator of second messenger gases," *Annual Review of Pharmacology and Toxicology*, vol. 37, pp. 517–554, 1997.

[7] M. D. Maines, G. M. Trakshel, and R. K. Kutty, "Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible," *Journal of Biological Chemistry*, vol. 261, no. 1, pp. 411–419, 1986.

[8] Y. Hu, N. Ma, M. Yang, and R. Semba, "Expression and distribution of heme oxygenase-2 mRNA and protein in rat kidney," *Journal of Histochemistry and Cytochemistry*, vol. 46, no. 2, pp. 249–256, 1998.

[9] K. A. Nath, "Heme oxygenase-1: a provenance for cytoprotective pathways in the kidney and other tissues," *Kidney International*, vol. 70, no. 3, pp. 432–443, 2006.

[10] M. Shepard, P. Dhulipala, S. Kabaria, N. G. Abraham, and E. A. Lianos, "Heme oxygenase-1 localization in the rat nephron," *Nephron*, vol. 92, no. 3, pp. 660–664, 2002.

[11] E. M. Sikorski, T. Hock, N. Hill-Kapturczak, and A. Agarwal, "The story so far: molecular regulation of the heme oxygenase-1 gene in renal injury," *American Journal of Physiology*, vol. 286, no. 3, pp. F425–F441, 2004.

[12] N. G. Abraham, F. T. Botros, R. Rezzani, L. Rodella, R. Bianchi, and A. I. Goodman, "Differential effect of cobalt protoporphyrin on distributions of heme oxygenase in renal structure and on blood pressure in SHR," *Cellular and Molecular Biology*, vol. 48, no. 8, pp. 895–902, 2002.

[13] F. Rodriguez, R. Kemp, M. Balazy, and A. Nasjletti, "Effects of exogenous heme on renal function: role of heme oxygenase and cyclooxygenase," *Hypertension*, vol. 42, no. 4, pp. 680–684, 2003.

[14] N. Li, F. Yi, E. A. Dos Santos, D. K. Donley, and P. L. Li, "Role of renal medullary heme oxygenase in the regulation of pressure natriuresis and arterial blood pressure," *Hypertension*, vol. 49, no. 1, pp. 148–154, 2007.

[15] F. T. Botros and L. G. Navar, "Interaction between endogenously produced carbon monoxide and nitric oxide in regulation of renal afferent arterioles," *American Journal of Physiology*, vol. 291, no. 6, pp. H2772–H2778, 2006.

[16] C. Thorup, C. L. Jones, S. S. Gross, L. C. Moore, and M. S. Goligorsky, "Carbon monoxide induces vasodilation and nitric oxide release but suppresses endothelial NOS," *American Journal of Physiology*, vol. 277, no. 6, pp. F882–F889, 1999.

[17] T. Aizawa, N. Ishizaka, J. I. Taguchi et al., "Heme oxygenase-1 is upregulated in the kidney of angiotensin II-induced hypertensive rats: possible role in renoprotection," *Hypertension*, vol. 35, no. 3, pp. 800–806, 2000.

[18] B. Arregui, B. López, M. G. Salom, F. Valero, C. Navarro, and F. J. Fenoy, "Acute renal hemodynamic effects of dimanganese decacarbonyl and cobalt protoporphyrin," *Kidney International*, vol. 65, no. 2, pp. 564–574, 2004.

[19] T. L. O'Donaughy and B. R. Walker, "Renal vasodilatory influence of endogenous carbon monoxide in chronically hypoxic rats," *American Journal of Physiology*, vol. 279, no. 6, pp. H2908–H2915, 2000.

[20] F. Rodriguez, F. Zhang, S. Dinocca, and A. Nasjletti, "Nitric oxide synthesis influences the renal vascular response to heme oxygenase inhibition," *American Journal of Physiology*, vol. 284, no. 6, pp. F1255–F1262, 2003.

[21] A. P. Zou, H. Billington, N. Su, and A. W. Cowley, "Expression and actions of heme oxygenase in the renal medulla of rats," *Hypertension*, vol. 35, no. 1, pp. 342–347, 2000.

[22] R. Stocker, Y. Yamamoto, and A. F. McDonagh, "Bilirubin is an antioxidant of possible physiological importance," *Science*, vol. 235, no. 4792, pp. 1043–1046, 1987.

[23] J. E. Clark, R. Foresti, C. J. Green, and R. Motterlini, "Dynamics of haem oxygenase-1 expression and bilirubin production in cellular protection against oxidative stress," *Biochemical Journal*, vol. 348, no. 3, pp. 615–619, 2000.

[24] S. Doré, M. Takahashi, C. D. Ferris, L. D. Hester, D. Guastella, and S. H. Snyder, "Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 5, pp. 2445–2450, 1999.

[25] J. Neuzil and R. Stocker, "Free and albumin-bound bilirubin are efficient co-antioxidants for α- tocopherol, inhibiting plasma and low density lipoprotein lipid peroxidation," *Journal of Biological Chemistry*, vol. 269, no. 24, pp. 16712–16719, 1994.

[26] S. R. Datla, G. J. Dusting, T. A. Mori, C. J. Taylor, K. D. Croft, and F. Jiang, "Induction of heme oxygenase-1 in vivo suppresses NADPH oxidase-derived oxidative stress," *Hypertension*, vol. 50, no. 4, pp. 636–642, 2007.

[27] A. Pflueger, A. J. Croatt, T. E. Peterson et al., "The hyperbilirubinemic Gunn rat is resistant to the pressor effects of angiotensin II," *American Journal of Physiology*, vol. 288, no. 3, pp. F552–F558, 2005.

[28] R. M. LeBlanc, L. G. Navar, and F. T. Botros, "Bilirubin exerts renoprotective effects in angiotensin II-hypertension," *American Journal of the Medical Sciences*, vol. 340, no. 2, pp. 144–146, 2010.

[29] T. Vera, J. P. Granger, and D. E. Stec, "Inhibition of bilirubin metabolism induces moderate hyperbilirubinemia and attenuates ANG II-dependent hypertension in mice," *American Journal of Physiology*, vol. 297, no. 3, pp. R738–R743, 2009.

[30] F. T. Botros, M. Laniado-Schwartzman, and N. G. Abraham, "Regulation of cyclooxygenase- and cytochrome P450-derived eicosanoids by heme oxygenase in the rat kidney," *Hypertension*, vol. 39, no. 2, pp. 639–644, 2002.

[31] W. C. Sessa, N. G. Abraham, B. Escalante, and M. L. Schwartzman, "Manipulation of cytochrome P-450 dependent renal thromboxane synthase activity in spontaneously hypertensive rats," *Journal of Hypertension*, vol. 7, no. 1, pp. 37–42, 1989.

[32] L. Yang, S. Quan, A. Nasjletti, M. Laniado-Schwartzman, and N. G. Abraham, "Heme oxygenase-1 gene expression modulates angiotensin II-induced increase in blood pressure," *Hypertension*, vol. 43, no. 6, pp. 1221–1226, 2004.

[33] D. E. Stec, T. Vera, M. V. Storm, G. R. McLemore, and M. J. Ryan, "Blood pressure and renal blow flow responses in heme oxygenase-2 knockout mice," *American Journal of Physiology*, vol. 297, no. 6, pp. R1822–R1828, 2009.

[34] M. E. Patterson, C. R. Mouton, J. J. Mullins, and K. D. Mitchell, "Interactive effects of superoxide anion and nitric oxide on blood pressure and renal hemodynamics in transgenic rats with inducible malignant hypertension," *American Journal of Physiology*, vol. 289, no. 4, pp. F754–F759, 2005.

[35] C. T. Wang, L. G. Navar, and K. D. Mitchell, "Proximal tubular fluid angiotensin II levels in angiotensin II-induced hypertensive rats," *Journal of Hypertension*, vol. 21, no. 2, pp. 353–360, 2003.

[36] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[37] M. J. Tracz, J. Alam, and K. A. Nath, "Physiology and pathophysiology of heme: implications for kidney disease," *Journal of the American Society of Nephrology*, vol. 18, no. 2, pp. 414–420, 2007.

[38] F. T. Botros and L. G. Navar, "Heme oxygenase in regulation of renal function and blood pressure," *Current Hypertension Reviews*, vol. 5, no. 1, pp. 13–23, 2009.

[39] K. E. Jackson, D. W. Jackson, S. Quadri, M. J. Reitzell, and L. G. Navar, "Inhibition of heme oxygenase augments tubular sodium reabsorption," *American Journal of Physiology*, vol. 300, no. 4, pp. F941–F946, 2011.

[40] R. Wang, R. Shamloul, X. Wang, Q. Meng, and L. Wu, "Sustained normalization of high blood pressure in spontaneously hypertensive rats by implanted hemin pump," *Hypertension*, vol. 48, no. 4, pp. 685–692, 2006.

[41] T. Vera, S. Kelsen, L. L. Yanes, J. F. Reckelhoff, and D. E. Stec, "HO-1 induction lowers blood pressure and superoxide production in the renal medulla of angiotensin II hypertensive mice," *American Journal of Physiology*, vol. 292, no. 4, pp. R1472–R1478, 2007.

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Review Article

Therapeutic Potential of Heme Oxygenase-1/Carbon Monoxide in Lung Disease

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Heme oxygenase (HO), a catabolic enzyme, provides the rate-limiting step in the oxidative breakdown of heme, to generate carbon monoxide (CO), iron, and biliverdin-IXα. Induction of the inducible form, HO-1, in tissues is generally regarded as a protective mechanism. Over the last decade, considerable progress has been made in defining the therapeutic potential of HO-1 in a number of preclinical models of lung tissue injury and disease. Likewise, tissue-protective effects of CO, when applied at low concentration, have been observed in many of these models. Recent studies have expanded this concept to include chemical CO-releasing molecules (CORMs). Collectively, salutary effects of the HO-1/CO system have been demonstrated in lung inflammation/acute lung injury, lung and vascular transplantation, sepsis, and pulmonary hypertension models. The beneficial effects of HO-1/CO are conveyed in part through the inhibition or modulation of inflammatory, apoptotic, and proliferative processes. Recent advances, however, suggest that the regulation of autophagy and the preservation of mitochondrial homeostasis may serve as additional candidate mechanisms. Further preclinical and clinical trials are needed to ascertain the therapeutic potential of HO-1/CO in human clinical disease.

1. Introduction

Stress-inducible protein systems represent a common and ubiquitous strategy that eukaryotic cells and tissues employ to maintain cellular homeostasis in adverse environments. Of these, the heat shock proteins (HSPs), whose synthesis increases with heat stress, and whose accumulation in turn confers survival advantage to cells undergoing heat stress, were among the first to be identified [1–3]. HSPs act as protein chaperones which play multifunctional roles in protein trafficking and in the clearance of denatured protein aggregates [3]. Although not strictly heat inducible in all cell types, the increased expression of a low-molecular-weight stress protein (32–34 kDa) has emerged as a general response to chemical and physical stress in cultured cells [4–6]. Although the agents that induce this response belong to seemingly disparate chemical and physical classes, a common feature is their potential to evoke cellular oxidative stress (i.e., altered redox homeostasis), and/or to stimulate the inflammatory response [4–10]. The 32–34 kDa protein was identified as identical to heme oxygenase-1 [4], (HO, E.C. 1.14.99.3), a catabolic enzyme, which provides the rate-limiting step in the oxidative breakdown of heme. In the presence of O_2 and the electron donor, NADPH: cytochrome p-450 reductase, HO converts heme to biliverdin-IXα, which is then converted to bilirubin-IXα by biliverdin reductase [11] (Figure 1). Additionally, ferrous iron and carbon monoxide (CO) are released during heme degradation [11].

The lung represents a critical organ for toxicological studies, since it provides essential life-sustaining functions in the transfer of molecular oxygen (O_2) to the circulatory system for ultimate use in respiration and energy generation, and at the same time can act as a major portal of entry for xenobiotic and pathogen exposure [12]. The expression of

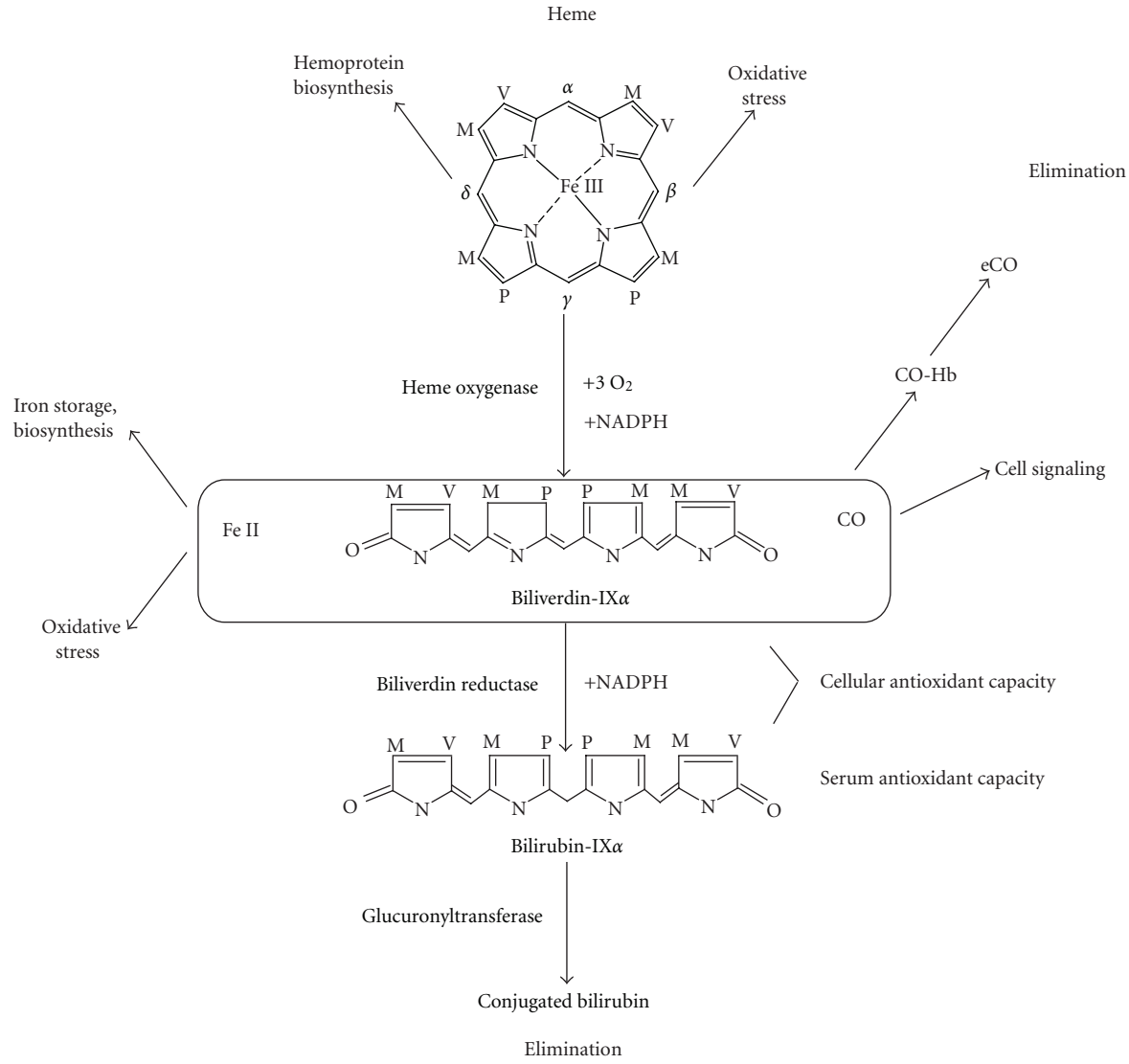

Figure 1: The heme oxygenase reaction. Heme oxygenase-1 catalyzes the rate-limiting step in heme degradation. The reaction produces biliverdin-IXα, carbon monoxide (CO), and ferrous iron (Fe II), at the expense of molecular oxygen and NADPH. Biliverdin-IXα produced in the HO reaction is then converted to bilirubin-IXα by biliverdin reductase. (Side chains are labeled as M: methyl, V: vinyl, P: propionate). The reactants and products of these enzymatic reactions have numerous and diverse biological sequelae. Heme is a vital molecule used in biosynthesis of cytochromes and other hemoproteins. Accumulation of this metabolite may promote deleterious oxidative reactions. Biliverdin-IXα and bilirubin-IXα may serve as cellular antioxidants, whereas circulating bilirubin may also provide antioxidant benefit in plasma. Bilirubin-IXα is conjugated by hepatic glucuronyltransferases and secreted by the biliary fecal route. CO has numerous signal transduction effects as outlined in this review. Systemic CO forms bind hemoglobin to form carboxyhemoglobin (CO-Hb). CO eventually diffuses to the lung where it is eliminated as exhaled CO (eCO). Fe (II) represents a potentially toxic metabolite of heme degradation. A potential metabolic fate of the released iron is sequestration by the iron storage protein ferritin.

HO-1 is now believed to act as a general protective mechanism of the lung in response to stress stimuli, especially those involving oxidative or inflammatory components [13–16].

HO-1 has in recent years been demonstrated to confer protection in a number of preclinical animal models of tissue injury and disease [13–20] (reviewed in [21]). This review will highlight those aspects of HO-1 tissue protection relevant to lung disease. Furthermore, accumulating studies over the past decade have shown that the exogenous application of the HO-1 end-product CO, when administered at low concentrations, or alternatively, by pharmacological application of carbon-releasing molecules (CORMs), can also confer protective effects in models of inflammatory stress or tissue injury [22–24] (reviewed in [21, 25]). Tissue protection has also been described for the exogenous application of bile pigments, biliverdin-IXα, and bilirubin-IXα, which represent the end products of the heme degradation pathway [26–28].

Many of the studies concerning HO-1/CO-dependent cytoprotection cite mechanisms involve the modulation of the inflammatory response, including, but not limited to,

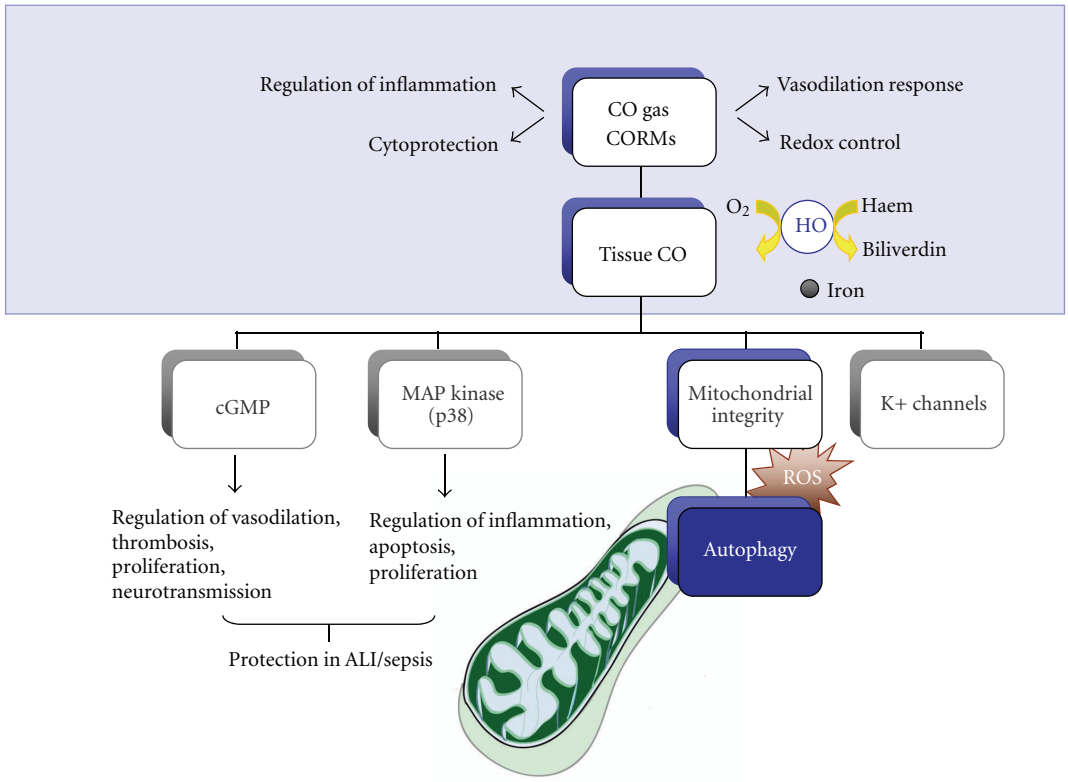

Figure 2: Overview of the signaling pathways relevant to the cytoprotective effects of CO. HO-1 and CO can confer cyto-/tissue-protection in models of acute lung injury (ALI) and sepsis. The homeostatic and beneficial effects of CO gas and CO-releasing molecules (CORMs) in animal models of ALI/sepsis occur through multiple cellular and molecular mechanisms that include regulation of the redox state, inflammation, the vasodilation response. CO gas and CORMs regulate different signaling pathways including cyclic guanosine monophosphate (cGMP), mitogen-activated protein (MAP), kinase signaling pathways, and potassium (K^+) ion channels. Autophagy is regulated by HO-1/CO levels in a cell-type-specific manner and has a role in the maintenance of mitochondrial integrity and modulation of reaction oxygen species (ROS) production.

downregulation of proinflammatory cytokine(s) production [22, 23], as well as the modulation of programmed cell death (i.e., apoptosis)[29, 30], and cell proliferation [31–34], depending on cell type and experimental context (Figure 2). More recent studies, as outlined in this review, suggest additional novel candidate mechanisms for CO-dependent protection, including the regulation of cellular macroautophagy, the maintenance of mitochondrial integrity, and mitochondrial biogenesis. This review will summarize recent findings on the role of HO-1/CO in lung injury and pulmonary disease, with an emphasis on disease pathogenesis and potential therapeutic applications.

1.1. The Heme Oxygenase Enzyme System. The microsomal enzyme heme oxygenase (HO, E.C. 1:14:99:3) exerts a vital metabolic function in the regulation of cellular and tissue heme homeostasis and consequently affects intracellular and tissue iron distribution [35]. The HO enzyme was originally discovered (*ca.* 1968-1969) as an NADPH-dependent enzymatic activity present in hepatic microsomal membrane preparations that is responsible for heme degradation [11]. HO is distinct from cytochrome p450, the major hepatic microsomal drug- and steroid-metabolizing system [36]. The two systems share some common features, including a requirement for electron mobilization from the reductase component of cytochrome p450 [37–40]. Similar to cytochrome p450, the HO enzyme reaction utilizes an activated oxygen molecule (O_2) bound to the ferrous iron of a heme coenzyme to catalyze substrate oxidation [38]. In contrast, p450 oxidizes a bound substrate (steroid or xenobiotic compound) [37], whereas HO specifically degrades heme [11, 41, 42]. The association of heme with the HO enzyme is transient, such that the bound heme uniquely serves as both catalytic cofactor, and substrate [11, 41, 42].

HO catalyzes the selective ring opening of heme at the α-methane bridge carbon to form the open chain tetrapyrrole biliverdin-IXα. The reaction proceeds through three oxidation cycles, requiring three moles of O_2 per heme oxidized [11, 43]. In each oxidation cycle, electrons from NADPH are utilized to reduce the heme iron to the ferrous form, which is permissive of O_2 binding, and subsequently, to activate the bound O_2 [43]. For each molecule of heme oxidized, one mole each of ferrous iron and carbon monoxide (CO) are also released [11]. In catalyzing the breakdown of heme, HO provides the major source of endogenous biological CO production [11]. The HO reaction, which is rate limiting for the pathway, is generally regarded as a detoxification reaction, in that heme, a potentially deleterious prooxidant

is processed for subsequent elimination steps. The cytosolic enzyme, NAD(P)H: biliverdin reductase, reduces biliverdin-IXα to the hydrophobic pigment bilirubin-IXα [44]. Bilirubin IXα accumulates in serum, where it circulates in a protein-bound form, and acts as a physiological antioxidant [45, 46]. Circulating bilirubin IXα is conjugated to water-soluble glucuronide derivatives by hepatic microsomal phase II enzymes and then subsequently eliminated through the bile and feces [47].

1.2. HO Isozymes. HO can exist in two distinct isozymes: the inducible form, heme oxygenase-1 (HO-1), and the constitutively expressed isozyme, heme oxygenase-2 (HO-2) [48]. The inducible isozyme HO-1 is a ubiquitous mammalian shock protein (identified by molecular-cloning strategies as identical to the major 32 kDa mammalian stress inducible protein) [4]. HO-1 is regulated at the transcriptional level by environmental stress agents. The myriad of inducing conditions that elicit this response is not limited to xenobiotic exposure (i.e., heavy metals, sulfhydryl reactive substances, oxidants) but also includes endogenous mediators (i.e., prostaglandins, nitric oxide, cytokines, heme), physical or mechanical stresses (i.e., shear stress, ultraviolet-A radiation), and extremes in O_2 availability (hyperoxia or hypoxia), as reviewed in [21, 49]. The induction of HO-1 occurs as a general response to oxidative stress [4, 5, 50]. High levels of HO-1 expression occur in the spleen and other tissues responsible in the degradation of senescent red blood cells [11, 51]. With the exception of these tissues, HO-1 expression is generally low in systemic tissues in the absence of stress. Furthermore, the induction of HO-1 is a common response to elevated temperature in rat organs [52].

The constitutively expressed form, HO-2, is expressed abundantly in the nervous and cardiovascular systems [16]. HO-2 catalyzes the identical biochemical reaction as HO-1 but represents a product of a distinct gene and differs from HO-1 in primary structure, molecular weight, and kinetic parameters [53, 54]. HO-2 contains additional noncatalytic heme-binding domains which are not present in HO-1 [55]. The transcriptional regulation of HO-2 is typically refractory to most inducing agents with the exception of glucocorticoids, which stimulate HO-2 transcription in the nervous tissue [56, 57].

1.3. Heme Oxygenase-1: A Cytoprotective Molecule. It is now well established in cell culture and animal studies that HO-1 expression provides a general cyto- and tissue-protective effect, which is elicited as a generalized protective response to environmental derangements. From published studies, it is generally concluded that HO-1 can defend against oxidative stress conditions *in vitro* and *in vivo* by modulating apoptotic and inflammatory pathways [13, 18, 22, 58, 59]. However, the molecular processes and mechanisms, in which HO-1 provides cellular and tissue protection, remain only partially understood. The direct removal of heme may serve an antioxidative function, since heme acts as a prooxidant compound on the basis of its iron functional group [60, 61]. Hypothetically, a buildup of heme from the denaturation of cellular hemoproteins, or from the impaired biosynthesis or assembly of hemoproteins, may result in oxidative stress to the cell, through the promotion of iron-dependent free radical reactions (i.e., Fenton reaction). However, the extent to which the "free" heme pool is mobilized during stress remains unknown. Heme is well known as a lipid peroxidation catalyst in model systems [60, 61] and may cause endothelial cell injury [62]. By breaking down heme, HO liberates heme iron, which can itself represent a deleterious catalytic byproduct with excessive overexpression [63]. HO-derived iron has been shown to drive the synthesis of ferritin, which serves as a protective sink for intracellular redox-active iron [64]. In addition to iron, the reaction products of the HO system, namely, biliverdin/bilirubin, and CO may also contribute to cytoprotection. Evidence for this is based largely on exogenous or pharmacological application of CO or biliverdin/bilirubin as described in detail in the sections below, and it remains incompletely clear whether these mechanisms can account entirely for the cytoprotective properties of the natural enzyme. An emerging consensus is that the pleiotropic effects of HO-1 summarized by the collective effects of the generation and distribution of bioactive products and their downstream sequelae collectively contribute to HO-dependent cytoprotection. In this regard, HO-2 likely also serves as a protective agent against oxidative stress by reducing intracellular heme concentrations and by increasing levels of bilirubin and ferritin, both of which are potent antioxidants [56]. However, HO-2 does not typically respond to transcriptional activation via environmental stimuli, although some posttranscriptional modulation of expression has been described [57, 65].

The critical role of HO-1 in systemic homeostasis was illustrated in the only documented case of HO-1 deficiency in a human subject, who presented with extensive endothelial cell damage, anemia, and abnormal tissue iron accumulation [66]. In addition, knockout mice with the *Hmox1*$^{-/-}$ genotype revealed hepatic and renal iron deposition, anemia and increased vulnerability to oxidative stress [35, 67].

1.4. Biliverdin/Bilirubin Mediators of HO-Dependent Cytoprotection. The cytoprotective effects of HO-1 have been postulated to involve the generation of its end products. The open-chain tetrapyrroles biliverdin and bilirubin exert antioxidant properties *in vitro* [45, 46], which have been demonstrated to confer cytoprotective and antiproliferative properties [27, 28, 68, 69] (reviewed in [70, 71]). Increasing evidence suggests that bilirubin plays an important physiological role as an antioxidant in serum [38, 39]. Increases of serum BR have been correlated with vascular protection and resistance to oxidative stress *in vivo* [72]. Hyperbilirubinemic Gunn rats display reduced plasma biomarkers of oxidative stress following exposure to hyperoxia, relative to normal controls, suggesting that hyperbilirubinemia may confer protection against oxidative stress [72]. Recent clinical studies indicate a relationship between circulating bilirubin levels and risk of vascular disease. Serum BR levels were indicated as an independent, inverse risk factor for coronary artery disease and peripheral vascular disease [73, 74]. In a large-scale prospective study of men, subjects in the midrange of serum

BR concentration were at the lowest incidence of ischemic heart disease relative to those subjects displaying the lowest or highest fifth of serum BR distribution [75]. In healthy subjects, serum BR levels were inversely correlated with two indicators for atherosclerosis [76]. Patients with Gilbert's syndrome, who have increased levels of circulating unconjugated bilirubin due to reduced glucuronyltransferase activity, displayed reduced incidence of ischemic heart disease when compared to the general population [77]. Serum samples from Gilbert's patients were further shown to have increased antioxidant capacity and resistance to oxidation [78]. It should be noted that bilirubin also may exert toxicological consequences at supraphysiological levels, as implicated in the neurological injury associated with neonatal jaundice [79].

2. Protective Effects of HO-1/CO in Lung Injury and Disease

2.1. HO-1/CO in Endotoxemia and Sepsis. HO-1, as an inducible cytoprotective molecule, has been implicated as a modulator of the acute inflammatory response, as demonstrated using *in vitro* and *in vivo* models of inflammatory stress [14, 15, 22]. HO-1 gene expression via adenovirus-mediated gene delivery inhibited the bacterial lipopolysaccharide- (LPS-) induced production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and macrophage inflammatory protein-1β (MIP-1β) in cultured macrophages *in vitro*, and increased the anti-inflammatory cytokine interleukin-10 (IL-10) levels during LPS challenge [22].

HO-1 has also exhibited anti-inflammatory effects through *in vivo* models of inflammatory diseases. Additional studies have shown that enhanced gene expression of HO-1 in rat lungs via intratracheal adenoviral-mediated gene transfer limited murine acute lung injury following influenza virus infection [14] and ameliorated LPS-induced lung injury in mice via increased IL-10 production [15, 22]. Furthermore, administration of biliverdin, a direct product of HO degradation, resulted in a significant decrease of proinflammatory cytokines, such as IL-6, upregulation of IL-10 levels, and reduction of lung injury markers in LPS-treated rats. Thus, biliverdin protected against systemic inflammation and lung injury after lethal exposure to LPS. This defense against LPS-induced injury applied to cultured lung endothelial cells as well as macrophages [80]. HO-1 has also displayed anti-inflammatory effects in various models of tissue injury besides the lung, which include enhanced protection during cardiac [81], renal [82], and liver [83] transplantation.

Several recent studies have implicated a protective role for HO-1 during microbial sepsis [84–87]. Using the cecal ligation and puncture (CLP) technique to induce sepsis, HO-1-deficient mice ($Hmox1^{-/-}$) suffered higher mortality rates compared with HO-1 sufficient mice. These mice were also shown to have an increased level of free circulating heme rendering them more susceptible to death from sepsis [85].

Conversely, targeted overexpression of HO-1 to smooth muscle cells and myofibroblasts, and bowel protected against sepsis-induced mortality associated with *Enterococcus faecalis* infection, enhanced bacterial clearance by increasing phagocytosis and the endogenous antimicrobial response [84].

High-mobility group box-1 (HMGB1) protein can mediate various cellular responses, including chemotaxis and accumulation of proinflammatory cytokines. Thus, this molecule may represent a key target in strategies to limit inflammation. With respect to potential mechanisms for HO-1-mediated protection in sepsis, several studies have demonstrated that circulating levels of HMGB1 contribute to LPS-induced mortality in $Hmox1^{-/-}$ mice [86, 87]. Furthermore, the pharmacological administration of HO-1-inducing compounds (i.e., heme) significantly reduced plasma levels of HMGB1 in mice challenged with LPS or CLP, which was also associated with the reduction of serum TNF-α, and IL-1β levels [86, 87]. Transfection of HO-1 or induction of HO-1-derived CO resulted in a significant reduction in the translocation and release of high-mobility group box 1 (HMGB1) in CLP-induced sepsis *in vivo*. In conclusion, HO-1-derived CO significantly attenuated HMGB1 release during sepsis, and this inhibition is a necessary step of CO in protection against sepsis [87].

In vitro experiments showed that pretreatment with HO-1 inducers, or transfection of HO-1, significantly inhibited HMGB1 release, translocation of HMGB1 from nucleus to cytosol, and release of proinflammatory cytokines (i.e., TNF-α, IL-1β, and IFN-β) in RAW264.7 cells stimulated with LPS. These effects were mimicked by CO donor compounds and reversed by CO scavengers [87]. Thus, inhibition of HMGB1 release via HO-1 treatment may represent a potential application for therapeutic intervention against sepsis [87].

Hemin administration was shown to protect mice from lethal endotoxemia and sepsis induced by LPS or CLP, respectively [87]. In this context, heme administration was used as a pharmacological agent to induce HO-1 in healthy animals before applying sepsis. In contrast however, a recent study has suggested that heme-driven tissue damage contributes to the pathogenesis of severe sepsis. The authors demonstrate that the exacerbated mortality of $Hmox1^{-/-}$ mice subjected to low-grade polymicrobial infection induced by CLP correlated with the accumulation of free heme in the plasma. Administration of free heme to wild-type ($Hmox1^{+/+}$) mice subjected to low-grade microbial infection (nonlethal) was sufficient to elicit a lethal form of severe sepsis. The development of lethal forms of severe sepsis after high-grade infection was associated with reduced serum concentrations of the heme-sequestering protein hemopexin (HPX), a protein produced by the body to scavenge free heme, whereas HPX administration after high-grade infection prevented tissue damage and lethality. Further, the lethal outcome of septic shock in patients was associated with reduced levels of serum HPX concentrations, suggesting that targeting free heme by modulation of HPX might be used therapeutically to treat severe sepsis. Therefore, in a clinical setting, monitoring the patients' levels of circulating heme and/or HPX might be used to predict the likelihood of a fatal outcome in each case of severe sepsis [85].

CO also plays a role in the protection against lung inflammation and injury in rodents. In mice, low doses of CO (250 ppm), as well as HO-1 expression, when administered with a sublethal dose of LPS, selectively inhibited the expression of LPS-induced proinflammatory cytokines including TNFα, IL-1β, and MIP-1β [22]. CO dose-dependently increased LPS-inducible IL-10 [22]. Similar effects were observed in cultured macrophages exposed to CO [22]. The p38 mitogen-activated protein kinase (MAPK) pathway was shown to be important for the CO-mediated effect in these cells [22].

The anti-inflammatory protection against LPS-induced organ injury conferred by CO was also observed in association with inhibition of inducible nitric oxide synthase (iNOS) expression and activity in the lung. In contrast, while CO also protected against LPS-induced hepatic injury, an enhancement of iNOS expression and activity by CO was observed in this organ [88]. Studies of primary lung macrophages and hepatocytes *in vitro* revealed a similar effect; CO inhibited LPS-induced cytokine production in lung macrophages while reducing LPS-induced iNOS expression, and protected hepatocytes from apoptosis while augmenting iNOS expression [88]. It remains unclear to which extent these changes in iNOS contribute to the cytoprotection conferred by CO, as it appears that the functional consequences of iNOS regulation by CO differ in an organ-specific fashion.

Anti-inflammatory effects of CO were also recently demonstrated in a swine model of endotoxin challenge. CO reduced the development of disseminated intravascular coagulation and diminished serum levels of the proinflammatory IL-1β in response to LPS and induced IL-10 after LPS challenge [89]. Recent studies evaluated the efficacy of inhaled CO in reducing LPS-induced lung inflammation in cynomolgus macaques (a nonhuman primate model). CO exposure (500 ppm, 6 h) following LPS inhalation decreased TNF-α release in the bronchioalveolar lavage fluid (BALF) but did not affect IL-6 and IL-8 release, in addition to reducing pulmonary neutrophilia (not observed at lower concentrations of CO). This reduction of pulmonary neutrophilia was as efficacious as pretreatment with a well-characterized inhaled corticosteroid. However, the therapeutic efficacy of CO required relatively high doses that resulted in high carboxyhemoglobin (CO-Hb) levels (>30%). This work highlights the complexity of interspecies variation of dose-response relationships of CO to CO-Hb levels and to the anti-inflammatory functions of CO [90]. This study is the first to examine the therapeutic index and dose-response relationships of CO therapy in nonhuman primates, and this warrants further investigations in humans [90].

2.2. HO-1/CO in High Oxygen Stress. O_2 is required to sustain aerobic life, but paradoxically, due to its biradical nature and reactivity, and consequently its ability to participate in electron transfer reactions, can also be harmful to life [91]. Supraphysiological concentrations of O_2 (hyperoxia) are routinely used in the clinic to prevent or treat hypoxemia and acute respiratory failure [92]. However, prolonged exposure to hyperoxia can result in tissue damage in many organs, including lungs, and lead to the development of both acute and chronic lung injury [92]. Hyperoxia-induced damage in mice is characterized by an alveolar-capillary barrier dysfunction, impaired gas exchange, and pulmonary edema [13, 93]. Elevated HO-1 protein expression was reported in lungs of mice and in cultured epithelial cells subjected to hyperoxia [93]. The expression of *ho-1* in rat lungs by intratracheal adenoviral-mediated gene transfer, which increased HO-1 expression in the bronchiolar epithelium, protected against the development of pulmonary damage during hyperoxia exposure [13]. Rats infected with *ho-1* prior to hyperoxia displayed reductions in lung injury markers, neutrophil infiltration, and apoptosis, and a marked increase in survival against hyperoxic stress when compared to control-infected rats [13]. *In vitro*, HO-1 overexpression also protected epithelial cells against hyperoxia-induced cytotoxicity [58].

Similarly, low doses of CO have been shown to provide protection against hyperoxic lung injury. The administration of CO (250 ppm) during hyperoxia exposure prolonged the survival of rats and mice subjected to a lethal dose of hyperoxia and dramatically reduced histological indices of lung injury, including airway neutrophil infiltration, fibrin deposition, alveolar proteinosis, pulmonary edema, and apoptosis, relative to animals exposed to hyperoxia alone [23, 94]. In mice, hyperoxia was shown to induce the expression of proinflammatory cytokines (i.e., TNFα, IL-1β, IL-6) and activate major MAPK pathways in lung tissue. The protection afforded by CO treatment against the lethal effects of hyperoxia correlated with the inhibited release of proinflammatory cytokines in BALF. Genetic studies in mice revealed that the anti-inflammatory effect of CO depended on the MKK3/p38β MAPK pathway [94]. Corresponding *in vitro* studies of oxidative lung cell injury have also indicated protective effects of low-dose CO application (250 ppm). CO inhibited hyperoxia-induced apoptosis of cultured epithelial cells, which required the activation of the MKK3/p38β MAPK pathway [94] as well as the STAT3 pathway [95]. Further mechanistic studies in pulmonary endothelial cells revealed that low-dose CO application inhibited the initiation and propagation of extrinsic apoptotic pathways in mouse lung endothelial cells subjected to hyperoxia [96]. CO inhibited O_2-induced activation of the death inducing signal complex (DISC) and downstream activation of apoptogenic factors, including caspases (-8, -9, -3) and Bid, thereby affording protection against cell death. CO also diminished membrane-dependent reactive oxygen species (ROS) production during hyperoxia by inhibiting the ERK1/2 MAPK pathway [96].

2.3. HO-1/CO in Ventilator-Induced Lung Injury. Mechanical ventilation is commonly used clinically for the maintenance of critically ill patients. However, this therapeutic tool can lead to the development of acute lung injury (ALI)/and acute respiratory distress syndrome (ARDS). Despite reductions in tidal volume currently implemented during mechanical ventilation in the clinic, the complications of ALI/ARDS continue to present a high rate of mortality (~40%) [97, 98]. The lung damage incurred by mechanical ventilation is referred to as ventilator-induced lung injury (VILI) and

involves a sterile inflammatory response to cyclic stretching of the tissue [99]. An anti-inflammatory effect of CO was first described in a two-hit model of VILI in which rats were subjected to an injurious high tidal volume ventilator setting combined with intraperitoneal endotoxin injection. This model caused increased expression of HO-1 in the lung. The inclusion of low-concentration CO (250 ppm) in the ventilator circuit reduced the inflammatory cell count in BALF. In the absence of cardiovascular derangements, CO dose-dependently decreased TNFα and increased IL-10 content in the BALF [100]. CO application was also found to confer tissue protection in a mouse model of VILI, using moderate tidal volume settings [101, 102]. In the mouse model, mechanical ventilation caused lung injury reflected by increases in protein concentration, and total cell and neutrophil counts in the BALF. CO reduced ventilation-induced cytokine and chemokine production and prevented lung injury during ventilation, as reflected by the inhibition of ventilation-induced increases in BALF protein concentration and cell count, lung neutrophil influx, and pulmonary edema formation [101, 102]. CO also prevented the HO-1 response to mechanical ventilation, indicating a tissue-protective effect that preceded and did not necessarily depend on secondary activation of stress proteins [101]. Inclusion of CO during ventilation increased the expression of the tumor-suppressor protein caveolin-1 in mouse lung epithelium. Mice genetically deficient in caveolin-1 (*Cav-1*$^{-/-}$) were reported to be more susceptible to VILI than their wild-type counterparts. Furthermore, CO ventilation failed to confer protection against mechanical ventilation-induced lung injury in *cav-1*$^{-/-}$ mice, indicating a requirement for caveolin-1 in the protective effects of CO [101]. Mechanical ventilation was also shown to increase the expression of the proinflammatory transcriptional regulator early growth response protein-1 (Egr-1) in the lungs of mice, which in turn was inhibited by CO ventilation. The *Egr-1*$^{-/-}$ mice resisted lung injury during ventilation, relative to their wild-type counterparts, affirming that Egr-1 acts as a proinflammatory mediator in VILI [102].

In lung macrophages, peroxisome proliferator activated receptor-γ (PPAR-γ), a nuclear regulator, has been demonstrated to act as an anti-inflammatory mediator by counteracting the proinflammatory effects of Egr-1 [103]. CO exposure was found to increase PPAR-γ in cultured macrophages. Furthermore, chemical inhibition of PPAR-γ *in vivo* reversed the protective effects of CO in this model with respect to Egr-1 regulation and lung injury parameters [102]. These studies in VILI models are supportive of general protective effects of CO in the maintenance of the alveolar-capillary barrier. CO has also been demonstrated to inhibit alveolar fluid clearance [104], and these effects should also be further studied when implementing CO for pulmonary therapies. These studies collectively suggest that mechanical ventilation in the presence of CO may provide protection in animal models of VILI. Further research is needed to better understand the pathogenesis of VILI as well as the protective potential of CO and other so-called therapeutic gases in these models. It remains unclear whether the protective effects of these gases as observed in the mouse would ultimately translate to clinical effectiveness in humans.

2.4. HO-1/CO in Pulmonary Ischemia Reperfusion Injury and Lung Transplantation. The therapeutic potential of HO-1/CO in ischemia/reperfusion (I/R) injury models has been described extensively in rodent systems. Lung I/R caused by occlusion of the pulmonary artery was shown to cause lung apoptosis, as evidenced by biochemical markers including caspase activation, expression changes in Bcl_2 family proteins, cleavage of PARP, and mitochondrial cytochrome-c release [105]. CO conferred tissue protection in rodents subjected to lung I/R injury, as evidenced by reduced markers of apoptosis, which depended on activation of the MKK3/p38α MAPK pathway [106]. Mechanistic studies from the same laboratory revealed that CO conferred similar antiapoptotic protection in cultured pulmonary artery endothelial cells against anoxia reoxygenation stress, which was dependent on activation of the MKK3/p38α MAPK pathway [106, 107]. Additional proposed pathway mechanisms included the activation of the phosphatidylinositol-3-kinase/Akt pathway and downstream induction of the signal transducer and activator of transcription (STAT)-3 [107].

In vivo studies using homozygous *ho-1* knockout mice (*hmox-1*$^{-/-}$) demonstrated that HO-1 deficiency conferred sensitivity to the lethal effects of lung I/R injury. Application of exogenous CO by inhalation compensated for the HO-1 deficiency in *hmox-1*$^{-/-}$ mice and improved survival subsequent to pulmonary I/R [108]. The protection provided by CO involved the stimulation of fibrinolysis, by the cGMP-dependent inhibition of plasminogen activator inhibitor-1, a macrophage-derived activator of smooth muscle cell proliferation [108]. CO also inhibited fibrin deposition and improved circulation in ischemic lungs [109]. These protective effects were related to the inhibited expression of the proinflammatory transcription factor Egr-1, and the subsequent downregulation of Egr-1 target genes, which contribute to inflammatory or prothrombotic processes. The downregulation of Egr-1 depended on the enhancement of cGMP signaling by CO treatment, leading to the inhibition of the ERK1/2 MAPK pathway [109].

I/R injury also represents an important causative component of graft rejection after lung transplantation. During orthotopic left lung transplantation in rats, the transplanted lungs were shown to develop severe intra-alveolar hemorrhage and intravascular coagulation. The application of continuous CO exposure (500 ppm) markedly preserved the graft and reduced hemorrhage, fibrosis, and thrombosis after transplantation. Furthermore, CO inhibited lung cell apoptosis and downregulated lung and proinflammatory cytokine and growth factor production which were induced during transplantation [110]. Additional studies revealed that protection against I/R and inflammatory injury was reduced in syngeneic rat orthotopic lung transplantation by inhalation exposure to either the donor or the recipient [111]. Delivery of CO to lung grafts by saturation of the preservation media reduced I/R injury and inflammation in syngeneic rat orthotopic lung transplantation [112].

2.5. Protective Role of CO in Vascular Injury. A protective role for CO in vascular injury has been reported. In this study, inhaled CO prevented arteriosclerotic lesions that occur following aorta transplantation in rodent models. Exposure to a low level of CO (250 ppm) for 1 hour before injury was sufficient to suppress intimal hyperplasia arising from balloon injury [32]. The protective effect of CO was associated with inhibition of graft leukocyte infiltration/activation as well as with inhibition of smooth muscle cell proliferation [32]. A more recent study has shown that intravenous injection of CO-saturated saline caused immediate vasodilation and increased blood flow in the hamster skin microcirculation, an effect that lasted up to 90 mins [113]. These changes were related to increased cardiac output and local cGMP levels. This study supports the possible use of CO-saturated solutions as a vasodilator in critical conditions; however, dosage appears to be critical, since higher and lower dosages by a factor of two were ineffective [113].

2.6. Carbon Monoxide and Pulmonary Arterial Hypertension (PAH). Pulmonary arterial hypertension (PAH) is a terminal disease characterized by a progressive increase in pulmonary vascular resistance leading to right ventricular failure. Several studies suggest that HO-1 or CO can exert protective effects in the context of pulmonary hypertension, and reverse hypoxic pulmonary vasoconstriction. The *hmox-1*$^{-/-}$ null mice displayed an exaggerated response to chronic hypoxia relative to wild-type mice, as exemplified by marked right heart hypertrophy, which included right ventricular infarcts and the formation of mural thrombi [114]. Chemical induction of HO-1 inhibited the development of PAH in rat lungs in response to chronic hypoxia [17]. Furthermore, transgenic mice with lung-specific overexpression of HO-1 displayed reduced lung inflammation, pulmonary hypertension, and vascular hypertrophy during chronic-hypoxia treatment, relative to wild-type mice [18]. In monocrotaline- (MCT-) induced hypertension, protective effects were observed by treatment with the antiproliferative agent rapamcyin, which were associated with the induction of HO-1 [115]. *In vitro,* the antiproliferative effect of rapamycin on smooth muscle cells also depended in part on HO-1 expression, as it was diminished in smooth muscle cells derived from *ho-1*$^{-/-}$ mice [115].

Inhalation of CO has been shown to attenuate the development of hypoxia-induced PAH in rats, by a mechanism possibly involving activation of Ca^{2+} -activated K^{+} channels [116] and NO generation [34]. In hypoxia and monocrotaline-induced PAH in rodents, daily CO exposure (250 ppm, 1 h) reversed established PAH and right ventricular hypertrophy and restored right ventricular and pulmonary arterial pressures. CO treatment restored pulmonary vascular architecture to a near-normal condition [34]. The protective effect of CO was endothelial cell dependent and associated with increased apoptosis and decreased cellular proliferation of vascular smooth muscle cells [34]. The ability of CO to reverse PAH was further shown to require endothelial nitric oxide synthase (eNOS) and NO production, as indicated by the inability of CO to reverse chronic hypoxia-induced PAH in eNOS$^{-/-}$ mice [34]. Biliverdin and bilirubin have also been shown to exert antiproliferative effects on vascular smooth muscle and thus may also have therapeutic potential in PAH and other diseases involving aberrant vascular cell proliferation [27, 28].

3. Role of HO-1/CO in the Regulation of Autophagy

In addition to classical mechanisms such as apoptosis and inflammation, several recent intriguing studies suggest that HO-1, and its byproduct CO, can possibly impact the regulation of autophagy, a vital cellular process, which may in part contribute to the cytoprotective mechanism. Macroautophagy (autophagy) is a regulated cellular pathway for the turnover of organelles and proteins by lysosomal-dependent processing. The autophagy mechanism involves double-membrane vesicles, called autophagosomes or autophagic vacuoles, that target and engulf cytosolic material, which may include damaged organelles or denatured proteins. The autophagosomes fuse with lysosomes to form single-membrane autolysosomes. Lysosomal enzymes facilitate a degradation process to regenerate metabolic precursor molecules (i.e., amino acids, fatty acids), which can be used for anabolic pathways and ATP production [117–124]. This process may thereby prolong cellular survival during starvation. During infection, autophagy assists in the immune response by providing a mechanism for the intracellular degradation of invading pathogens, such as bacteria, and may also contribute to adaptive immune mechanisms [123]. At least 30 autophagy-related (*Atg*) genes have been determined, primarily in yeast. The homologues of many of these Atg genes have been shown to participate in the regulation of autophagy [125, 126]. Among these, Beclin 1 (the mammalian homolog of yeast Atg6) represents a major autophagic regulator [126]. Beclin 1 associates with a macromolecular complex that includes the class III phosphatidylinositol-3 kinase (Vps34). The Beclin 1 complex produces phosphatidylinositol-3-phosphate, a second messenger that regulates autophagosomal nucleation [124, 125]. The microtubule-associated protein-1 light chain-3B (LC3B), the mammalian homologue of Atg8 is an important mediator of autophagosome formation, which is found in association with the autophagosomal membrane [127].

Autophagy has been shown to be both protective and injurious in a variety of different models, suggesting that its role in human diseases is complex. Autophagy is generally considered to be protective when it is induced in response to stress, reducing the activation of lethal signal transduction cascades, and maintaining crucial levels of ATP that allow for the generation of proteins and other biosynthetic reactions. Autophagy also facilitates the elimination of potentially toxic protein aggregates, helping to limit the accumulation of ubiquitinylated proteins that otherwise would inhibit proteasome function. Induction of autophagy affects the progression of the cell cycle (and vice versa), suggesting that autophagy can influence cellular sensitivity to cell cycle-dependent toxins [128].

Autophagy is rarely considered a suicidal mechanism as it usually precedes apoptosis or necrosis [128]. Nevertheless, autophagy has been proposed to contribute to Type-II programmed cell death (PCD), a morphologically distinct form of PCD that involves excess levels of cellular autophagy, degradation of irreversibly damaged organelles, and preservation of cytoskeletal elements. Autophagic cell death occurs during development, in a number of homeostatic processes in adulthood that require the elimination of large amounts of cells, and during the neonatal period in order to maintain cellular energy homeostasis and survival [129]. However, there is still no conclusive evidence that a specific mechanism of autophagic cell death exists, as this phenomenon seems to occur only in cells that cannot die by conventional apoptotic mechanisms [130]. Apoptosis can occur at the same time as autophagy in the same cells suggesting a common regulatory mechanism; however, the precise crosstalk between these two processes remains to be elucidated. Several proapoptotic signaling molecules known to induce autophagy include TRAIL [131], TNF [132], FADD, DRP-I (dynamin-related protein-1), and DAPK (death-associated protein kinase) [133]. Ca^{2+} is a major intracellular second messenger involved in mediating both apoptosis and autophagy, where elevated Ca^{2+} induces autophagy which can be inhibited by ER-associated Bcl-2 [134]. The Bcl-2 proteins are also known to be important in both autophagy and apoptosis signaling. Beclin 1 has been shown to interact with Bcl-2 resulting in the inhibition of Beclin 1-mediated autophagy in response to starvation [135, 136]. Further evidence for a cross-talk between apoptosis and autophagy is also supported by a recent study on Atg5. A truncated form of Atg5 (cleaved by calpains 1 and 2) participates in apoptosis regulation and translocates from the cytosol to mitochondria to trigger cytochrome c release and caspase activation [134]. This Atg5 fragment has been shown to bind to Bcl-X_L, displacing Bcl-X_L-Bax complexes, to inactivate Bcl-X_L antiapoptotic activity, thereby promoting Bax-Bax complex formation, which suggests that Atg5 may be an independent key player in both apoptosis and autophagy. Functional mitochondria are also needed for autophagic induction [137]. Mitochondria have been proposed to act as a platform for controlling the crosstalk between stress responses, autophagy, and programmed cell death, however, the exact mechanisms through which autophagy can intercept lethal signaling remain unknown.

The role of autophagy, whether protective or deleterious, in human diseases, or specifically in chronic lung disease remains obscure. Recently, we demonstrated a pivotal role for autophagy in cigarette smoke-induced apoptosis and emphysema. We have observed increased autophagy in mouse lungs subjected to chronic cigarette smoke exposure, and in pulmonary epithelial cells exposed to cigarette smoke extract (CSE). Knockdown of autophagic proteins inhibited apoptosis in response to cigarette smoke exposure *in vitro*, suggesting that increased autophagy was associated with epithelial cell death. We have also observed increased morphological and biochemical markers of autophagy in human lung specimens from patients with chronic COPD, suggestive of novel therapeutic targets for COPD treatment [138].

HO-1 has been associated with both the cytoprotective and cytotoxic functions of autophagy induction (Figure 3). HO-1 induces a cytoprotective role for autophagy in lung epithelial cells in response to cigarette smoke by downregulating apoptosis and autophagy-related signaling [139]. CSE increased the processing of LC3B-I to LC3B-II (the lipidated active form), within 1 hr of exposure in Beas-2B cells. Increased LC3B-II was associated with increased autophagic activity, since inhibitors of lysosomal proteases and of autophagosome-lysosome fusion further increased LC3B-II levels during CSE exposure. CSE concurrently induced extrinsic apoptosis in Beas-2B cells involving early activation of death-inducing-signaling-complex (DISC) formation and downstream activation of caspases (-8, -9, -3). HO-1 protected against such CSE-induced effects; adenoviral-mediated expression of HO-1 inhibited DISC formation and caspase-3/9 activation in CSE-treated epithelial cells, diminished the expression of Beclin 1, and partially inhibited the processing of LC3B-I to LC3B-II. These studies were the first to demonstrate a relationship between autophagic and apoptogenic signaling in CSE-induced cell death, and their coordinated downregulation by HO-1 [139].

We have also shown that HO-1 mRNA expression was elevated in the lungs of mice chronically exposed to cigarette smoke [139], implying that HO-1 is upregulated in response to cigarette smoke. In addition, HO-1 was shown to localise to mitochondria in response to hemin, lipopolysaccharide, and CSE in human alveolar (A549), or bronchial epithelial cells (Beas-2B) [140]. These studies suggest that the intracellular location of HO-1, in this case, translocation to the mitochondria may be important for its role in remediating cellular stress and cell death.

In other models, HO-1 has been shown to upregulate autophagy in hepatocytes, leading to protection against hepatocyte cell death and hepatic injury from infection-induced sepsis in mice [141]. HO-1 and autophagy are both upregulated in the liver in response to sepsis and LPS and have been shown to limit cell death. Pharmacological inhibition of HO-1 activity or knockdown of HO-1 prevents the induction of autophagic signaling in this model and resulted in increased hepatocellular injury, apoptosis, and death [141]. Finally, HO-1 dependent autophagic signaling has also been shown to have anti-inflammatory effects in LPS-stimulated macrophages where HO-1 and autophagy collectively serve to limit cytokine production [142]. HO-1 is integral to regulating and dampening the inflammatory response, as demonstrated by the expressed pro-inflammatory phenotype found in HO-1 knockout mice. Many of the anti-inflammatory effects of HO-1 have been attributed to CO which, when provided exogenously, is known to decrease inflammation in macrophages and other cells.

On the contrary, HO-1 has been shown to promote autophagy and consequent cell death in a number of models. HO-1 overexpression results in the activation of mitochondrial-selective autophagy (mitophagy) resulting in the accumulation of iron-laden cytoplasmic inclusions [143] in Alzheimer's disease and Parkinson's disease. HO-1 has also been implicated in the inhibition of autophagosome

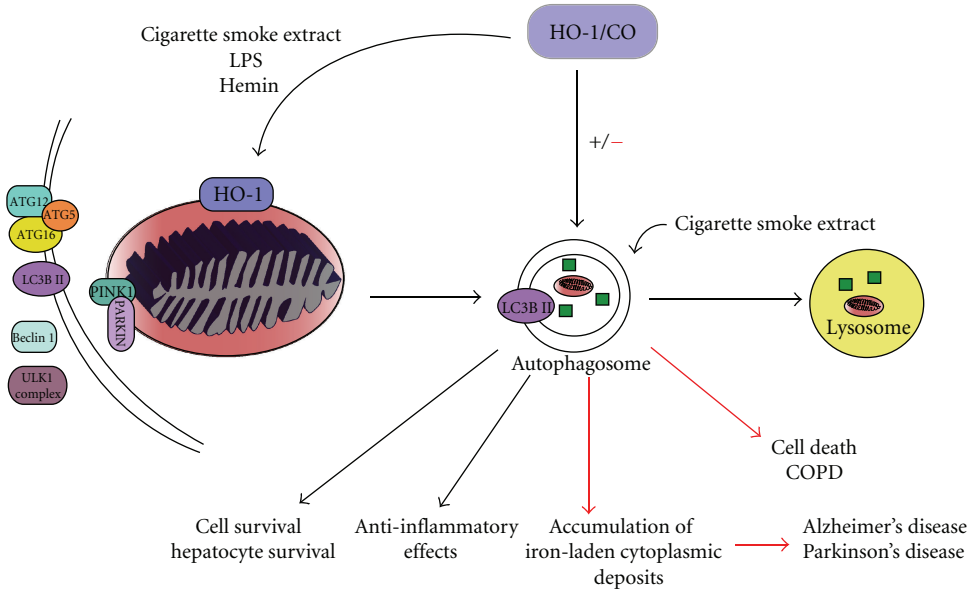

Figure 3: HO-1 as a regulator of autophagy. Autophagic machinery is mobilized in response to stress signals that result in mitochondrial perturbation or accumulations of protein aggregates. A number of proteins have been identified as signaling molecules in preautophagosomal assembly. These include master regulators such as the ULK1 complex, the Beclin-1/Vps34 complex, as well as the autophagic proteins LC3B (Atg8), Atgs 5, 12, 16 which transiently associate with the nascent autophagosome. In inflammation models, HO-1 has been implicated as an inducer of autophagy leading to cell survival and anti-inflammatory effects. In this regard, HO-1 may preserve mitochondrial integrity through the activation of mitochondrial-selective autophagy (mitophagy) which enhances cell survival. In models of neurodegeneration, overexpression of HO-1 leading to activation of autophagy/mitophagy may be detrimental and contribute to neuronal cell death. In lung epithelial cells, HO-1 prevents the induction of autophagy in response to cigarette smoke, leading to cell survival and inhibition of cell death pathways. Overall, the role of HO-1 in controlling cell fate through autophagy is complex. In limited studies to date, the effect of HO-1 on autophagy varies in a cell-type and inducer-specific fashion.

formation in renal tubular epithelial cells exposed to cisplatin promoting their survival. The absence of HO-1 in renal epithelial cells treated with cisplatin results in impaired autophagy and increased apoptosis. Restoring HO-1 expression in these cells reversed the impaired autophagic response and decreased susceptibility to cisplatin-induced apoptosis, validating the importance of HO-1 expression during cisplatin injury [144]. These data suggest that the role of HO-1 in the control of autophagy is specific to differences in stimulus and cell type; however, in general, HO-1 induction and signaling is an adaptive response to restore cellular homeostasis, much like autophagy. This dual nature of autophagy and HO-1 and the increasing number of pathologies they are associated with highlights of the importance of studying the regulation and effects of autophagy and its control by HO-1 during lung injury.

Our recent studies suggest that CO exposure alone has the potential to induce autophagy in epithelial cells. CO treatment increased the expression and activation of the autophagic protein LC3B in mouse lung, and in cultured human alveolar or bronchial epithelial cells, in a time-dependent manner [145]. Furthermore, CO exposure elicited increased autophagosome formation in epithelial cells, as determined by electron microscopy and GFP-LC3 puncta assays. Recent studies indicate that ROS plays an important role in the activation of autophagy. CO upregulated mitochondria-specific generation of ROS in epithelial cells. Furthermore, CO-dependent induction of LC3B expression was inhibited by the general antioxidant N-acetyl-L-cysteine and the mitochondria-targeting antioxidant Mito-TEMPO, suggesting that CO promotes the autophagic process through mitochondrial ROS generation. We further examined the relationships between autophagic proteins and CO-dependent cytoprotection, using a model of hyperoxic stress. CO protected against hyperoxia-induced cell death and inhibited hyperoxia-associated ROS production. The ability of CO to protect against hyperoxia-induced cell death and caspase-3 activation was compromised in epithelial cells infected with LC3B-siRNA, indicating a role for autophagic proteins [145]. These studies uncover a potentially new candidate mechanism for the protective action of CO in lung cells which has not been previously explored. Further investigations are now underway to investigate elucidate the role of autophagy in lung disease and injury, and in the therapeutic potential of HO-1/CO.

4. Pharmacological CO

4.1. Carbon-Monoxide-Releasing Molecules. The development of transition metal-based carbon-monoxide-releasing compounds (CORMs) has provided a pharmacological method for delivery of CO as a promising alternative to inhalation. The CORMs used in experimental studies to date include Mn_2CO_{10} (CORM-1) and the ruthenium-based compounds tricarbonyldichlororuthenium-(II)-dimer (CORM-2) and tricarbonylchoro(glycinato)-ruthenium (II)

(CORM-3) [146, 147]. CORM-1 and CORM-2 are soluble in organic solvents, whereas CORM-3 dissolves in water and rapidly releases CO in physiological fluids. A nontransition metallic water-soluble boron-containing CORM (CORM-A1) has also been developed, which slowly releases CO in a pH and temperature-dependent fashion (half-life of 21 min) [148]. This chemical difference dictates how CO causes vasorelaxation and hypotension as CORM-3 elicits a prompt and rapid vasodilatory effect, whereas CORM-A1 promotes mild vasorelaxation and hypotension [149]. Recently, a novel light-sensitive CORM has been developed [150]. Interestingly, and in contrast to inhaled CO, CORMs appear to deliver CO directly to the tissues without significant formation of CO-Hb. There is an abundance of preclinical evidence in large and small animals showing the beneficial effects of CO, administered as a gas or as CORM, in cardiovascular disease, sepsis, and shock; cancer, acute and chronic rejection of a transplanted organ; kidney, liver injury, and some published reports in the acute lung injury field.

The chemistry of transition metals carbonyls is varied, highly versatile, and not restricted to the above-described compounds. Several subclasses of metal carbonyl compounds containing either manganese, iron, cobalt, molybdenum, or ruthenium have been synthesized and tested for their ability to act as CORMs [151]. Though the discovery of these CORM compounds opens up new possibilities, there are still several issues to overcome for medical applications, particularly those in which downstream tissue sites draining the injection site are targeted. These small molecular drugs diffuse rapidly within the body after administration and may liberate CO prior to reaching these target tissues. Thus, there is considerable need for developing a safe and efficient CO-delivery system. Future work in this area should be directed to the synthesis of CORMs which, beyond an effective therapeutic action and low toxicity, need molecular characteristics with appropriate absorption, distribution, metabolism, and excretion properties [25]. A recent report by Kretschmer demonstrates the synthesis of a new CORM (CORM-S1) based on iron and cysteamine, which is soluble in water and releases CO under irradiation with visible light, while it is widely stable in the dark [150]. This is the first example of a light-induced CO release from water-soluble iron-based CORMs, which has low toxicity, compared to that of boron-containing compounds. Hubbell and colleagues have developed micelle forms of metal carbonyl complexes that displayed slowed diffusion in tissues and better ability to target distal tissue drainage sites [152]. The CO release of the micelles was slower than that of CORM-3. CO-releasing micelles efficiently attenuated the LPS-induced NF-κB activation of human monocytes while CORM-3 did not show any beneficial effects. This novel CO-delivery system based on CO-releasing micelles may be useful for therapeutic applications of CO. Efforts in medicinal chemistry development of metal carbonyl compounds are actively ongoing, which should help establish these compounds as a new class of drugs in the near future.

4.2. CORMs and Sepsis. CORM compounds are capable of delivering small amounts of CO to biological systems in a controlled manner and are emerging as a potential therapy for sepsis. In terms of lung physiology, most studies to date have focused on the therapeutic effects of CORMs in sepsis models. For example, CORMs reduce cytokine release in LPS-stimulated macrophages [24] and decrease inflammatory response and oxidative stress in LPS-stimulated endothelial cells [153]. *In vivo,* CORMs attenuate systemic inflammation and proadhesive vascular cell properties in septic and thermally injured mice by reducing nuclear factor-κB activation, protein expression of ICAM-1, and tissue granulocyte infiltration [154, 155]. CORM-3 has been shown to prevent reoccurrence of sepsis, CORM-2 prolongs survival and reduces inflammation, while CORM-3 reduces liver injury after CLP [155, 156]. These studies taken together have demonstrated that the CORM-dependent release of CO can reduce mortality in septic mice, suggesting that CORMs could be used therapeutically to prevent organ dysfunction and death in sepsis. As with inhaled CO, full consideration of the toxicological and physiological properties of the released CO, including possible effects on hemodynamics, must be understood before proceeding with CORMs as clinical therapy, with additional considerations for the biological properties of the chemical backbone and transition metal components.

4.3. CORM and Ion Channels. Over the last decade, ion channels have been recognized as important effectors in the actions of CO and may play roles in some of the beneficial effects of CO. Members of several ion channel families are molecular targets for the action of CO and/or CORMs and include: (i) the large-conductance, voltage-, and Ca^{2+}-activated K^+ channels [157–163]; (ii) the purinergic P2X2 receptor [164]; (iii) the tandem P domain channel, TREK1 [165]. Interestingly, CORM-2 inhibits the purinergic P2X4 receptor [166] and K$_v$2.1 [167]. Possible mechanisms by which CO regulates ion channels may include sGC-dependent signaling [168], direct binding of CO to the polypeptide as proposed by Wang and Wu [157], indirect binding via heme [161], or modulation of cellular redox state and mitochondrial function [167, 169]. The precise details of how CO differentially regulates each of these ion channels is beginning to be elucidated but still warrants further investigation and contradictory data has been reported for each channel [170]. For example, the most widely studied ion channel target of CO is the large-conductance, voltage-, and Ca^{2+}-activated K^+ channel, BK_{Ca}. While a number of mechanisms have been proposed to explain how CO activates BK_{Ca} channels, the exact mechanism of action is unknown. Direct binding of CO to extracellular histidines has been reported [157] but mutagenesis of these residues did not fully abolish the ability of CO to activate the ion channel [160, 162]. CO has been proposed to bind to a high-affinity, channel-associated heme moiety on the α-subunit [160], yet mutation of the key histidine residue required for heme binding does not affect CO activation of the channel [162]. Clearly, further investigation is required to determine the exact mechanisms of action.

Two studies, with opposing outcomes, have reported the regulation of voltage-activated, L-type Ca^{2+} channels.

A study by Scragg et al. demonstrated that CO, applied either as the dissolved gas or from the donor molecule CORM-2, inhibits both native (rat) and recombinant (human) cardiac L-type Ca^{2+} channels [169]. This effect arose due to the ability of CO to bind to mitochondria, presumably by interacting at complex IV causing electron leak specifically from complex III. Such leak leads to rapid formation of ROS which causes channel inhibition through a specific interaction with three cytosine residues in the C-terminal tail of the channel's major, pore-forming subunit. Therefore, CO evokes channel modulation in the heart via production of mitochondrial ROS [169]. In another study, the opposite results were reported. Human recombinant intestinal smooth-muscle L-type Ca^{2+} channels were shown to be activated by CO via an NO-dependent mechanism [171]. The reasons for these contrary observations remain unclear but may reflect tissue-specific splice variation of L-type Ca^{2+} channels, as seen for O_2 regulation of L-type channels [172].

In conclusion, CO modulates ion channels via multiple mechanisms, and it is hoped that these pathways and targets may be exploited for therapeutic intervention in the treatment of a number of important and diverse clinical conditions.

4.4. CORM-3 and Mitochondrial Dynamics. The notion that mitochondria serve as important targets in transducing the beneficial signaling properties of CO has been proposed [173]. Recent studies indicate that increased mitochondrial biogenesis is part of the mechanisms by which CO gas and CORMs exert protective effects against cardiomyopathy and cardiac dysfunction in sepsis [174, 175]. Studies by Lancel et al. investigated the potential of CORMs to preserve mitochondrial function in the CLP model of sepsis. CORM-3 treatment in CLP-induced mice prevented the decline in mitochondrial function. Administration of CORM-3 during sepsis also stimulated mitochondrial biogenesis with corresponding increases in (PPAR-γ-) coactivator-1α protein expression and mitochondrial DNA copy number. CLP was found to impair mitochondrial energetic metabolism and reduce mitochondrial biogenesis in mice [175].

Recent work by Iacono et al. shows that low-micromolar concentrations of CO, delivered to isolated heart mitochondria by the water-soluble CORM-3, uncouple mitochondrial respiration, consequently modulating both ROS production and bioenergetic parameters. In addition, CORM-3 decreased mitochondrial membrane potential at concentrations that did not inhibit cytochrome c oxidase [176]. The CO-mediated effects were attenuated by pharmacological agents known to inhibit mitochondrial uncoupling. Taken together, this work demonstrates that CORM-3, through the liberation of CO, represents a novel regulator of mitochondrial respiration, which in addition to fatty acids and thyroid and steroid hormones could play a crucial role in those pathological conditions for which strategies aimed at targeting mitochondrial uncoupling and metabolism are developed for therapeutic interventions.

5. Clinical Aspects of CO

Studies have shown that CO exerts direct anti-inflammatory effects after LPS challenge *in vitro* and in an *in vivo* mouse model [22]. Mice exposed to 250 ppm CO for 1 hour before LPS administration responded with significantly lower levels of proinflammatory cytokines (TNFα and IL-1β) and higher levels of IL-10 than control mice. As a consequence of this work, the role of CO in various rodent models has since been investigated (reviewed in [25]). On the basis of the rationale provided by these animal studies, Mayr and colleagues studied the effects of CO inhalation on systemic inflammation during experimental human endotoxemia. Specifically, in a randomized, double-blinded, placebo-controlled, two-way crossover trial, experimental endotoxemia was induced in healthy volunteers by injection of 2 ng/kg LPS. The potential anti-inflammatory effects of CO inhalation were investigated by inhalation of 500 ppm CO (leading to an increase in CO-Hb from 1.2% to 7%) versus synthetic air as a placebo for 1 h. CO inhalation had no effect on the inflammatory response as measured by systemic cytokine production (TNF-α, IL-6, IL-8, IL-1α, and IL-1β). In this study, no adverse side effects of CO inhalation were observed [177]. However, given the limited scope of this initial trial, and the protective characteristics of CO application in many animal models of sepsis, further more detailed clinical trials are urgently needed to reach a verdict on the efficacy of CO for reducing inflammation in septic patients. In contrast, a recent clinical trial demonstrates the feasibility of administering inhaled CO to humans with chronic obstructive pulmonary disease (COPD) [178]. In this study, exsmoking patients with stable COPD were subjected to CO inhalation (100–125 ppm for 2 hours/day for 4 days), which increased CO-Hb levels to 4.5%. Inhalation of CO by patients with stable COPD led to trends in reduction of sputum eosinophils and improvement of methacholine responsiveness [178]. In summary, the protective phenotype of CO in rodents in protecting against lung disease has not been recapitulated in human trial studies to date. One possibility is that differences in lung physiological responses to CO exist between different species. Further experiments are required to confirm the safety and efficacy of CO inhalation as a treatment for inflammatory lung diseases.

6. Final Remarks

The overexpression of HO-1 by gene transfer has now been shown to confer protection in several models of lung and vascular injury and disease, as well as systemic inflammatory diseases (i.e., sepsis). Potential clinical application of HO-1 would imply targeted gene delivery or pharmacological manipulation of gene expression [179]. The development of vectors for tissue-specific delivery of HO-1 in humans may facilitate gene therapy approaches [179].

Likewise, similar protective effects have been reported for inhalation CO in models of acute lung injury and sepsis. The demonstrated protective properties of low-dose CO in preclinical rodent models continue to suggest promising therapeutic applications for CO (reviewed in [21, 25, 180]).

More recent studies imply the stabilization of mitochondrial function and the stimulation of cellular autophagy as potential candidate mechanisms.

It should be noted that there are limitations, such that some studies have been disputed, and some negative findings reported [181, 182]. However, experimental work showing therapeutic potential of CO has now been extended to large animal models such as swine and nonhuman primates [89, 90].

As an alternative to inhalation of CO, pharmacological application of CO using CORMs may provide a promising therapeutic strategy [25]. Targeted delivery of CORMs may reduce the systemic effects associated with inhaled CO, resulting from CO-Hb elevation, while retaining therapeutic potential. Whether direct application of CO by CORMs administration or inhalation will provide a safe and effective modality for the treatment of human disease requires further research directed at understanding the pharmacokinetics and toxicology of CO or CORMs application in humans [25].

Ultimately, the goal of this experimentation remains to translate the therapeutic potential of CO, whether inhaled or administered through prodrugs, to possible medicinal application in human disease. Although some obstacles remain, limited human experimentation is now underway. Pilot clinical trials to date have indicated either negative efficacy for human CO therapy in endotoxemia or partial efficacy in COPD, while several other trials involving organ transplantation await completion [177, 178]. Currently, new clinical studies in fibrosis and sepsis are projected to begin shortly. Despite the success in animal models, which do not always directly translate to human disease, the therapeutic benefit of CO therapies has yet to be validated in humans.

Abbreviations

Atg:	Autophagy related gene
BALF:	Bronchio-alveolar lavage fluid
CLP:	Cecal ligation and puncture
CO:	Carbon monoxide
CO-Hb:	Carboxyhemoglobin
CORM:	Carbon monoxide-releasing molecule
COPD:	Chronic obstructive pulmonary disease
CSE:	Cigarette smoke extract
DISC:	Death inducing signaling complex
GFP-LC3:	Green fluorescence protein conjugated LC3B
HMGB-1:	High-mobility group box 1
HO-1:	Heme oxygenase-1
HO-2:	Heme oxygenase-2
HPX:	Hemopexin
IL:	Interleukin
LC3B:	Microtubule-associated protein-1 light chain-3B
LPS:	Lipopolysaccharide
MAPK:	Mitogen activated protein kinase
ROS:	Reactive oxygen species
siRNA:	Small-interfering ribonucleic acid
VILI:	Ventilator-induced lung injury.

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References

[1] B. Wu, C. Hunt, and R. Morimoto, "Structure and expression of the human gene encoding major heat shock protein HSP70," *Molecular and Cellular Biology*, vol. 5, no. 2, pp. 330–341, 1985.

[2] R. P. Beckmann, L. A. Mizzen, and W. J. Welch, "Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly," *Science*, vol. 248, no. 4957, pp. 850–854, 1990.

[3] G. C. Li and Z. Werb, "Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 79, no. 10, pp. 3218–3222, 1982.

[4] S. M. Keyse and R. M. Tyrrell, "Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 1, pp. 99–103, 1989.

[5] S. M. Keyse and R. M. Tyrrell, "Both near ultraviolet radiation and the oxidizing agent hydrogen peroxide induce a 32-kDa stress protein in normal human skin fibroblasts," *The Journal of Biological Chemistry*, vol. 262, no. 30, pp. 14821–14825, 1987.

[6] L. A. Applegate, P. Luscher, and R. M. Tyrrell, "Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells," *Cancer Research*, vol. 51, no. 3, pp. 974–978, 1991.

[7] M. Rizzardini, M. Terao, F. Falciani, and L. Cantoni, "Cytokine induction of haem oxygenase mRNA in mouse liver: interleukin 1 transcriptionally activates the haem oxygenase gene," *Biochemical Journal*, vol. 290, no. 2, pp. 343–347, 1993.

[8] C. M. Terry, J. A. Clikeman, J. R. Hoidal, and K. S. Callahan, "Effect of tumor necrosis factor-α and interleukin-1α on heme oxygenase-1 expression in human endothelial cells," *American Journal of Physiology*, vol. 274, no. 3, pp. H883–H891, 1998.

[9] S. L. Camhi, J. Alam, L. Otterbein, S. L. Sylvester, and A. M. Choi, "Induction of heme oxygenase-1 gene expression by lipopolysaccharide is mediated by AP-1 activation," *American Journal of Respiratory Cell and Molecular Biology*, vol. 13, no. 4, pp. 387–398, 1995.

[10] S. L. Camhi, J. Alam, G. W. Wiegand, B. Y. Chin, and A. M. K. Choi, "Transcriptional activation of the HO-1 gene by lipopolysaccharide is mediated by 5′ distal enhancers: role of reactive oxygen intermediates and AP-1," *American Journal of Respiratory Cell and Molecular Biology*, vol. 18, no. 2, pp. 226–234, 1998.

[11] R. Tenhunen, H. S. Marver, and R. Schmid, "Microsomal heme oxygenase. Characterization of the enzyme," *The*

Journal of Biological Chemistry, vol. 244, no. 23, pp. 6388–6394, 1969.

[12] D.B. Menzel and M.O. Amdur, "Toxic response of the respiratory system," in *Casarett & Doull's Toxicology: The Basic Science of Poisons*, K. Klaassen, M. O. Amdur, and J. Doull, Eds., pp. 330–358, MacMillan, New York, NY, USA, 3rd edition, 1986.

[13] L. E. Otterbein, J. K. Kolls, L. L. Mantell, J. L. Cook, J. Alam, and A. M. K. Choi, "Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury," *Journal of Clinical Investigation*, vol. 103, no. 7, pp. 1047–1054, 1999.

[14] T. Hashiba, M. Suzuki, Y. Nagashima et al., "Adenovirus-mediated transfer of heme oxygenase-1 cDNA attenuates severe lung injury induced by the influenza virus in mice," *Gene Therapy*, vol. 8, no. 19, pp. 1499–1507, 2001.

[15] S. Inoue, M. Suzuki, Y. Nagashima et al., "Transfer of heme oxygenase 1 cDNA by a replication-deficient adenovirus enhances interleukin 10 production from alveolar macrophages that attenuates lipopolysaccharide-induced acute lung injury in mice," *Human Gene Therapy*, vol. 12, no. 8, pp. 967–979, 2001.

[16] H. J. Duckers, M. Boehm, A. L. True et al., "Heme oxygenase-1 protects against vascular constriction and proliferation," *Nature Medicine*, vol. 7, no. 6, pp. 693–698, 2001.

[17] H. Christou, T. Morita, C. M. Hsieh et al., "Prevention of hypoxia-induced pulmonary hypertension by enhancement of endogenous heme oxygenase-1 in the rat," *Circulation Research*, vol. 86, no. 12, pp. 1224–1229, 2000.

[18] T. Minamino, H. Christou, C. M. Hsieh et al., "Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 15, pp. 8798–8803, 2001.

[19] S. H. Juan, T. S. Lee, K. W. Tseng et al., "Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein e-deficient mice," *Circulation*, vol. 104, no. 13, pp. 1519–1525, 2001.

[20] K. Ishikawa, D. Sugawara, X. P. Wang et al., "Heme oxygenase-1 inhibits atherosclerotic lesion formation in LDL-receptor knockout mice," *Circulation Research*, vol. 88, no. 5, pp. 506–512, 2001.

[21] S. W. Ryter, J. Alam, and A. M. K. Choi, "Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications," *Physiological Reviews*, vol. 86, no. 2, pp. 583–650, 2006.

[22] L. E. Otterbein, F. H. Bach, J. Alam et al., "Carbon monoxide has anti-inflammatory effects involving the mitogen- activated protein kinase pathway," *Nature Medicine*, vol. 6, no. 4, pp. 422–428, 2000.

[23] L. E. Otterbein, L. L. Mantell, and A. M. K. Choi, "Carbon monoxide provides protection against hyperoxic lung injury," *American Journal of Physiology*, vol. 276, no. 4, pp. L688–L694, 1999.

[24] P. Sawle, R. Foresti, B. E. Mann, T. R. Johnson, C. J. Green, and R. Motterlini, "Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages," *British Journal of Pharmacology*, vol. 145, no. 6, pp. 800–810, 2005.

[25] R. Motterlini and L. E. Otterbein, "The therapeutic potential of carbon monoxide," *Nature Reviews Drug Discovery*, vol. 9, no. 9, pp. 728–743, 2010.

[26] C. Fondevila, X. D. Shen, S. Tsuchiyashi et al., "Biliverdin therapy protects rat livers from ischemia and reperfusion injury," *Hepatology*, vol. 40, no. 6, pp. 1333–1341, 2004.

[27] A. Nakao, N. Murase, C. Ho, H. Toyokawa, T. R. Billiar, and S. Kanno, "Biliverdin administration prevents the formation of intimal hyperplasia induced by vascular injury," *Circulation*, vol. 112, no. 4, pp. 587–591, 2005.

[28] R. Öllinger, M. Bilban, A. Erat et al., "A natural inhibitor of vascular smooth muscle cell proliferation," *Circulation*, vol. 112, no. 7, pp. 1030–1039, 2005.

[29] S. Brouard, L. E. Otterbein, J. Anrather et al., "Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis," *Journal of Experimental Medicine*, vol. 192, no. 7, pp. 1015–1025, 2000.

[30] R. Song, Z. Zhou, P. K. M. Kim et al., "Carbon monoxide promotes Fas/CD95-induced apoptosis in Jurkat cells," *The Journal of Biological Chemistry*, vol. 279, no. 43, pp. 44327–44334, 2004.

[31] T. Morita, S. A. Mitsialis, H. Koike, Y. Liu, and S. Kourembanas, "Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells," *The Journal of Biological Chemistry*, vol. 272, no. 52, pp. 32804–32809, 1997.

[32] L. E. Otterbein, B. S. Zuckerbraun, M. Haga et al., "Carbon monoxide suppresses arteriosclerotic lesions associated with chronic graft rejection and with balloon injury," *Nature Medicine*, vol. 9, no. 2, pp. 183–190, 2003.

[33] H. P. Kim, X. Wang, A. Nakao et al., "Caveolin-1 expression by means of p38β mitogen-activated protein kinase mediates the antiproliferative effect of carbon monoxide," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 32, pp. 11319–11324, 2005.

[34] B. S. Zuckerbraun, Y. C. Beek, B. Wegiel et al., "Carbon monoxide reverses established pulmonary hypertension," *Journal of Experimental Medicine*, vol. 203, no. 9, pp. 2109–2119, 2006.

[35] K. D. Poss and S. Tonegawa, "Heme oxygenase 1 is required for mammalian iron reutilization," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 20, pp. 10919–10924, 1997.

[36] M. D. Maines and A. Kappas, "Cobalt induction of hepatic heme oxygenase; with evidence that cytochrome P 450 is not essential for this enzyme activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 71, no. 11, pp. 4293–4297, 1974.

[37] F. P. Guengerich, D. P. Ballou, and M. J. Coon, "Purified liver microsomal cytochrome P 450. Electron accepting properties and oxidation reduction potential," *The Journal of Biological Chemistry*, vol. 250, no. 18, pp. 7405–7414, 1975.

[38] T. Yoshida, M. Noguchi, and G. Kikuchi, "Oxygenated form of heme . heme oxygenase complex and requirement for second electron to initiate heme degradation from the oxygenated complex," *The Journal of Biological Chemistry*, vol. 255, no. 10, pp. 4418–4420, 1980.

[39] T. Yoshinaga, S. Sassa, and A. Kappas, "The occurrence of molecular interactions among NADPH-cytochrome c reductase, heme oxygenase, and biliverdin reductase in heme degradation," *The Journal of Biological Chemistry*, vol. 257, no. 13, pp. 7786–7793, 1982.

[40] M. Noguchi, T. Yoshida, and G. Kikuchi, "Specific requirement of NADPH-cytochrome c reductase for the microsomal heme oxygenase reaction yielding biliverdin IXα," *FEBS Letters*, vol. 98, no. 2, pp. 281–284, 1979.

[41] T. Yoshida and G. Kikuchi, "Features of the reaction of heme degradation catalyzed by the reconstituted microsomal heme

oxygenase system," *The Journal of Biological Chemistry*, vol. 253, no. 12, pp. 4230–4236, 1978.

[42] G. Kikuchi and T. Yoshida, "Heme catabolism by the reconstituted heme oxygenase system," *Annals of Clinical Research*, vol. 8, no. 17, pp. 10–17, 1976.

[43] T. Yoshida and G. Kikuchi, "Sequence of the reaction of heme catabolism catalyzed by the microsomal heme oxygenase system," *FEBS Letters*, vol. 48, no. 2, pp. 256–261, 1974.

[44] R. Tenhunen, M. E. Ross, H. S. Marver, and R. Schmid, "Reduced nicotinamide-adenine dinucleotide phosphate dependent biliverdin reductase: partial purification and characterization," *Biochemistry*, vol. 9, no. 2, pp. 298–303, 1970.

[45] R. Stocker, Y. Yamamoto, A. F. McDonagh, A. N. Glazer, and B. N. Ames, "Bilirubin is an antioxidant of possible physiological importance," *Science*, vol. 235, no. 4792, pp. 1043–1046, 1987.

[46] R. Stocker, A. N. Glazer, and B. N. Ames, "Antioxidant activity of albumin-bound bilirubin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 16, pp. 5918–5922, 1987.

[47] C. D. King, G. R. Rios, M. D. Green, and T. R. Tephly, "UDP-Glucuronosyltransferases," *Current Drug Metabolism*, vol. 1, no. 2, pp. 143–161, 2000.

[48] M. D. Maines, G. M. Trakshel, and R. K. Kutty, "Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible," *The Journal of Biological Chemistry*, vol. 261, no. 1, pp. 411–419, 1986.

[49] M. D. Maines, "The heme oxygenase system: a regulator of second messenger gases," *Annual Review of Pharmacology and Toxicology*, vol. 37, pp. 517–554, 1997.

[50] L. A. Applegate, P. Luscher, and R. M. Tyrrell, "Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells," *Cancer Research*, vol. 51, no. 3, pp. 974–978, 1991.

[51] R. Tenhunen, H. S. Marver, and R. Schmid, "The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin," *The Journal of Laboratory and Clinical Medicine*, vol. 75, no. 3, pp. 410–421, 1970.

[52] V. S. Raju and M. D. Maines, "Coordinated expression and mechanism of induction of HSP32 (heme oxygenase-1) mRNA by hyperthermia in rat organs," *Biochimica et Biophysica Acta*, vol. 1217, no. 3, pp. 273–280, 1994.

[53] I. Cruse and M. D. Maines, "Evidence suggesting that the two forms of heme oxygenase are products of different genes," *The Journal of Biological Chemistry*, vol. 263, no. 7, pp. 3348–3353, 1988.

[54] G. M. Trakshel, R. K. Kutty, and M. D. Maines, "Purification and characterization of the major constitutive form of testicular heme oxygenase. The noninducible isoform," *The Journal of Biological Chemistry*, vol. 261, no. 24, pp. 11131–11137, 1986.

[55] W. K. McCoubrey, T. J. Huang, and M. D. Maines, "Heme oxygenase-2 is a hemoprotein and binds heme through heme regulatory motifs that are not involved in heme catalysis," *The Journal of Biological Chemistry*, vol. 272, no. 19, pp. 12568–12574, 1997.

[56] M. D. Maines, B. C. Eke, and X. Zhao, "Corticosterone promotes increased heme oxygenase-2 protein and transcript expression in the newborn rat brain," *Brain Research*, vol. 722, no. 1-2, pp. 83–94, 1996.

[57] V. S. Raju, W. K. McCoubrey, and M. D. Maines, "Regulation of heme oxygenase-2 by glucocorticoids in neonatal rat brain: characterization of a functional glucocorticoid response element," *Biochimica et Biophysica Acta*, vol. 1351, no. 1-2, pp. 89–104, 1997.

[58] P. J. Lee, J. Alam, G. W. Wiegand, and A. M. K. Choi, "Overexpression of heme oxygenase-1 in human pulmonary epithelial cells results in cell growth arrest and increased resistance to hyperoxia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 19, pp. 10393–10398, 1996.

[59] I. Petrache, L. E. Otterbein, J. Alam, G. W. Wiegand, and A. M. K. Choi, "Heme oxygenase-1 inhibits TNF-α-induced apoptosis in cultured fibroblasts," *American Journal of Physiology*, vol. 278, no. 2, pp. L312–L319, 2000.

[60] G. Carlin, R. Djursäter, and K. E. Arfors, "Inhibition of heme-promoted enzymatic lipid peroxidation by desferrioxamine and EDTA," *Upsala Journal of Medical Sciences*, vol. 93, no. 3, pp. 215–223, 1988.

[61] A. L. Tappel, "The mechanism of the oxidation of unsaturated fatty acids catalyzed by hematin compounds," *Archives of Biochemistry and Biophysics*, vol. 44, no. 2, pp. 378–395, 1953.

[62] J. Balla, H. S. Jacob, G. Balla, K. Nath, J. W. Eaton, and G. M. Vercellotti, "Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 20, pp. 9285–9289, 1993.

[63] D. M. Suttner and P. A. Dennery, "Reversal of HO-1 related cytoprotection with increased expression is due to reactive iron," *FASEB Journal*, vol. 13, no. 13, pp. 1800–1809, 1999.

[64] G. F. Vile and R. M. Tyrrell, "Oxidative stress resulting from ultraviolet A irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin," *The Journal of Biological Chemistry*, vol. 268, no. 20, pp. 14678–14681, 1993.

[65] J. Z. He, J. J. D. Ho, S. Gingerich, D. W. Courtman, P. A. Marsden, and M. E. Ward, "Enhanced translation of heme oxygenase-2 preserves human endothelial cell viability during hypoxia," *The Journal of Biological Chemistry*, vol. 285, no. 13, pp. 9452–9461, 2010.

[66] A. Yachie, Y. Niida, T. Wada et al., "Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency," *Journal of Clinical Investigation*, vol. 103, no. 1, pp. 129–135, 1999.

[67] K. D. Poss and S. Tonegawa, "Reduced stress defense in heme oxygenase 1-deficient cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 20, pp. 10925–10930, 1997.

[68] S. Doré, M. Takahashi, C. D. Ferris, L. D. Hester, D. Guastella, and S. H. Snyder, "Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 5, pp. 2445–2450, 1999.

[69] T. W. Wu, J. Wu, R. K. Li, D. Mickle, and D. Carey, "Albumin-bound bilirubins protect human ventricular myocytes against oxyradical damage," *Biochemistry and Cell Biology*, vol. 69, no. 10-11, pp. 683–688, 1991.

[70] R. Öllinger, H. Wang, K. Yamashita et al., "Therapeutic applications of bilirubin and biliverdin in transplantation," *Antioxidants and Redox Signaling*, vol. 9, no. 12, pp. 2175–2185, 2007.

[71] R. Foresti, C. J. Green, and R. Motterlini, "Generation of bile pigments by haem oxygenase: a refined cellular strategy in

response to stressful insults," *Biochemical Society Symposium*, vol. 71, pp. 177–192, 2004.

[72] P. A. Dennery, A. F. McDonagh, D. R. Spitz, and P. A. Rodgers, "Hyperbilirubinemia results in reduced oxidative injury in neonatal Gunn rats exposed to hyperoxia," *Free Radical Biology and Medicine*, vol. 19, no. 4, pp. 395–404, 1995.

[73] H. A. Schwertner, W. G. Jackson, and G. Tolan, "Association of low serum concentration of bilirubin with increased risk of coronary artery disease," *Clinical Chemistry*, vol. 40, no. 1, pp. 18–23, 1994.

[74] L. H. Breimer, K. A. Spyropolous, A. F. Winder, D. P. Mikhailidis, and G. Hamilton, "Is bilirubin protective against coronary artery disease?" *Clinical Chemistry*, vol. 40, no. 10, pp. 1987–1988, 1994.

[75] L. H. Breimer, G. Wannamethee, S. Ebrahim, and A. G. Shaper, "Serum bilirubin and risk of ischemic heart disease in middle-aged British men," *Clinical Chemistry*, vol. 41, no. 10, pp. 1504–1508, 1995.

[76] D. Erdogan, H. Gullu, E. Yildirim et al., "Low serum bilirubin levels are independently and inversely related to impaired flow-mediated vasodilation and increased carotid intima-media thickness in both men and women," *Atherosclerosis*, vol. 184, no. 2, pp. 431–437, 2006.

[77] L. Vítek, M. Jirsa Jr., M. Brodanová et al., "Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels," *Atherosclerosis*, vol. 160, no. 2, pp. 449–456, 2002.

[78] A. C. Bulmer, J. T. Blanchfield, I. Toth, R. G. Fassett, and J. S. Coombes, "Improved resistance to serum oxidation in Gilbert's syndrome: a mechanism for cardiovascular protection," *Atherosclerosis*, vol. 199, no. 2, pp. 390–396, 2008.

[79] J. F. Watchko, "Hyperbilirubinemia and bilirubin toxicity in the late preterm infant," *Clinics in Perinatology*, vol. 33, no. 4, pp. 839–852, 2006.

[80] J. K. Sarady-Andrews, F. Liu, D. Gallo et al., "Biliverdin administration protects against endotoxin-induced acute lung injury in rats," *American Journal of Physiology*, vol. 289, no. 6, pp. L1131–L1137, 2005.

[81] S. G. Tullius, M. Nieminen-Kelha, and U. Bachmann, "Induction of heme-oxygenase-1 prevents ischemia/reperfusion injury and improves long-term graft outcome in rat renal allografts," *Transplantation Proceedings*, vol. 33, no. 1-2, pp. 1286–1287, 2001.

[82] Y. Avihingsanon, N. Ma, E. Csizmadia et al., "Expression of protective genes in human renal allografts: a regulatory response to injury associated with graft rejection," *Transplantation*, vol. 73, no. 7, pp. 1079–1085, 2002.

[83] F. Amersi, R. Buelow, H. Kato et al., "Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury," *Journal of Clinical Investigation*, vol. 104, no. 11, pp. 1631–1639, 1999.

[84] S. W. Chung, X. Liu, A. A. Macias, R. M. Baron, and M. A. Perrella, "Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice," *Journal of Clinical Investigation*, vol. 118, no. 1, pp. 239–247, 2008.

[85] R. Larsen, R. Gozzelino, V. Jeney et al., "A central role for free heme in the pathogenesis of severe sepsis," *Science Translational Medicine*, vol. 2, no. 51, Article ID 51ra71, 2010.

[86] R. Takamiya, C. C. Hung, S. R. Hall et al., "High-mobility group box 1 contributes to lethality of endotoxemia in heme oxygenase-1-deficient mice," *American Journal of Respiratory Cell and Molecular Biology*, vol. 41, no. 2, pp. 129–135, 2009.

[87] K. Tsoyi, Y. L. Tae, S. L. Young et al., "Heme-oxygenase-1 induction and carbon monoxide-releasing molecule inhibit lipopolysaccharide (LPS)-induced high-mobility group box 1 release in vitro and improve survival of mice in LPS- and cecal ligation and puncture-induced sepsis model in vivo," *Molecular Pharmacology*, vol. 76, no. 1, pp. 173–182, 2009.

[88] J. K. Sarady, B. S. Zuckerbraun, M. Bilban et al., "Carbon monoxide protection against endotoxic shock involves reciprocal effects on iNOS in the lung and liver," *The FASEB Journal*, vol. 18, no. 7, pp. 854–856, 2004.

[89] S. Mazzola, M. Forni, M. Albertini et al., "Carbon monoxide pretreatment prevents respiratory derangement and ameliorates hyperacute endotoxic shock in pigs," *FASEB Journal*, vol. 19, no. 14, pp. 2045–2047, 2005.

[90] L. A. Mitchell, M. M. Channell, C. M. Royer, S. W. Ryter, A. M. K. Choi, and J. D. McDonald, "Evaluation of inhaled carbon monoxide as an anti-inflammatory therapy in a nonhuman primate model of lung inflammation," *American Journal of Physiology*, vol. 299, no. 6, pp. L891–L897, 2010.

[91] K. J. Davies, "Oxidative stress: the paradox of aerobic life," *Biochemical Society symposium*, vol. 61, pp. 1–31, 1995.

[92] R. M. Jackson, "Molecular, pharmacologic, and clinical aspects of oxygen-induced lung injury," *Clinics in Chest Medicine*, vol. 11, no. 1, pp. 73–86, 1990.

[93] P. J. Lee, J. Alam, S. L. Sylvester, N. Inamdar, L. Otterbein, and A. M. K. Choi, "Regulation of heme oxygenase-1 expression in vivo and in vitro in hyperoxic lung injury," *American Journal of Respiratory Cell and Molecular Biology*, vol. 14, no. 6, pp. 556–568, 1996.

[94] L. E. Otterbein, S. L. Otterbein, E. Ifedigbo et al., "MKK3 mitogen activated protein kinase pathway mediates carbon monoxide-induced protection against oxidant induced lung injury," *American Journal of Pathology*, vol. 163, no. 6, pp. 2555–2563, 2003.

[95] X. Zhang, P. Shan, G. Jiang et al., "Endothelial STAT3 is essential for the protective effects of HO-1 in oxidant-induced lung injury," *The FASEB Journal*, vol. 20, no. 12, pp. 2156–2158, 2006.

[96] X. Wang, Y. Wang, H. P. Kim, K. Nakahira, S. W. Ryter, and A. M. K. Choi, "Carbon monoxide protects against hyperoxia-induced endothelial cell apoptosis by inhibiting reactive oxygen species formation," *The Journal of Biological Chemistry*, vol. 282, no. 3, pp. 1718–1726, 2007.

[97] R. G. Brower, M. A. Matthay, A. Morris, D. Schoenfeld, B. T. Thompson, and A. Wheeler, "Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome," *The New England Journal of Medicine*, vol. 342, no. 18, pp. 1301–1308, 2000.

[98] K. Tsushima, L. S. King, N. R. Aggarwal, A. De Gorordo, F. R. D'Alessio, and K. Kubo, "Acute lung injury review," *Internal Medicine*, vol. 48, no. 9, pp. 621–630, 2009.

[99] A. S. Slutsky, "Lung injury caused by mechanical ventilation," *Chest*, vol. 116, pp. 9S–15S, 1999.

[100] T. Dolinay, M. Szilasi, M. Liu, and A. M. K. Choi, "Inhaled carbon monoxide confers antiinflammatory effects against ventilator-induced lung injury," *American Journal of Respiratory and Critical Care Medicine*, vol. 170, no. 6, pp. 613–620, 2004.

[101] A. Hoetzel, R. Schmidt, S. Vallbracht et al., "Carbon monoxide prevents ventilator-induced lung injury via caveolin-1," *Critical Care Medicine*, vol. 37, no. 5, pp. 1708–1715, 2009.

[102] A. Hoetzel, T. Dolinay, S. Vallbracht et al., "Carbon monoxide protects against ventilator-induced lung injury via PPAR-γ and inhibition of Egr-1," *American Journal of Respiratory and Critical Care Medicine*, vol. 177, no. 11, pp. 1223–1232, 2008.

[103] M. Bilban, F. H. Bach, S. L. Otterbein et al., "Carbon monoxide orchestrates a protective response through PPARγ," *Immunity*, vol. 24, no. 5, pp. 601–610, 2006.

[104] M. Althaus, M. Fronius, Y. Buchäckert et al., "Carbon monoxide rapidly impairs alveolar fluid clearance by inhibiting epithelial sodium channels," *American Journal of Respiratory Cell and Molecular Biology*, vol. 41, no. 6, pp. 639–650, 2009.

[105] X. Zhang, P. Shan, J. Alam, R. J. Davis, R. A. Flavell, and P. J. Lee, "Carbon monoxide modulates Fas/Fas ligand, caspases, and Bcl-2 family proteins via the p38α mitogen-activated protein kinase pathway during ischemia-reperfusion lung injury," *The Journal of Biological Chemistry*, vol. 278, no. 24, pp. 22061–22070, 2003.

[106] X. Zhang, P. Shan, L. E. Otterbein et al., "Carbon monoxide inhibition of apoptosis during ischemia-reperfusion lung injury is dependent on the p38 mitogen-activated protein kinase pathway and involves caspase 3," *The Journal of Biological Chemistry*, vol. 278, no. 2, pp. 1248–1258, 2003.

[107] X. Zhang, P. Shan, J. Alam, X. Y. Fu, and P. J. Lee, "Carbon monoxide differentially modulates STAT1 and STAT3 and inhibits apoptosis via a phosphatidylinositol 3-kinase/Akt and p38 kinase-dependent STAT3 pathway during anoxia-reoxygenation injury," *The Journal of Biological Chemistry*, vol. 280, no. 10, pp. 8714–8721, 2005.

[108] T. Fujita, K. Toda, A. Karimova et al., "Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis," *Nature Medicine*, vol. 7, no. 5, pp. 598–604, 2001.

[109] S. Mishra, T. Fujita, V. M. Lama et al., "Carbon monoxide rescues ischemic lungs by interrupting MAPK-driven expression of early growth response 1 gene and its downstream target genes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 13, pp. 5191–5196, 2006.

[110] R. Song, M. Kubo, D. Morse et al., "Carbon monoxide induces cytoprotection in rat orthotopic lung transplantation via anti-inflammatory and anti-apoptotic effects," *American Journal of Pathology*, vol. 163, no. 1, pp. 231–242, 2003.

[111] J. Kohmoto, A. Nakao, T. Kaizu et al., "Low-dose carbon monoxide inhalation prevents ischemia/reperfusion injury of transplanted rat lung grafts," *Surgery*, vol. 140, no. 2, pp. 179–185, 2006.

[112] J. Kohmoto, A. Nakao, R. Sugimoto et al., "Carbon monoxide-saturated preservation solution protects lung grafts from ischemia-reperfusion injury," *Journal of Thoracic and Cardiovascular Surgery*, vol. 136, no. 4, pp. 1067–1075, 2008.

[113] N. Hangai-Hoger, A. G. Tsai, P. Cabrales, M. Suematsu, and M. Intaglietta, "Microvascular and systemic effects following top load administration of saturated carbon monoxide-saline solution," *Critical Care Medicine*, vol. 35, no. 4, pp. 1123–1132, 2007.

[114] S. F. Yet, M. A. Perrella, M. D. Layne et al., "Hypoxia induces severe right ventricular dilatation and infarction in heine oxygenase-1 null mice," *Journal of Clinical Investigation*, vol. 103, no. 8, pp. R23–R29, 1999.

[115] H. Zhou, H. Liu, S. L. Porvasnik et al., "Heme oxygenase-1 mediates the protective effects of rapamycin in monocrotaline-induced pulmonary hypertension," *Laboratory Investigation*, vol. 86, no. 1, pp. 62–71, 2006.

[116] E. Dubuis, M. Potier, R. Wang, and C. Vandier, "Continuous inhalation of carbon monoxide attenuates hypoxic pulmonary hypertension development presumably through activation of BKCa channels," *Cardiovascular Research*, vol. 65, no. 3, pp. 751–761, 2005.

[117] A. Kelekar, "Autophagy," *Annals of the New York Academy of Sciences*, vol. 1066, pp. 259–271, 2005.

[118] C. He and D. J. Klionsky, "Regulation mechanisms and signaling pathways of autophagy," *Annual Review of Genetics*, vol. 43, pp. 67–93, 2009.

[119] B. Levine and D. J. Klionsky, "Development by self-digestion: molecular mechanisms and biological functions of autophagy," *Developmental Cell*, vol. 6, no. 4, pp. 463–477, 2004.

[120] N. Mizushima, B. Levine, A. M. Cuervo, and D. J. Klionsky, "Autophagy fights disease through cellular self-digestion," *Nature*, vol. 451, no. 7182, pp. 1069–1075, 2008.

[121] D. J. Klionsky and S. D. Emr, "Autophagy as a regulated pathway of cellular degradation," *Science*, vol. 290, no. 5497, pp. 1717–1721, 2000.

[122] T. Yorimitsu and D. J. Klionsky, "Autophagy: molecular machinery for self-eating," *Cell Death and Differentiation*, vol. 12, no. 2, pp. 1542–1552, 2005.

[123] B. Levine, N. Mizushima, and H. W. Virgin, "Autophagy in immunity and inflammation," *Nature*, vol. 469, no. 7330, pp. 323–335, 2011.

[124] B. Ravikumar, S. Sarkar, J. E. Davies et al., "Regulation of mammalian autophagy in physiology and pathophysiology," *Physiological Reviews*, vol. 90, no. 4, pp. 1383–1435, 2010.

[125] Z. Yang and D. J. Klionsky, "Mammalian autophagy: core molecular machinery and signaling regulation," *Current Opinion in Cell Biology*, vol. 22, no. 2, pp. 124–131, 2010.

[126] X. H. Liang, S. Jackson, M. Seaman et al., "Induction of autophagy and inhibition of tumorigenesis by beclin 1," *Nature*, vol. 402, no. 6762, pp. 672–676, 1999.

[127] I. Tanida, T. Ueno, and E. Kominami, "LC3 conjugation system in mammalian autophagy," *International Journal of Biochemistry and Cell Biology*, vol. 36, no. 12, pp. 2503–2518, 2004.

[128] D. R. Green, L. Galluzzi, and G. Kroemer, "Mitochondria and the autophagy-inflammation-cell death axis in organismal aging," *Science*, vol. 333, no. 6046, pp. 1109–1112, 2011.

[129] B. Levine and J. Yuan, "Autophagy in cell death: an innocent convict?" *Journal of Clinical Investigation*, vol. 115, no. 10, pp. 2679–2688, 2005.

[130] Y. Tsujimoto and S. Shimizu, "Another way to die: autophagic programmed cell death," *Cell Death and Differentiation*, vol. 12, no. 2, supplement, pp. 1528–1534, 2005.

[131] J. Debnath, K. R. Mills, N. L. Collins, M. J. Reginato, S. K. Muthuswamy, and J. S. Brugge, "The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini," *Cell*, vol. 111, no. 1, pp. 29–40, 2002.

[132] L. Jia, R. R. Dourmashkin, P. D. Allen, A. B. Gray, A. C. Newland, and S. M. Kelsey, "Inhibition of autophagy abrogates tumour necrosis factor α induced apoptosis in human T-lymphoblastic leukaemic cells," *British Journal of Haematology*, vol. 98, no. 3, pp. 673–685, 1997.

[133] B. Inbal, S. Bialik, I. Sabanay, G. Shani, and A. Kimchi, "DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death," *Journal of Cell Biology*, vol. 157, no. 3, pp. 455–468, 2002.

[134] F. Zhou, Y. Yang, and D. Xing, "Bcl-2 and Bcl-xL play important roles in the crosstalk between autophagy and apoptosis," *FEBS Journal*, vol. 278, no. 3, pp. 403–413, 2011.

[135] V. M. Aita, X. H. Liang, V. V. V. S. Murty et al., "Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21," *Genomics*, vol. 59, no. 1, pp. 59–65, 1999.

[136] S. Pattingre, A. Tassa, X. Qu et al., "Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy," *Cell*, vol. 122, no. 6, pp. 927–939, 2005.

[137] S. Shimizu, T. Kanaseki, N. Mizushima et al., "Role of Bcl-2 family proteins in a non-apoptopic programmed cell death dependent on autophagy genes," *Nature Cell Biology*, vol. 6, no. 12, pp. 1221–1228, 2004.

[138] Z. H. Chen, H. C. Lam, Y. Jin et al., "Autophagy protein microtubule-associated protein 1 light chain-3B (LC3B) activates extrinsic apoptosis during cigarette smoke-induced emphysema," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 44, pp. 18880–18885, 2010.

[139] H. P. Kim, X. Wang, Z. H. Chen et al., "Autophagic proteins regulate cigarette smoke-induced apoptosis:Protective role of heme oxygenase-1," *Autophagy*, vol. 4, no. 7, pp. 887–895, 2008.

[140] D. J. Slebos, S. W. Ryter, M. van der Toorn et al., "Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death," *American Journal of Respiratory Cell and Molecular Biology*, vol. 36, no. 4, pp. 409–417, 2007.

[141] E. H. Carchman, J. Rao, P. A. Loughran, M. R. Rosengart, and B. S. Zuckerbraun, "Heme oxygenase-1-mediated autophagy protects against hepatocyte cell death and hepatic injury from infection/sepsis in mice," *Hepatology*, vol. 53, no. 6, pp. 2053–2062, 2011.

[142] P. Waltz, E. H. Carchman, A. C. Young et al., "Lipopolysaccaride induces autophagic signaling in macrophages via a TLR4, heme oxygenase-1 dependent pathway," *Autophagy*, vol. 7, no. 3, pp. 315–320, 2011.

[143] H. Zukor, W. Song, A. Liberman et al., "HO-1-mediated macroautophagy: a mechanism for unregulated iron deposition in aging and degenerating neural tissues," *Journal of Neurochemistry*, vol. 109, no. 3, pp. 776–791, 2009.

[144] S. Bolisetty, A. M. Traylor, J. Kim et al., "Heme oxygenase-1 inhibits renal tubular macroautophagy in acute kidney injury," *Journal of the American Society of Nephrology*, vol. 21, no. 10, pp. 1702–1712, 2010.

[145] S. J. Lee, S. W. Ryter, J. F. Xu et al., "Carbon monoxide activates autophagy via mitochondrial reactive oxygen species formation," *American Journal of Respiratory Cell and Molecular Biology*, vol. 45, no. 4, pp. 867–873, 2011.

[146] R. Motterlini, B. E. Mann, and R. Foresti, "Therapeutic applications of carbon monoxide-releasing molecules," *Expert Opinion on Investigational Drugs*, vol. 14, no. 11, pp. 1305–1318, 2005.

[147] R. Foresti, M. G. Bani-Hani, and R. Motterlini, "Use of carbon monoxide as a therapeutic agent: promises and challenges," *Intensive Care Medicine*, vol. 34, no. 4, pp. 649–658, 2008.

[148] R. Motterlini, P. Sawle, J. Hammad et al., "CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule," *FASEB Journal*, vol. 19, no. 2, pp. 284–286, 2005.

[149] R. Foresti, J. Hammad, J. E. Clark et al., "Vasoactive properties of CORM-3, a novel water-soluble carbon monoxide-releasing molecule," *British Journal of Pharmacology*, vol. 142, no. 3, pp. 453–460, 2004.

[150] R. Kretschmer, G. Gessner, H. Görls, S. H. Heinemann, and M. Westerhausen, "Dicarbonyl-bis(cysteamine)iron(II): a light induced carbon monoxide releasing molecule based on iron (CORM-S1)," *Journal of Inorganic Biochemistry*, vol. 105, no. 1, pp. 6–9, 2011.

[151] G. L. Bannenberg and H. L. A. Vieira, "Therapeutic applications of the gaseous mediators carbon monoxide and hydrogen sulfide," *Expert Opinion on Therapeutic Patents*, vol. 19, no. 5, pp. 663–682, 2009.

[152] B. Sun, X. Zou, Y. Chen, P. Zhang, and G. Shi, "Preconditioning of carbon monoxide releasing molecule-derived CO attenuates LPS-induced activation of HUVEC," *International Journal of Biological Sciences*, vol. 4, no. 5, pp. 270–278, 2008.

[153] U. Hasegawa, A. J. van der Vlies, E. Simeoni, C. Wandrey, and J. A. Hubbell, "Carbon monoxide-releasing micelles for immunotherapy," *Journal of the American Chemical Society*, vol. 132, no. 51, pp. 18273–18280, 2010.

[154] B. Sun, H. Sun, C. Liu, J. Shen, Z. Chen, and X. Chen, "Role of CO-releasing molecules liberated CO in attenuating leukocytes sequestration and inflammatory responses in the lung of thermally injured mice," *Journal of Surgical Research*, vol. 139, no. 1, pp. 128–135, 2007.

[155] G. Cepinskas, K. Katada, A. Bihari, and R. F. Potter, "Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice," *American Journal of Physiology*, vol. 294, no. 1, pp. G184–G191, 2007.

[156] S. Mizuguchi, J. Stephen, R. Bihari et al., "CORM-3-derived CO modulates polymorphonuclear leukocyte migration across the vascular endothelium by reducing levels of cell surface-bound elastase," *American Journal of Physiology*, vol. 297, no. 3, pp. H920–H929, 2009.

[157] R. Wang and L. Wu, "The chemical modification of K(Ca) channels by carbon monoxide in vascular smooth muscle cells," *The Journal of Biological Chemistry*, vol. 272, no. 13, pp. 8222–8226, 1997.

[158] A. M. Riesco-Fagundo, M. T. Pérez-García, C. González, and J. R. López-López, "O_2 modulates large-conductance Ca^{2+}-dependent K^+ channels of rat chemoreceptor cells by a membrane-restricted and CO-sensitive mechanism," *Circulation Research*, vol. 89, no. 5, pp. 430–436, 2001.

[159] S. E. J. Williams, P. Wootton, H. S. Mason et al., "Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel," *Science*, vol. 306, no. 5704, pp. 2093–2097, 2004.

[160] S. E. Williams, S. P. Brazier, N. Baban et al., "A structural motif in the C-terminal tail of slo1 confers carbon monoxide sensitivity to human BKCa channels," *Pflugers Archiv*, vol. 456, no. 3, pp. 561–572, 2008.

[161] J. H. Jaggar, A. Li, H. Parfenova et al., "Heme is a carbon monoxide receptor for large-conductance Ca 2+-activated K+ channels," *Circulation Research*, vol. 97, no. 8, pp. 805–812, 2005.

[162] S. Hou, R. Xu, S. H. Heinemann, and T. Hoshi, "The RCK1 high-affinity Ca^{2+} sensor confers carbon monoxide sensitivity to Slo1 BK channels," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 10, pp. 4039–4043, 2008.

[163] V. Telezhkin, S. P. Brazier, R. Mears, C. T. Müller, D. Riccardi, and P. J. Kemp, "Cysteine residue 911 in C-terminal tail of human BKCaα channel subunit is crucial for its activation

by carbon monoxide," *Pflugers Archiv European Journal of Physiology*, vol. 461, no. 6, pp. 665–675, 2011.

[164] W. Wilkinson, H. C. Gadeberg, A. W. J. Harrison, N. D. Allen, D. Riccardi, and P. J. Kemp, "Carbon monoxide is a rapid modulator of recombinant and native P2X 2 ligand-gated ion channels," *British Journal of Pharmacology*, vol. 158, no. 3, pp. 862–871, 2009.

[165] M. L. Dallas, J. L. Scragg, and C. Peers, "Modulation of hTREK-1 by carbon monoxide," *NeuroReport*, vol. 19, no. 3, pp. 345–348, 2008.

[166] W. J. Wilkinson and P. J. Kemp, "The carbon monoxide donor, CORM-2, is an antagonist of ATP-gated, human P2X4 receptors," *Purinergic Signalling*, vol. 7, no. 1, pp. 57–64, 2011.

[167] M. L. Dallas, J. P. Boyle, C. J. Milligan et al., "Carbon monoxide protects against oxidant-induced apoptosis via inhibition of Kv2.1," *FASEB Journal*, vol. 25, no. 5, pp. 1519–1530, 2011.

[168] A. Rich, G. Farrugia, and J. L. Rae, "Carbon monoxide stimulates a potassium-selective current in rabbit corneal epithelial cells," *American Journal of Physiology*, vol. 267, no. 2, pp. C435–C442, 1994.

[169] J. L. Scragg, M. L. Dallas, J. A. Wilkinson, G. Varadi, and C. Peers, "Carbon monoxide inhibits L-type Ca^{2+} channels via redox modulation of key cysteine residues by mitochondrial reactive oxygen species," *The Journal of Biological Chemistry*, vol. 283, no. 36, pp. 24412–24419, 2008.

[170] W. J. Wilkinson and P. J. Kemp, "Carbon monoxide: an emerging regulator of ion channels," *Journal of Physiology*, vol. 589, no. 13, pp. 3055–3062, 2011.

[171] I. Lim, S. J. Gibbons, G. L. Lyford et al., "Carbon monoxide activates human intestinal smooth muscle L-type Ca^{2+} channels through a nitric oxide-dependent mechanism," *American Journal of Physiology*, vol. 288, no. 1, pp. G7–G14, 2005.

[172] I. M. Fearon, G. Varadi, S. Koch, I. Isaacsohn, S. G. Ball, and C. Peers, "Splice variants reveal the region involved in oxygen sensing by recombinant human L-type Ca^{2+} channels," *Circulation Research*, vol. 87, no. 7, pp. 537–539, 2000.

[173] M. Desmard, J. Boczkowski, J. Poderoso, and R. Motterlini, "Mitochondrial and cellular heme-dependent proteins as targets for the bioactive function of the heme oxygenase/carbon monoxide system," *Antioxidants and Redox Signaling*, vol. 9, no. 12, pp. 2139–2155, 2007.

[174] H. B. Suliman, M. S. Carraway, A. S. Ali, C. M. Reynolds, K. E. Welty-Wolf, and C. A. Piantadosi, "The CO/HO system reverses inhibition of mitochondrial biogenesis and prevents murine doxorubicin cardiomyopathy," *Journal of Clinical Investigation*, vol. 117, no. 12, pp. 3730–3741, 2007.

[175] S. Lancel, S. M. Hassoun, R. Favory, B. Decoster, R. Motterlini, and R. Neviere, "Carbon monoxide rescues mice from lethal sepsis by supporting mitochondrial energetic metabolism and activating mitochondrial biogenesis," *Journal of Pharmacology and Experimental Therapeutics*, vol. 329, no. 2, pp. 641–648, 2009.

[176] L. Lo Iacono, J. Boczkowski, R. Zini et al., "A carbon monoxide-releasing molecule (CORM-3) uncouples mitochondrial respiration and modulates the production of reactive oxygen species," *Free Radical Biology and Medicine*, vol. 50, no. 11, pp. 1556–1564, 2011.

[177] F. B. Mayr, A. Spiel, J. Leitner et al., "Effects of carbon monoxide inhalation during experimental endotoxemia in humans," *American Journal of Respiratory and Critical Care Medicine*, vol. 171, no. 4, pp. 354–360, 2005.

[178] E. Bathoorn, D. J. Slebos, D. S. Postma et al., "Anti-inflammatory effects of inhaled carbon monoxide in patients with COPD: a pilot study," *European Respiratory Journal*, vol. 30, no. 6, pp. 1131–1137, 2007.

[179] N. G. Abraham, A. Asija, G. Drummond, and S. Peterson, "Heme oxygenase -1 gene therapy: recent advances and therapeutic applications," *Current Gene Therapy*, vol. 7, no. 2, pp. 89–108, 2007.

[180] S. W. Ryter and A. M. K. Choi, "Heme oxygenase-1/carbon monoxide: from metabolism to molecular therapy," *American Journal of Respiratory Cell and Molecular Biology*, vol. 41, no. 3, pp. 251–260, 2009.

[181] S. Ghosh, M. R. Wilson, S. Choudhury et al., "Effects of inhaled carbon monoxide on acute lung injury in mice," *American Journal of Physiology*, vol. 288, no. 6, pp. L1003–L1009, 2005.

[182] C. E. Clayton, M. S. Carraway, H. B. Suliman et al., "Inhaled carbon monoxide and hyperoxic lung injury in rats," *American Journal of Physiology*, vol. 281, no. 4, pp. L949–L957, 2001.

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Research Article

Heme Induction with Delta-Aminolevulinic Acid Stimulates an Increase in Water and Electrolyte Excretion

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Purpose. Studies were performed to examine hemodynamic and renal function before and after acute induction of the endogenous CO system with delta-aminolevulinic acid (DALA), which drives HO activity. *Methods*. *In vivo* studies were conducted on Inactin-anesthetized male Sprague Dawley rats (250–300 g) either with or without chronic pretreatment with L-NAME (50 mg/Kg, q12 hours x4d). *Results*. DALA (80 μmol/Kg, IV bolus) administration acutely increased endogenous CO production and HO-1 protein. In untreated and L-NAME-pretreated rats, DALA did not alter BP, GFR, or RBF but increased UF, $U_{Na}V$, and U_KV (untreated: $\Delta 108.8 \pm 0.28\%$, $172.1 \pm 18.4\%$, and $165.2 \pm 45.9\%$; pretreated: $\Delta 109.4 \pm 0.29\%$, $187.3 \pm 26.9\%$, and $197.2 \pm 45.7\%$). Acute administration of biliverdin (20 mg/kg, IV) and bilirubin (30 mg/kg, IV) to similarly treated animals did not alter UF, $U_{Na}V$, and U_KV. *Conclusion*. These results demonstrate that heme oxygenase induction increases urine and electrolyte excretion and suggest a direct tubular action of endogenous carbon monoxide.

1. Introduction

Metabolic degradation of heme by heme oxygenase (HO) yields three products; biliverdin, ferrous iron, and carbon monoxide (CO) [1]. Currently, two major isoforms of the HO enzyme have been recognized, the inducible HO-1 and the constitutive HO-2. Both isoforms have been reported to be present in the kidneys [1–3]. Several biological stressors, such as oxidative stress, ischemia, and hypertension, are known to increase HO-1 levels [4–6]. In contrast, the HO-2 isoform is constitutively expressed and is present in high concentrations in the kidney, as well as in other vascular beds and tissues [7]. Alterations in HO levels have been demonstrated to alter CO concentration, in addition to having profound effects on vascular tone [8, 9].

Current literature supports both an endothelial-dependent vasoconstrictor effect of CO and an endothelial-independent vasorelaxation [10, 11]. CO-mediated vasoconstriction is via inhibition of nitric oxide synthase (NOS) [11, 12]. CO also promotes endothelium-independent vasodilation through the activation of soluble guanylyl cyclase, stimulation of K channels, and inhibition of the cytochrome-P450-dependent monooxygenase system in vascular smooth muscle cells [10, 13]. Increases in endogenous CO levels produce a decrease in blood pressure in several forms of hypertension, while HO inhibition increases arterial blood pressure [4, 14–17]. Regional differences in renal blood flow (RBF) have been demonstrated with increases in the medulla without significant increases in cortical blood flow during heme-induced increases in CO [13]. Other studies have not shown significant alterations in renal vascular resistance during alterations in CO levels, thus controversy does exist in the literature as it relates to the ability of CO to regulate renal vascular resistance [18].

Increases in HO activity via heme administration promote vasorelaxation and produce diuresis and natriuresis [19]. In addition, several studies have identified an antioxidant role for bilirubin and biliverdin during stress [20, 21]. However, the mechanisms of HO-mediated effects on renal function have yet to be elucidated. Because the HO-mediated diuretic and natriuretic effects were observed concomitantly with an increase in RBF, it is possible that alterations in renal hemodynamics mediate the increase in UF and sodium excretion. It has also been reported that renal medullary HO plays a key role in the regulation of pressure natriuresis and, thus, the control of arterial blood pressure [22]. Macula densa cells have been reported to express HO-1 and HO-2, and stannous mesoporphyrin, an inhibitor of HO, was shown to augment tubuloglomerular feedback in both *in vitro* and *in vivo* studies [23]. In addition, we recently reported that CO inhibition promotes antidiuresis and antinatriuresis independent of vascular or systemic changes [24]. Therefore, we hypothesized that increased levels of endogenous CO promote natriuresis and diuresis independent of inhibition of nitric oxide synthase (NOS) and alterations in RBF. To examine this hypothesis, the potential direct tubular effects of a heme precursor, delta-aminolevulinic acid (DALA), which drives HO activity, were studied using a dose of DALA that does not elicit changes in renal hemodynamic function in control and L-NAME treated rats.

2. Methods

2.1. Materials. DALA was purchased from Frontier Scientific (Logan, UT, USA). Inactin (thiobutabarbital sodium), N-Nitro-L-Arginine Methyl Ester (L-NAME), bilirubin, and para-aminohippuric acid (PAH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Albumin was purchased from EMD Biosciences Inc. (San Diego, CA, USA). Inulin was purchased from Fresenius Kabi UK Ltd. (Runcorn, Cheshire). Plasma Renin Activity (PRA) kits were purchased from Diasorin Inc. (Stillwater, MN, USA). Biliverdin was purchased from MP Biochemicals, LLC (Solon, OH, USA). All other chemicals were purchased from Fisher Scientific (Houston, TX, USA). DALA stock solution (800 mmol/L) was prepared in saline on the day of the experiments. L-NAME (50 mmol/L) was dissolved in saline immediately before intraperitoneal injection. All other solutions were freshly prepared on the day of the experiment.

2.2. Animals. Male Sprague-Dawley rats (250–350 g; n = 146, Harlan, Indianapolis, IN, USA) were used (n = 32). This protocol was approved by the Tulane School of Medicine and University of Louisiana at Monroe Institutional Animal Care and Use Committee. Prior to experiments, rats were housed in a controlled environment and had free access to commercial rat chow and tap water. Subsets of animals were chronically treated every 12 hours for four days with an inhibitor of NOS [25], L-NAME (50 mg/kg, IP). To minimize postprandial sodium excretion variability, animals were deprived of food for 12 hours before experiments.

Subsets of animals were chronically treated with L-NAME every 12 hours for four days. After anesthetization with Inactin and surgical preparation, rats were allowed to stabilize for 45 min. After this initial stabilization period, a 30-minute control period was performed and urine was collected. L-NAME-treated and -untreated animals were then acutely administered DALA (80 μmol/kg, IV), biliverdin (20 mg/kg, IV), bilirubin (30 mg/kg, IV), or vehicle (1 mL saline, IV), and an additional 30-minute treatment period was performed. The doses of biliverdin and bilirubin were chosen from previous studies where an antioxidant effect was observed [20, 21]. Mean arterial pressures (MAP), heart rates (HR), and RBF were measured during both the 30-minute control and treatment periods. After the experimental protocols were completed, renal vascular resistance (RVR) was calculated as the pressure to flow ratio and expressed as "mmHg/(mL/min)".

2.3. Experimental Procedures. Rats were anesthetized with a single injection of thiobutabarbital sodium (120 mg/kg; IP), and a tracheal tube was inserted to maintain an open airway. Fluid filled catheters (PE-50 tubing filled with heparinized saline) were inserted into a carotid artery and a jugular vein to allow for continuous monitoring of MAP and HR, and for intravenous administration of drugs, respectively. The arterial catheter was connected to a pressure transducer (model TSD104A, Biopac Systems, Santa Barbara, CA, USA), and the venous catheter was connected to a Sage microinfusion pump (Orion Research, Inc., model M361, Boston, MA, USA) set at 1 mL/hr saline infusion rate. A bladder cannula was inserted to allow urine collection for determination of urine flow and concentrations of sodium and potassium (Flame Photometry; Instrumentation Laboratories, IL 943). A flank incision was made to expose the left kidney and renal artery. RBF was measured with a renal flow probe (Transonic, Ithaca, NY, USA) placed around the renal artery and connected to a Transonic-T206 synchronized flow meter coupled to a polygraph system (model MP100, Biopac System).

2.4. Glomerular Filtration Rate. In a subset (n = 24) of anesthetized rats, the experiments were repeated with an additional catheter inserted into the right femoral vein to infuse inulin, para-amino hippuric acid, and albumin. Plasma and urine sodium and potassium concentrations were determined by flame photometry, and inulin concentrations were measured colorimetrically to determine glomerular filtration rate (GFR) [26]. RVR and fractional sodium excretion (FE_{Na}) were calculated according to standard formulas. The renal excretion data from this subset of animals were not included in the final measures due to the different handling of these animals (additional catheter, and albumin, PAH, and inulin infusion). However, the excretory data from these animals followed the same trends as the reported data.

2.5. Plasma Renin Activity. Plasma renin activity (PRA) was measured with a commercially available assay kit (Gamma Coat PRA Assay Kit) [27]. Briefly, DALA (80 μmol/kg; IP) was infused into L-NAME- (50 mg/Kg; IP) pretreated or

untreated rats and PRA was measured to determine if altered CO levels had any effect on the renin-angiotensin system. PRA was determined by the radioimmunoassay generation of angiotensin I. Given the noted experimental difficulties with measuring PRA in whole animals, we did not perform clearance measurements or CO measurements in these animals.

2.6. Determination of the Effect of DALA to Increase CO Excretion. A subset of awake Sprague-Dawley rats ($n = 12$) that did not receive any surgical treatments were infused with DALA (80 μmol/kg, IV) to increase HO activity, both with and without chronic L-NAME pretreatment every 12 hours for 4 days. Animals were placed in an acrylic airtight chamber with the outflow leading to a heated mercuric oxide bed coupled with a gas chromatograph (Peak, Mountain View, CA, USA) for the determination of CO concentration, detailed elsewhere [28, 29]. The chamber was continuously purged with purified air and the outflow sampled for CO concentration at 2 min intervals. After a 10 min equilibration period, the average of four measurements was used to calculate the CO excretion rate for the whole animal.

2.7. Determination of Renal HO-1 Levels. In a subset ($n = 14$) of similarly treated anesthetized animals, the experimental protocols were repeated to determine the ability of DALA infusion to alter renal HO-1 levels. Renal HO-1 levels were measured by commercially available ELISA kits purchased from Stressgen. Kidneys from L-NAME-pretreated and untreated rats were removed and flash frozen in liquid nitrogen and suspended in 1X extraction reagent and protease inhibitor. Once the kidney tissues were homogenized, the ELISA sandwich immunoassay was preformed and the level of HO-1 protein present in the kidney was determined.

3. Data Analysis

Data were expressed as mean ± SEM. Data were analyzed by analysis of variance (ANOVA) followed by orthogonal contrast when appropriate (SYSTAT). Bonferroni correction was employed in the final analysis of completed series ($\alpha = 0.05$) [30].

4. Results

4.1. Whole Animal CO Excretion. Acute administration of the heme precursor, DALA (80 μmol/kg, IP), to untreated animals produced a significant increase in expired CO levels ($\Delta 63.9 \pm 1.6\%$, $n = 3$) (Figure 1). This effect was similar to a higher dose of DALA (800 μmol/kg, IP) (Jackson et al, unpublished results). This increase in expired CO was not affected by L-NAME (50 mg/Kg; IP) pretreatment ($\Delta 67.6 \pm 1.9\%$, $n = 3$) (Figure 1).

4.2. Renal HO-1 Levels. Acute administration of DALA (80 μmol/kg, IV) in untreated and L-NAME-pretreated anesthetized rats produced a significant increase in renal HO-1 levels in untreated ($\Delta 50 \pm 0.56\%$, $n = 7$) (Figure 2) and L-NAME-treated ($\Delta 60 \pm 0.64\%$, $n = 7$) (Figure 2)

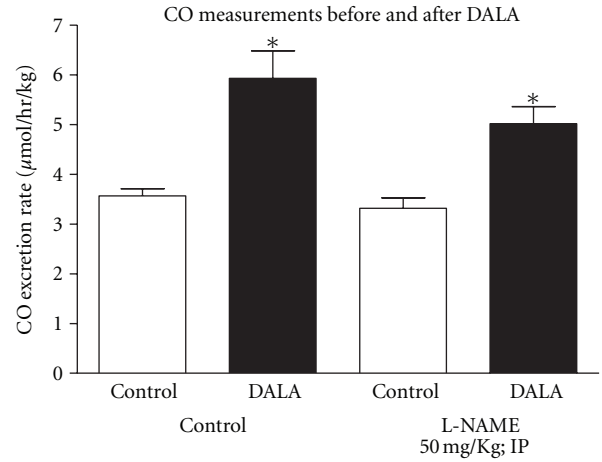

Figure 1: DALA (80 μmol/kg; IV) infusion acutely increased expired CO levels in L-NAME-treated and -untreated awake rats. Values are mean ± SE; $n = 6$ each.

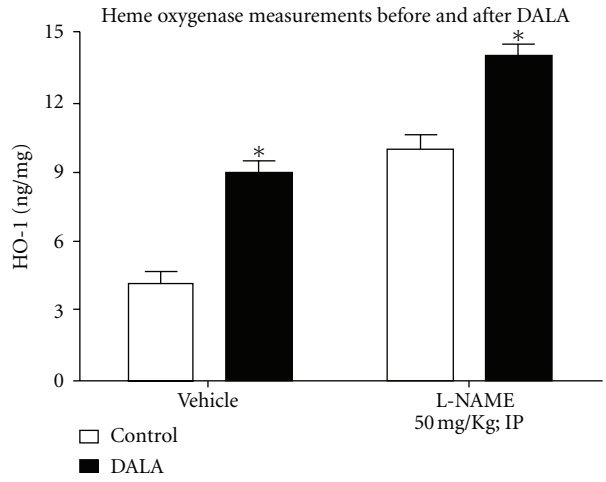

Figure 2: In anesthetized rats, DALA (80 μmol/kg; IV) acutely increased renal HO-1 levels in vehicle (left) and L-NAME-treated (right) rats. ($^*P < 0.05$, pre- versus 30 min post-DALA; $n = 6$ each).

rats. L-NAME pretreatment produced a significant increase in renal HO-1 levels, as compared to untreated animals (Figure 2); however, DALA increased renal HO-1 levels to a similar extent as in untreated animals. There were no significant differences in hematocrit pre- and post-DALA administration in both the L-NAME-pretreated and -untreated animals.

4.3. Renal Functional Responses. The subsequent values were obtained during the 30 min experimental period following administration of DALA (80 μmol/kg; IP), biliverdin (20 mg/kg), bilirubin (30 mg/kg), or vehicle in L-NAME-(50 mg/kg; IP) treated and untreated animals. In animals without pretreatment, DALA did not exert significant systemic or renal hemodynamic effects (Table 1), but there were significant increases in urine flow and sodium and potassium excretion ($\Delta 108.8 \pm 0.28\%$, $172.1 \pm 18.4\%$, and $165.2 \pm 45.9\%$: $n = 20$)

Table 1: Effects of DALA (80 μmol/kg, IV), biliverdin (20 mg/Kg, IV), and bilirubin (30 mg/Kg, IV) administration on heart rate (HR), mean arterial pressure (MAP), renal blood flow (RBF), and calculated renal vascular resistance (RVR).

	N	HR (bpm)	MAP (mmHg)	RBF (ml/min)	RVR (mmHg/ml/min)
No pretreatment					
Control		385 ± 0.13	110 ± 0.06	5.6 ± 0.12	19.6 ± 0.14
Vehicle	8	395 ± 0.16	114 ± 0.08	5.8 ± 0.14	19.7 ± 0.11
DALA	8	382 ± 0.12	118 ± 0.09	5.9 ± 0.16	20.0 ± 0.18
Biliverdin	8	396 ± 0.25	113 ± 0.04	6.1 ± 0.15	18.6 ± 0.21
Bilirubin	8	398 ± 0.18	109 ± 0.15	5.4 ± 0.14	20.1 ± 0.24
Chronic L-NAME					
Control		398 ± 0.19	153 ± 0.05	5.5 ± 0.14	27.8 ± 0.28
Vehicle	8	400 ± 0.23	150 ± 0.12	5.2 ± 0.18	28.8 ± 0.20
DALA	8	403 ± 0.14	155 ± 0.24	5.8 ± 0.21	28.2 ± 0.38
Biliverdin	8	396 ± 0.19	158 ± 0.15	5.5 ± 0.14	28.7 ± 0.47
Bilirubin	8	399 ± 0.24	152 ± 0.05	5.1 ± 0.14	29.2 ± 0.32

(Figure 3). Biliverdin (20 mg/kg) and bilirubin (30 mg/kg) did not cause significant systemic or renal hemodynamic effects (Table 1) and any significant changes in urine flow or sodium, and potassium excretion (Table 3). In rats pretreated chronically with L-NAME, there was a significant increase in MAP (100 mmHg versus 150 mmHg) but DALA administration had no significant effects on MAP, HR, RBF, or RVR (Table 1). However, DALA significantly increased urine flow and sodium and potassium excretion (Δ109.4 ± 0.29%, 187.3 ± 26.9%, and 197.2 ± 45.7%: $n = 20$) (Figure 4). Biliverdin (20 mg/kg) and bilirubin (30 mg/kg) did not exert significant effects on MAP, HR, RBF, or RVR (Table 1) and any significant effects on urinary volume, sodium and potassium excretion (Table 3). There were no significant differences between the urine flow and electrolyte excretion in the L-NAME-untreated and -treated animals. Vehicle treatment had no effect in either group. DALA had no effects on glomerular filtration in either L-NAME-treated or -untreated rats (Table 2: $n = 24$).

4.4. Plasma Renin Activity. In untreated rats given DALA, no significant differences in plasma renin activity (PRA) were evident (Figure 5; $n = 25$). Similarly DALA did not significantly alter PRA in L-NAME-pretreated animals (Figure 5; $n = 21$).

5. Discussion

The present study investigated the role of increases in endogenous CO on renal excretory function. The heme precursor, DALA, increased expired CO levels in both L-NAME-treated and -untreated animals. DALA, which promotes the generation of endogenous CO, increased volume and electrolyte excretion in both L-NAME-treated and -untreated animals. Acute increases in endogenous CO formation were not accompanied by any significant differences in systemic or renal hemodynamic function in that a low dose of DALA was specifically chosen to avoid alterations in renal or systemic hemodynamics. There were also no significant changes in GFR with DALA infusion in L-NAME-treated or -untreated animals.

Increases in heme oxygenase activity, promote an equimolar elevation in carbon monoxide, iron, and biliverdin [31]. Biliverdin is quickly converted to bilirubin [31]. Current literature would support an antioxidant role for both biliverdin and bilirubin [20, 21]. To examine the potential role of these heme products to alter renal excretory function, a subset of animals was given biliverdin or bilirubin and the study was repeated. However, no significant differences in renal or systemic hemodynamics were observed and, similarly, no significant differences in renal excretory function were observed, thus, suggesting that the observed increases in urine flow and sodium and potassium excretion were due to carbon monoxide. The negative results observed with biliverdin and bilirubin suggest that they are not involved in the heme-oxygenase-mediated diuretic effects; however, the current study cannot rule out the importance of these metabolites in the kidney in that renal intracellular concentrations of biliverdin and bilirubin were not measured.

DALA has been previously shown to increase HO activity in rats [32]. We have reported that DALA also increases expired CO levels, as well. Thus, DALA, a substrate that drives heme formation and increases HO activity, can produce significant increases in endogenous CO formation. DALA administration was observed to significantly increase HO-1 levels both in the presence and absence of an intact NO system. L-NAME administration increased baseline HO-1 levels, perhaps due to the observed elevation in MAP. Importantly, the ability of DALA administration to increase HO-1 levels was not affected by L-NAME.

The current study used DALA to drive CO formation in that iron loading can lead to effects on the vasculature that are independent of the CO system. Iron loading can occur, when one increases CO formation via heme administration or CO releasing molecules [32]. The current data support the hypothesis that CO increases water and electrolyte excretion independent of changes in systemic or renal hemodynamics.

Table 2: Effects of increases in endogenous CO (DALA 80 μmol/Kg, IV) on glomerular filtration rate (GFR), urine flow (UF), sodium excretion ($U_{Na}V$), fractional excretion of sodium (FE_{Na}), and urinary potassium (U_KV).

	N	UF (μl/min)	GFR (ml/min)	$U_{Na}V$ (μmol/min)	FE_{Na} (%)	U_KV (μmol/min)
No pretreatment						
Control	6	6.49 ± 0.47	1.11 ± 0.05	0.59 ± 0.15	0.50 ± 0.03	0.14 ± 0.04
Vehicle	6	6.49 ± 0.48	1.10 ± 0.08	0.61 ± 0.08	0.51 ± 0.16	0.15 ± 0.03
Control	6	6.51 ± 0.50	1.12 ± 0.11	0.60 ± 0.11	0.53 ± 0.12	0.14 ± 0.01
DALA	6	**13.99 ± 1.84***	1.10 ± 0.06	**1.19 ± 0.03***	**0.78 ± 0.01***	**0.90 ± 0.13***
Chronic L-NAME						
Control	6	7.37 ± 0.73	1.11 ± 0.02	0.68 ± 0.13	0.50 ± 0.01	0.23 ± 0.13
Vehicle	6	7.43 ± 0.78	1.15 ± 0.18	0.68 ± 0.04	0.49 ± 0.05	0.23 ± 0.02
Control	6	7.51 ± 0.54	1.12 ± 0.17	0.67 ± 0.11	0.50 ± 0.02	0.24 ± 0.04
DALA	6	**14.37 ± 0.41***	1.11 ± 0.10	**1.22 ± 0.02***	**0.80 ± 0.12***	**1.09 ± 0.19***

Table 3: Effects of biliverdin (20 mg/Kg, IV) and bilirubin (30 mg/Kg, IV) administration on urine flow (UF), sodium excretion ($U_{Na}V$) and urinary potassium (U_KV).

	N	UF (μl/min)	$U_{Na}V$ (μmol/min)	U_KV (μmol/min)
No pretreatment				
Control	6	2.50 ± 0.30	0.17 ± 0.02	0.49 ± 0.06
Biliverdin	6	2.10 ± 0.12	0.16 ± 0.01	0.46 ± 0.05
Control	6	2.80 ± 0.15	0.19 ± 0.12	0.46 ± 0.01
Bilirubin	6	2.90 ± 0.21	0.16 ± 0.04	0.41 ± 0.09
Chronic L-NAME				
Control	6	3.40 ± 0.25	0.15 ± 0.02	0.41 ± 0.06
Biliverdin	6	3.30 ± 0.39	0.17 ± 0.02	0.40 ± 0.35
Control	6	3.20 ± 0.51	0.18 ± 0.02	0.46 ± 0.05
Bilirubin	6	3.30 ± 0.37	0.18 ± 0.02	0.45 ± 0.03

The increases in urine flow and electrolyte excretion were still present during NOS inhibition by L-NAME, indicating once again that the CO effects on urine flow and electrolyte excretion are not simply due to alterations in the nitric oxide (NO) system. Furthermore, DALA administration did not significantly alter PRA, thus CO enhancement of renal excretion was not via suppression of the renin angiotensin system. We recently reported that inhibition of endogenous CO increased PRA in untreated rats [24]. This increase in PRA was abolished by L-NAME pretreatment [24]. However, endogenous CO effects on the juxtaglomerular cells could be maximal even at basal conditions; therefore, increasing CO formation by DALA would not affect PRA.

In previous research, an increase in CO concentration elicited through heme administration was shown to decrease RVR, increase RBF, and urine flow and sodium excretion [33]. Similar results can be observed with CO releasing molecules. Pretreatment with the HO inhibitor, SnMP, abolished the diuretic and natriuretic effects of heme but did not affect the increases in RBF. The heme-induced changes in renal hemodynamic parameters could perhaps be attributed to differences in agents (DALA versus heme) and/or concentrations. Regional differences in HO activity in the kidney have been reported, where medullary heme oxygenase contributes to pressure natriuresis and arterial blood pressure in the absence of any significant changes in cortical HO activity [22]. As previously stated, low concentrations of DALA were employed to avoid hemodynamic changes in the present study. Therefore, DALA-induced increases in urine flow and electrolyte excretion were not accompanied by any changes in renal hemodynamic function. However, medullary blood flow was not measured and we cannot exclude the possibility of small increases in medullary BF to the diuretic and natriuretic responses. Collectively these data suggest that CO alters water and electrolyte excretion independent of changes in NO and renal hemodynamic function and suggests that this response is due to a direct renal tubular effect.

Previous studies have demonstrated CO's ability to promote vasoconstriction via inhibition of NOS [19, 34]. However, in the present study, such an interaction between the two systems in acutely regulating water and electrolyte excretion was not observed. Thus, it is possible that in organ systems with a large capacity to autoregulate, such as the brain, heart, and kidney, CO inhibition of NO does not play a major role in establishing normal basal vascular tone. CO was able to promote water and electrolyte excretion without

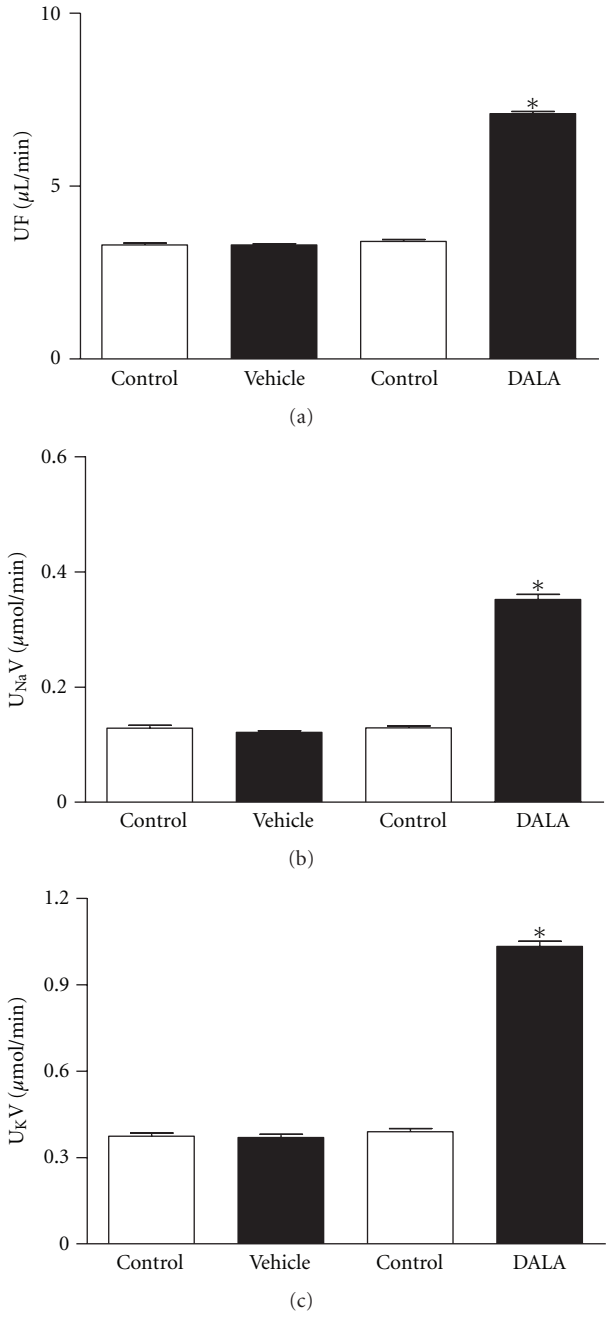

Figure 3: In anesthetized rats, DALA (80 μmol/kg) IV infusion acutely increased urine flow and sodium and potassium excretion in untreated rats. Values are mean ± SE; $n = 20$. $^*P < 0.05$ versus control (vehicle infusion).

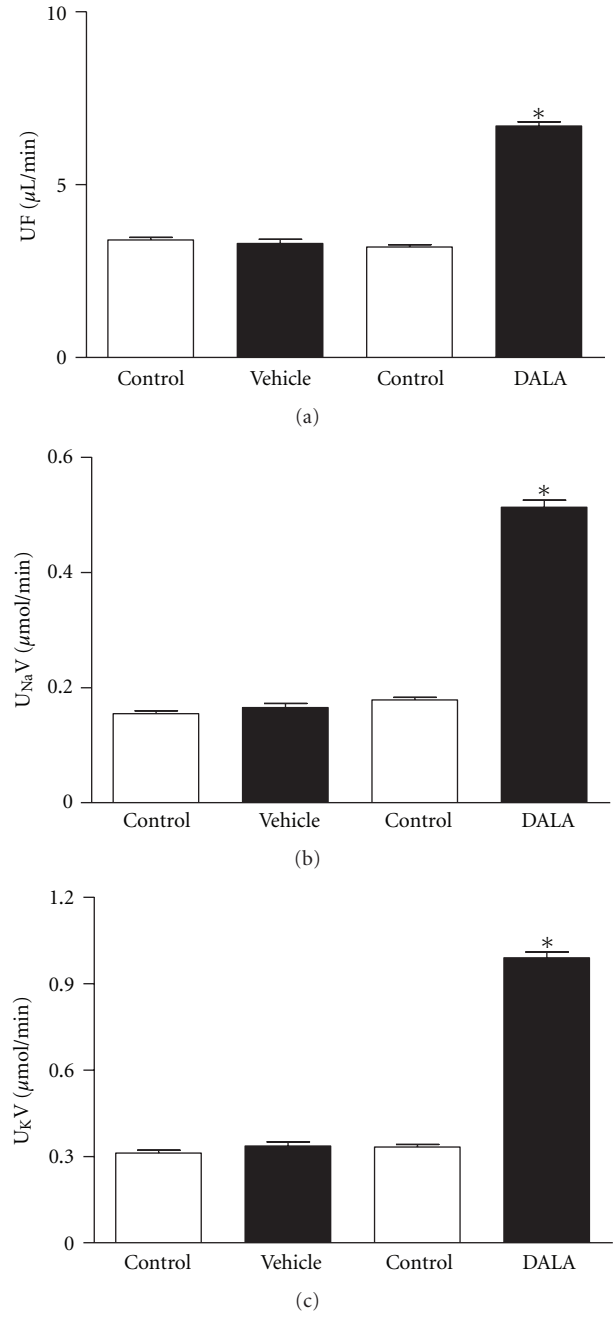

Figure 4: In anesthetized rats, acute IV infusion of DALA (80 μmol/kg) increased urine flow, urinary sodium, and urinary potassium excretion in L-NAME-pretreated animals. Values are mean ± SE; $n = 20$. $^*P < 0.05$ versus control (vehicle infusion).

affecting renal hemodynamics, which suggests an alternate pathway for CO regulation of renal excretory function. Thus, CO could have direct effects on the tubules to alter water and electrolyte excretion. As a low dose of DALA was administered to avoid altering renal hemodynamics, the results suggest that the alterations in renal excretory function are most likely mediated via a direct tubular effect to inhibit sodium transport in that sodium and potassium excretion were enhanced during DALA administration.

6. Significance of the Study

Previous studies have demonstrated that increases in HO activity can promote significant diuresis [33]. Since heme administration was accompanied by a significant increase in blood pressure, it could not be established if the observed diuresis was due to a direct tubular action or simply due to an increase in perfusion pressure. In addition, it was not shown if the diuresis was due to CO or one of the other HO

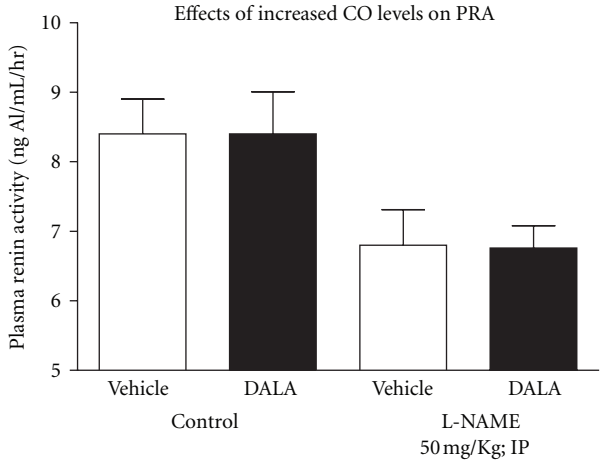

Figure 5: Acute administration of DALA (80 μmol/kg; IP) did not exert significant effects on plasma renin activity (PRA) in L-NAME-pretreated and -untreated rats. Vehicle and DALA changes in PRA were observed for 30-minute periods. Values are mean ± SE; $n = 46$.

metabolites. In the current study, we demonstrate a direct tubular action of HO induction in the absence of alterations in renal hemodynamic function. Furthermore, the negative results with biliverdin and bilirubin administration suggest a tubular role of CO as a novel diuretic and therapeutic target to treat hypertension.

7. Conclusion

In summary, the present data indicate that an induction in HO-1 increases water and electrolyte excretion in the absence of alterations in renal hemodynamics, PRA, GFR, or NO production, thus, suggesting a direct tubular role for endogenous CO in the control of sodium excretion.

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References

[1] M. D. Maines, "The heme oxygenase system: a regulator of second messenger gases," *Annual Review of Pharmacology and Toxicology*, vol. 37, pp. 517–554, 1997.

[2] N. G. Abraham, J. Cao, D. Sacerdoti, X. Li, and G. Drummond, "Heme oxygenase: the key to renal function regulation," *American Journal of Physiology*, vol. 297, no. 5, pp. F1137–F1152, 2009.

[3] J. L. Da Silva, B. A. Zand, L. M. Yang, H. E. Sabaawy, E. Lianos, and N. G. Abraham, "Heme oxygenase isoform-specific expression and distribution in the rat kidney," *Kidney International*, vol. 59, no. 4, pp. 1448–1457, 2001.

[4] T. Aizawa, N. Ishizaka, J. I. Taguchi et al., "Heme oxygenase-1 is upregulated in the kidney of angiotensin II-induced hypertensive rats: possible role in renoprotection," *Hypertension*, vol. 35, no. 3, pp. 800–806, 2000.

[5] E. N. Haugen, A. J. Croatt, and K. A. Nath, "Angiotensin ii induces renal oxidant stress in vivo and heme oxygenase-1 in vivo and in vitro," *Kidney International*, vol. 58, no. 1, pp. 144–152, 2000.

[6] S. Quan, L. Yang, S. Shnouda et al., "Expression of human heme oxygenase-1 in the thick ascending limb attenuates angiotensin II-mediated increase in oxidative injury," *Kidney International*, vol. 65, no. 5, pp. 1628–1639, 2004.

[7] Y. Hu, N. Ma, M. Yang, and R. Semba, "Expression and distribution of heme oxygenase-2 mRNA and protein in rat kidney," *Journal of Histochemistry and Cytochemistry*, vol. 46, no. 2, pp. 249–256, 1998.

[8] L. Grundemar, M. B. Johansson, M. Ekelund, and E. D. Högestätt, "Haem oxygenase activity in blood vessel homogenates as measured by carbon monoxide production," *Acta Physiologica Scandinavica*, vol. 153, no. 2, pp. 203–204, 1995.

[9] I. A. Sammut, R. Foresti, J. E. Clark et al., "Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of haeme oxygenase-1," *British Journal of Pharmacology*, vol. 125, no. 7, pp. 1437–1444, 1998.

[10] R. F. Furchgott and D. Jothianahdan, "Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light," *Blood Vessels*, vol. 28, no. 1–3, pp. 52–61, 1991.

[11] N. G. Abraham and A. Kappas, "Pharmacological and clinical aspects of heme oxygenase," *Pharmacological Reviews*, vol. 60, pp. 79–127, 2008.

[12] A. Matsuoka, D. J. Stuehr, J. S. Olson, P. Clark, and M. Ikeda-Saito, "L-arginine and calmodulin regulation of the heme iron reactivity in neuronal nitric oxide synthase," *Journal of Biological Chemistry*, vol. 269, no. 32, pp. 20335–20339, 1994.

[13] A. P. Zou, H. Billington, N. Su, and A. W. Cowley, "Expression and actions of heme oxygenase in the renal medulla of rats," *Hypertension*, vol. 35, no. 1, pp. 342–347, 2000.

[14] R. A. Johnson, M. Lavesa, K. Deseyn, M. J. Scholer, and A. Nasjletti, "Heme oxygenase substrates acutely lower blood pressure in hypertensive rats," *American Journal of Physiology*, vol. 271, no. 3, pp. H1132–H1138, 1996.

[15] F. Kozma, R. A. Johnson, and A. Nasjletti, "Role of carbon monoxide in heme-induced vasodilation," *European Journal of Pharmacology*, vol. 323, no. 2-3, pp. R1–R2, 1997.

[16] F. Kozma, R. A. Johnson, F. Zhang, C. Yu, X. Tong, and A. Nasjletti, "Contribution of endogenous carbon monoxide to regulation of diameter in resistance vessels," *American Journal of Physiology*, vol. 276, no. 4, pp. R1087–R1094, 1999.

[17] J. F. Ndisang, W. Zhao, and R. Wang, "Selective regulation of blood pressure by heme oxygenase-1 in hypertension," *Hypertension*, vol. 40, no. 3, pp. 315–321, 2002.

[18] F. T. Botros and L. G. Navar, "Interaction between endogenously produced carbon monoxide and nitric oxide in regulation of renal afferent arterioles," *American Journal of Physiology*, vol. 291, no. 6, pp. H2772–H2778, 2006.

[19] F. Rodriguez, F. Zhang, S. Dinocca, and A. Nasjletti, "Nitric oxide synthesis influences the renal vascular response to heme oxygenase inhibition," *American Journal of Physiology*, vol. 284, no. 6, pp. F1255–F1262, 2003.

[20] R. M. LeBlanc, L. G. Navar, and F. T. Botros, "Bilirubin exerts renoprotective effects in angiotensin II-hypertension," *American Journal of the Medical Sciences*, vol. 340, no. 2, pp. 144–146, 2010.

[21] D. E. Baranano, M. Rao, C. D. Ferris, and S. H. Snyder, "Biliverdin reductase: a major physiologic cytoprotectant," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 25, pp. 16093–16098, 2002.

[22] N. Li, F. Yi, E. A. Dos Santos, D. K. Donley, and P. L. Li, "Role of renal medullary heme oxygenase in the regulation of pressure natriuresis and arterial blood pressure," *Hypertension*, vol. 49, no. 1, pp. 148–154, 2007.

[23] H. Wang, J. L. Garvin, M. A. D'Ambrosio, J. R. Falck, P. Leung, and R. Liu, "Heme oxygenase metabolites inhibit tubuglomerular feedback in vivo," *American Journal of Physiology*, vol. 300, no. 4, pp. H1320–H1326, 2011.

[24] K. E. Jackson, D. W. Jackson, S. Quadri, M. J. Reitzell, and L. G. Navar, "Inhibition of heme oxygenase augments tubular sodium reabsorption," *American Journal of Physiology*, vol. 300, no. 4, pp. 941–946, 2011.

[25] R. A. Johnson and R. H. Freeman, "Sustained hypertension in the rat induced by chronic blockade of nitric oxide production," *American Journal of Hypertension*, vol. 5, no. 12 I, pp. 919–922, 1992.

[26] T. Takenaka, K. D. Mitchell, and L. G. Navar, "Contribution of angiotensin II to renal hemodynamic and excretory responses to nitric oxide synthesis inhibition in the rat," *Journal of the American Society of Nephrology*, vol. 4, no. 4, pp. 1046–1053, 1993.

[27] J. Fox, S. Guan, A. A. Hymel, and L. G. Navar, "Dietary Na and ACE inhibition effects on renal tissue angiotensin I and II and ACE activity in rats," *American Journal of Physiology*, vol. 262, no. 5, pp. F902–F909, 1992.

[28] H. J. Verman, R. J. Wong, and D. K. Stevenson, *Carbon Monoxide in Breath, Blood, and Other Tissues*, CRC Press, Boca Raton, Fla, USA, 2000.

[29] H. J. Vreman, R. J. Wong, T. Kadotani, and D. K. Stevenson, "Determination of carbon monoxide (CO) in rodent tissue: effect of heme administration and environmental CO exposure," *Analytical Biochemistry*, vol. 341, no. 2, pp. 280–289, 2005.

[30] G. W. Snedecor and W. G. Cocharan, *Statistical Methods*, Iowa State University Press, Ames, Iowa, USA, 8th edition, 1994.

[31] N. G. Abraham, J. H. C. Lin, M. L. Schwartzman, R. D. Levere, and S. Shibahara, "The physiological significance of heme oxygenase," *International Journal of Biochemistry*, vol. 20, no. 6, pp. 543–558, 1988.

[32] F. K. Johnson and R. A. Johnson, "Carbon monoxide promotes endothelium-dependent constriction of isolated gracilis muscle arterioles," *American Journal of Physiology*, vol. 285, no. 3, pp. R536–R541, 2003.

[33] F. Rodriguez, R. Kemp, M. Balazy, and A. Nasjletti, "Effects of exogenous heme on renal function: role of heme oxygenase and cyclooxygenase," *Hypertension*, vol. 42, no. 4, pp. 680–684, 2003.

[34] F. K. Johnson, W. Durante, K. J. Peyton, and R. A. Johnson, "Heme oxygenase inhibitor restores arteriolar nitric oxide function in dahl rats," *Hypertension*, vol. 41, no. 1, pp. 149–155, 2003.

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Research Article

Reciprocal Effects of Oxidative Stress on Heme Oxygenase Expression and Activity Contributes to Reno-Vascular Abnormalities in EC-SOD Knockout Mice

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Heme oxygenase (HO) system is one of the key regulators of cellular redox homeostasis which responds to oxidative stress (ROS) via HO-1 induction. However, recent reports have suggested an inhibitory effect of ROS on HO activity. In light of these conflicting reports, this study was designed to evaluate effects of chronic oxidative stress on HO system and its role in contributing towards patho-physiological abnormalities observed in extracellular superoxide dismutase (EC-SOD, SOD3) KO animals. Experiments were performed in WT and EC-SOD$^{(-/-)}$ mice treated with and without HO inducer, cobalt protoporphyrin (CoPP). EC-SOD$^{(-/-)}$ mice exhibited oxidative stress, renal histopathological abnormalities, elevated blood pressure, impaired endothelial function, reduced p-eNOS, p-AKT and increased HO-1 expression; although, HO activity was significantly ($P < 0.05$) attenuated along with attenuation of serum adiponectin and vascular epoxide levels ($P < 0.05$). CoPP, in EC-SOD$^{(-/-)}$ mice, enhanced HO activity ($P < 0.05$) and reversed aforementioned pathophysiological abnormalities along with restoration of vascular EET, p-eNOS, p-AKT and serum adiponectin levels in these animals. Taken together our results implicate a causative role of insufficient activation of heme-HO-adiponectin system in pathophysiological abnormalities observed in animal models of chronic oxidative stress such as EC-SOD$^{(-/-)}$ mice.

1. Introduction

Oxidative stress induces NRF2-dependent antioxidant enzymes including the heme-HO system [1], whose two isoforms HO-1 (inducible) and HO-2 (constitutive) catabolizes free heme to equimolar concentrations of biliverdin (BV), carbon monoxide (CO), and iron. Excess-free heme, due to its pro-oxidant and proinflammatory properties, contributes to an increase in free radical formation and cellular injury [1, 2], thus necessitating its catabolism by HO. Apart from restricting accumulation of pro-oxidant heme, antioxidant properties of heme-HO system arise from production of BV and bilirubin (BR), which have potent antioxidant properties [3, 4]. In addition, CO has been shown to exhibit antioxidant [5, 6] (reviewed by [1]), antiapoptotic [7], and vasomodulatory properties. These properties of the heme-HO system are pertinent to redox balance and its associated physiological ramifications, especially in the cardiovascular-renal systems [1, 8]. Further evidence of HO-mediated sustenance of renovascular homeostasis is provided by studies demonstrating HO-dependent activation of adiponectin release [9], which has antioxidant and anti-inflammatory properties [10] in addition to its renoprotective effects [11]. An increase in adiponectin has also been shown to lead to increased levels of mitochondrial transport carriers and cytochrome oxidases via an increase in Bcl-XL [12]. The Bcl-2 family of proteins,

consisting of anti- and proapoptotic proteins, along with serine-threonine kinase (Akt) (protein kinase B), are critical in cell death/survival pathways [13]. Akt is activated through phosphorylation at either threonine-308 or serine-473 [14]. Activated Akt inhibits ASK-1, a proapoptotic member of the MAP kinase kinase family, and protects against stress induced apoptosis in endothelial cells [15].

Apart from inducible NRF2-dependent genes, constitutive enzymes such as superoxide dismutases (SOD) regulate basal redox and prevent excess-free radical accumulation. SOD enzymes are known to exist in three isoforms: Cu-Zn SOD (SOD1), predominately located in the cytoplasm, Mn-SOD (SOD2) in the mitochondria, and EC-SOD (SOD3) in the extracellular space. Although SOD1 accounts for 60% to 80% of SOD activity *in vivo* [16, 17], SOD3 is highly expressed in renal and vascular tissues, particularly in the arterial wall, and its activity constitutes almost half of the total SOD activity in the human aorta [18]. Gene deletion of EC-SOD results in chronic oxidative stress, endothelial dysfunction, and increased blood pressure [17, 19, 20], implicating this enzyme in the regulation of redox homeostasis and preservation of cardiovascular and renal function. Where, in an event of increased oxidative stress HO-1 is rapidly induced, recent reports have emerged suggesting an inhibitory effect of the same on HO activity [21]. In this contradictory contexture, the present study was designed to examine the effects of chronic oxidative stress, as observed in EC-SOD$^{(-/-)}$ mice, on HO expression and activity with regards to the pathophysiological abnormalities observed in these animals. The study was performed in WT and EC-SOD$^{(-/-)}$ mice in the absence and in the presence of HO inducer, CoPP. Our results demonstrate that EC-SOD deficiency, although accompanied by oxidative stress and induction of HO-1, is characterized by attenuation of HO activity with the resultant attenuation of vascular epoxide and adiponectin levels. Phenotypic analysis of EC-SOD knockout mice revealed renal microvascular and corticomedullary damage along with elevated blood pressure and vascular endothelial dysfunction. Induction of HO-1 in SOD3-deficient mice not only restored HO activity and redox homeostasis, but also prevented renovascular injury and offset endothelial dysfunction and elevated blood pressure. These events are accompanied by the restoration of vascular epoxide and serum adiponectin levels with a concomitant increase in p-AKT and p-eNOS expression.

2. Research Design and Methods

2.1. Animal Treatment. Three-month-old homozygote, male, EC-SOD$^{(-/-)}$ and C57BL/6 genetic background EC-SOD$^{(+/+)}$ mice were used for this study. EC-SOD$^{(-/-)}$ mice were a gift from University of Pittsburgh, PA, USA. These mice had been backcrossed to C57BL/6 mice and are congenic with this line of mice [22]; as such, C57BL/6, purchased from The Jackson Laboratories, were used as WT controls. All animals were maintained on a standard laboratory diet and water, and every effort was made to minimize animal suffering according to the National Institutes of Health (NIH), Institutional Animal Care and Use Committee guidelines. The studies were approved by the animal use committee at New York Medical College.

Mice, both WT and KO, were divided into two groups (6 mice/group): controls, injected with saline solution, and the treatment group, treated with cobalt protoporphyrin (CoPP) 5 mg/Kg i.p. weekly for 6 weeks. CoPP was dissolved in trisma base as previously described [23]. The last injection of CoPP was made three days before sacrificing the animals. Mice were followed for 6 weeks (duration of the experiments), at which time they were sacrificed with CO_2 gas. Blood and tissues were collected and stored, as described, for appropriate experiments.

2.2. Morphology and Proteins Analysis. Renal cross-sections were fixed in formalin, paraffin-embedded, and processed for histology using standard techniques. Tissue sections were cut at 3-4 micron thickness and stained with H&E and periodic acid Schiff (PAS). A portion of the tissues was snap-frozen and kept at -80° for proteins determinations and Western blot analysis.

2.3. Tissue Preparation for Western Blot, HO Activity, and O_2^- Levels. Sections of femoral arteries and whole kidneys were homogenized in homogenization buffer (255 mM sucrose, 20 mM tris-hcl, 1 mM EDTA, 0.1 mM PMSF, and 0.5% Nonidet P-40 at pH 7.4) containing a cocktail of protease inhibitors (Roche, Indiana, IN) and Halt, a phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL). The homogenates were centrifuged at 14000 g for 10 min at 4°C, supernatant was isolated and protein levels assayed using a Bio-Rad kit on the basis of the Bradford dye binding procedure.

2.4. Measurement of O_2^- Levels. Employing previously described methods [24], kidney and arterial samples were placed in glass scintillation mini vials containing 5 μM lucigenin, for the detection of O_2^- in a final volume of 1 mL of air-equilibrated Krebs solution buffered with 10 mM HEPES-NaOH (pH 7.4). Lucigenin chemiluminescence was measured in a liquid scintillation counter (LS6000IC, Beckman Instruments, and San Diego, CA), and data are reported as counts/minute/mg protein after background subtraction.

2.5. Measurement of HO Activity. Tissue HO activity was assayed as described previously [25] using a technique in which bilirubin, the end product of heme degradation, was extracted with chloroform, and its concentration was determined spectrophotometrically (dual UV/VIS beam spectrophotometer lambda 25; PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA) using the difference in absorbance at a wavelength from λ460 to λ530 nm with an absorption coefficient of 40 $mM^{-1}\,cm^{-1}$. Under these conditions, HO activity was linear with protein concentration, time-dependent, and substrate-dependent [26].

2.6. RT-PCR Analysis. Frozen arterial and renal segments were pulverized under liquid nitrogen and RNA extracted and used for RT/PCR. Reverse transcription (RT) was carried

out using the Advantage RT-for-PCR Kit (Clontech). Poly-d (T) n was used as the reverse transcription primer. Specific primers for amplifying EC-SOD and 18 s cDNA were employed. PCR was performed using a Taq PCR kit (Roche, Indianapolis, IN, USA). For each RT-PCR, a sample without reverse transcriptase was processed in parallel and served as a negative control. Cycling parameters for amplifying RT products were as follows: 95°C, 1′, 60°C, 1′, and 72°C, 1–3′, for 30 cycles, and then extended at 72°C for another 5′. After amplification, PCR products were electrophoresed on 1.2% agarose gel, stained with ethylene bromide, and visualized under UV light.

2.7. Assessment of Vascular Reactivity. The femoral artery was removed and placed in cold oxygenated Krebs-bicarbonate solution, cleaned of fat and loose connective tissue, and sectioned into rings of approximately 3 mm length. Two rings per artery were obtained, and each ring was mounted on stainless steel hooks and suspended in a 5-mL tissue DMT myograph bath (DMT, Atlanta, GA) filled with Krebs solution, pH 7.4, gassed with 95% O_2/5% CO_2, and maintained at 37°C. The rings were incubated under a passive tension of 0.2 g for 1 hour. The Krebs buffer solution was replaced every 15 minutes and the tension readjusted each time. Force was recorded from force displacement transducers via AD Instrument's Powerlab system, running Chart 5 software. At the end of the equilibration period, the maximal force generated by the addition of a depolarizing solution of KCl (60 mM) was determined. To evaluate acetylcholine-induced vasorelaxation, the rings were preconstricted with phenylephrine to obtain maximal contraction followed by cumulative dose-response curves to acetylcholine.

2.8. Blood Pressure Measurements. Blood pressure was measured by the tail cuff method before the start and at the completion of the experiment (6 wks). Prior to the experiment, mice were all acclimated to the tail cuff method. Mice were placed in a heat controlled box (36°C–38°C) for approximately 10 min. before applying the tail cuff. The mean of a minimum of 5 measurements was obtained from each mouse. All measurements were determined at the same time of day, between 9:00 and 13:00 hr. Systolic blood pressure is reported in mm of Hg.

2.9. Adiponectin Measurements. Adiponectin was determined using an ELISA assay (Pierce Biotechnology, Inc., Woburn, MA, USA).

2.10. Western Blot Analysis. The supernatant from tissue homogenates was used for evaluation of protein expression via Western blot analysis. Primary antibodies used are listed as following: HO-1 and HO-2 (Assay Designs, Inc.), ASK1 (Abcam), eNOS, Bcl-Xl and AKT (Cell Signaling Technologies, Inc.), p-eNOS-Thr495 (Cell Signaling Technologies, Inc.), and p-Akt-Ser473 (Cell Signaling Technologies, Inc.). B-actin (Sigma-Aldrich) was used as housekeeping gene for normalization. Antibodies were prepared in the following dilutions: HO-1 and HO-2 (1 : 1000); eNOS, p-eNOS, Bcl-xl, ASK1, and p-AKT antibodies (1 : 5000); β-actin (1 : 10000). Experimental protocol in brief: 20 μg of lysate supernatant was separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden) with a semidry transfer apparatus (Bio-Rad, Hercules, CA, USA). The membranes were incubated with 10% milk in tris-buffered saline with tween 20 (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) at 4°C, overnight. After incubation, the membranes were washed with tris-buffered saline with tween 20, and the membranes were then incubated with secondary antibodies for 1 h at room temperature with constant shaking. The filters were washed and subsequently probed with horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG (Amersham Biosciences). Chemiluminescence detection was performed with the Amersham Biosciences enhanced chemiluminescence detection kit, according to the manufacturer's instructions.

2.11. Measurement of EETs. Femoral arterial segments (two segments per tube) were incubated in 1 mL of oxygenated Krebs' buffer containing 1 mM NADPH at 37°C for 1 h. Thereafter, internal standards were added to each sample followed by acidification to pH $\approx$ 4.0 with glacial acetic acid. Eicosanoids were extracted with twice the volume of ethyl acetate (performed three times), dried under gentle stream of nitrogen, and stored at −80°C until further analysis. Identification and quantification of EETs and DiHETs was performed with a Q-trap 3200 linear ion trap quadruple liquid chromatography-tandem mass spectrometry system equipped with a turbo V ion source operated in negative electro spray mode (Applied Biosystems, Foster City, CA, USA). Extracted samples were suspended in 10 μL of methanol and injected into the high-performance liquid chromatography via an Agilent 1200 standard series auto sampler equipped with a thermostat set at 4°C (Agilent Technologies). The high-performance liquid chromatographic component consisted of an Agilent 1100 series binary gradient pump equipped with an Eclipse plus C18 column (50 × 4.6 mm; 1.8 mm) (Agilent Technologies). The column was eluted at a flow rate of 0.5 mL/min with 100% mobile phase A [methanol/water/acetic acid (60 : 40 : 0.01, v/v/v)] from 0 to 2 min and a gradient increasing to 100% B (100% methanol) at 13 min. Multiple reaction monitoring was used with a dwell time of 25 or 50 ms for each compound, with the following source parameters: ion spray voltage, −4500 V, curtain gas, 40 U, ion source gas flow rate 1, 65 U, ion source gas flow 2, 50 U, and temperature, 600°C. Synthetic standards were used to obtain standard curves (5–500 pg) for each compound. These standard curves were used and extrapolated to calculate the final EET concentrations, which are presented as nanograms per milligram of protein per hour [27].

2.12. Statistical Analysis. The data are presented as mean ± SE for the number of experiments. Statistical significance ($P \leq 0.05$) was determined by the Fisher method of multiple comparisons. For comparison between treatment groups, the Null hypothesis was tested by a single-factor analysis of

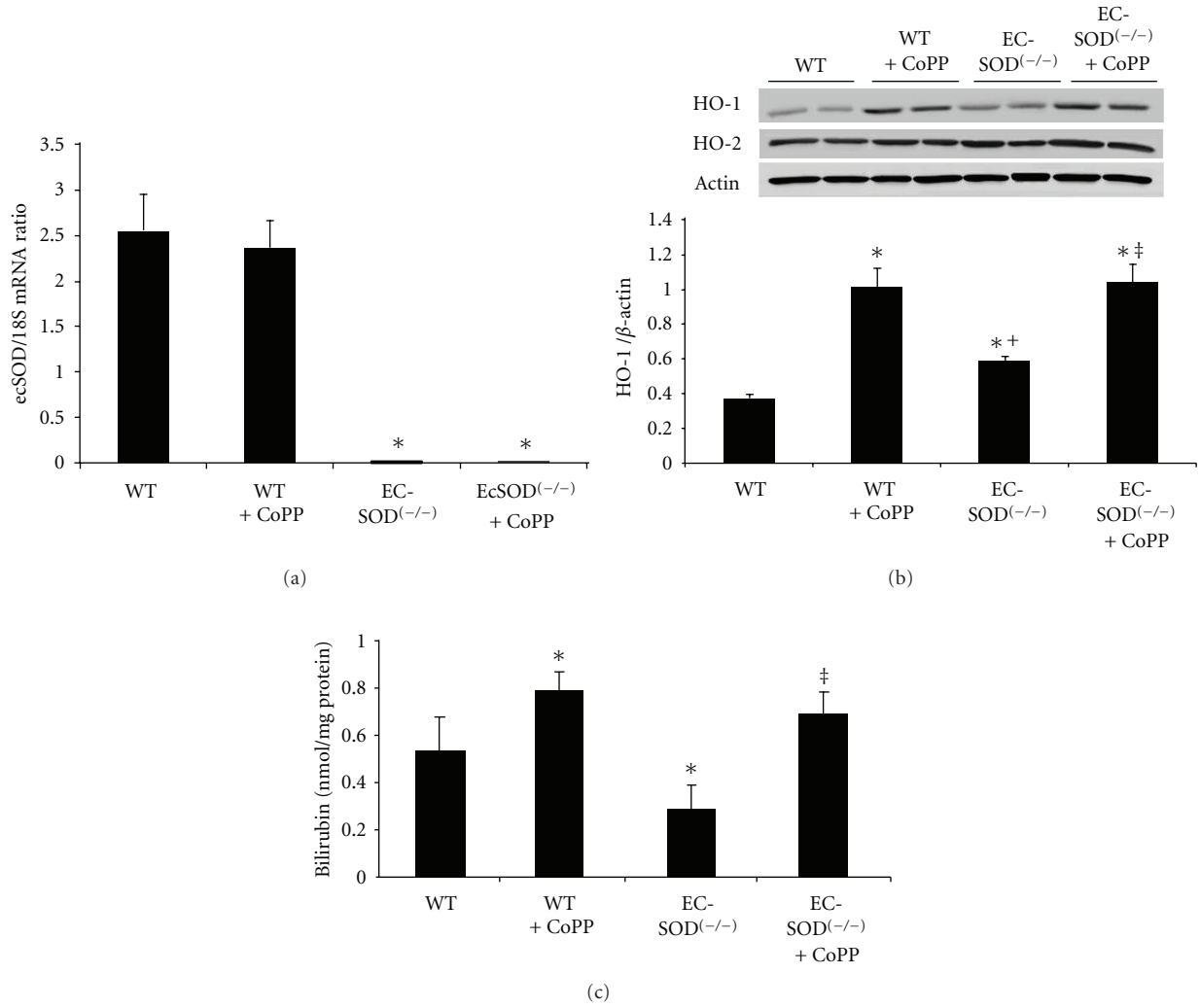

Figure 1: (a) Detection of EC-SOD mRNA transcripts by RT-PCR analysis in renal tissues. EC-SOD$^{(-/-)}$ animals demonstrated significant ($P < 0.05$) reduction in EC-SOD mRNA as compared to their WT counterparts, and the administration of CoPP had no significant effect on SOD3 mRNA levels in both WT and KO animals. Results are expressed as mean ± SE, $^{*}P < 0.01$ versus WT mice. (b) Western blot analysis of HO-1 and HO-2 proteins in kidney homogenates of WT and EC-SOD$^{(-/-)}$ mice. Immunoblots were performed using antibodies against mice HO-1 and HO-2 proteins. Blots are representative of six separate experiments. Results are expressed as mean ± SE, $^{*}P < 0.01$ versus WT, $^{+}P < 0.05$ versus WT + CoPP, $^{\ddagger}P < 0.05$ versus EC-SOD$^{(-/-)}$. (c) Bilirubin production in WT, EC-SOD$^{(-/-)}$ mice and EC-SOD$^{(-/-)}$ mice treated with CoPP. Values are expressed as the mean ± SD of 4 experiments, $^{*}P < 0.05$ versus WT mice, $^{\ddagger}P < 0.01$ versus EC-SOD$^{(-/-)}$.

variance (ANOVA) for multiple groups or unpaired t-test for two groups.

3. Results

3.1. Effect of SOD3 Gene Deletion on HO Expression and Activity. The effectiveness of EC-SOD gene knockdown was analyzed in renal homogenates using R.T-PCR. SOD3 KO animals demonstrated significant ($P < 0.05$) reduction in EC-SOD mRNA as compared to their WT counterparts (WT -2.55 ± 0.5 versus KO -0.36 ± 0.04). The administration of CoPP had no effect on SOD3 mRNA levels in both WT and KO animals (Figure 1(a)). Oxidative stress was confirmed in renal homogenates from SOD3 KO animals using lucigenin detectable chemiluminescence (WT -0.78 ± 0.1 versus KO -1.85 ± 0.14 cpm $\times$ 1000/mg, $P < 0.05$). Renal homogenates from EC-SOD$^{(-/-)}$ animals demonstrate significant ($P < 0.05$) induction of HO-1 which was further accentuated in animals undergoing CoPP treatment (Figure 1(b)). However, this HO-1 induction was accompanied by attenuation ($P < 0.05$) of HO activity in SOD3 KO animals, as measured by total bilirubin generation (WT -0.53 ± 0.12 versus KO -0.28 ± 0.09 nmol/mg protein). EC-SOD$^{(-/-)}$ mice concurrently exposed to CoPP demonstrated increased HO-1 expression which was accompanied by a corollary increase in ($P < 0.05$) HO activity in these animals (Figure 1(c)). That chronic oxidative stress induces HO expression but suppresses HO activity, is substantiated by these results which also suggest that porphyrin-induced overexpression of HO overwhelms this inhibitory effect and restores HO activity.

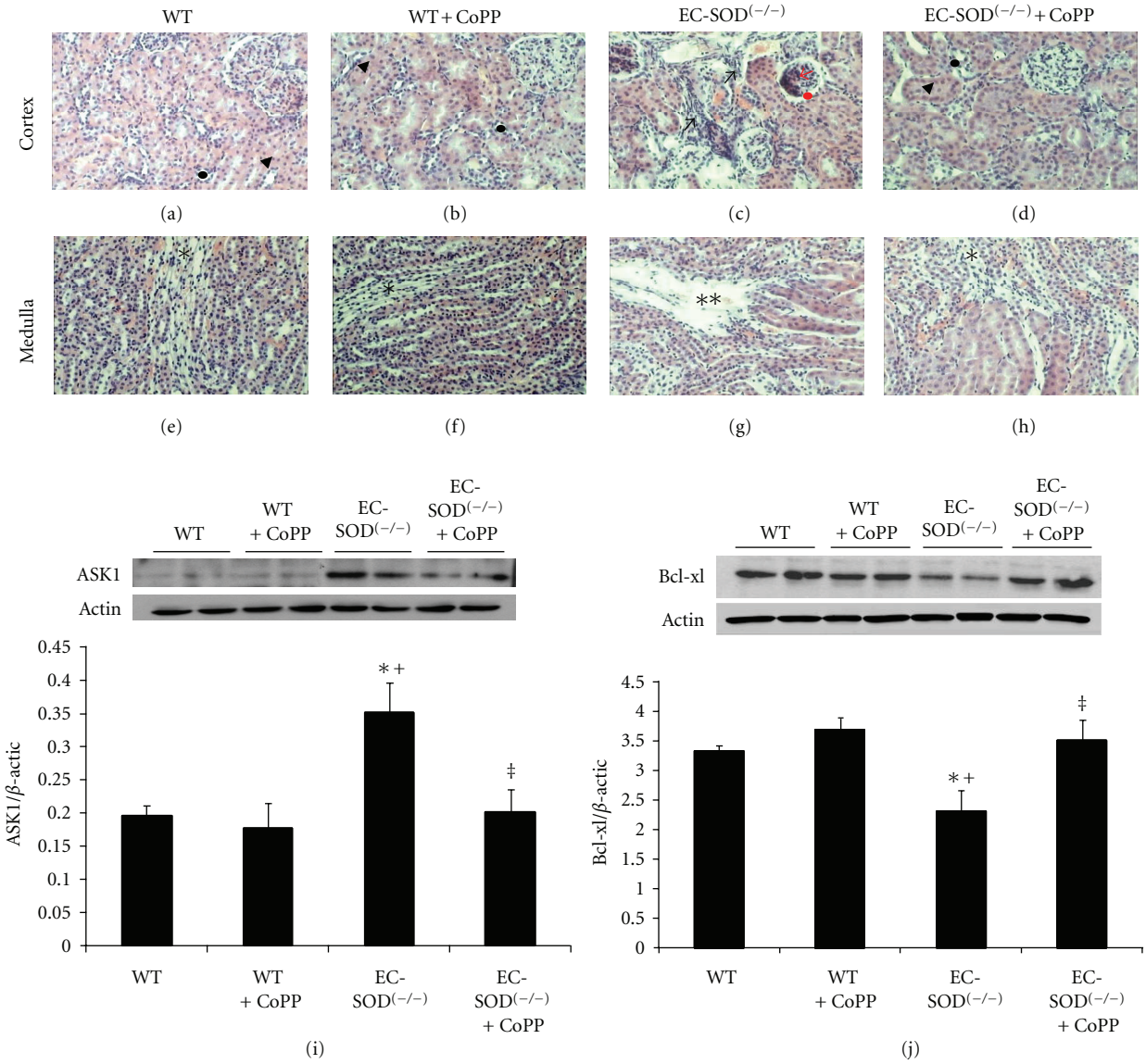

Figure 2: Representative images of renal parenchyma (haematoxylin-eosin staining, 200X) in EC-SOD$^{(-/-)}$ and WT mice. WT mice and WT mice treated with CoPP showed no significant morphologic alterations both in cortex (arrow's head for proximal tubules and black point for distal tubules) and medulla (asterisk for vasa recta) ((a), (b), (e), (f)). EC-SOD$^{(-/-)}$ mice displayed tubular damage, hyperproliferation in the glomerulii (red arrows) with Bowman's capsule dilatation (red point), infiltrates (black arrows) and breakage of vasa recta in outer and inner medulla (double asterisks). ((c), (g)). Tubular interstitial and microvascular pathology are abrogated in CoPP-treated EC-SOD$^{(-/-)}$ mice ((d), (h)). Western blot analysis of (i) ASK1 (j) BCL-xl protein expression in kidney of WTmic, EC-SOD$^{(-/-)}$ mice, and mice treated with CoPP. Quantitative densitometry evaluation of Bcl-xl, ASK-1-to-β-actin ratio was determined. Blots are representative of four separate experiments. Results are expressed as mean ± SE, $^{*}P < 0.05$ versus WT, $^{+}P < 0.01$ versus WT + CoPP, $^{\ddagger}P < 0.05$ versus EC-SOD$^{(-/-)}$.

3.2. Effects of HO Induction on Renal Histopathology in EC-SOD$^{(-/-)}$ Animals. HO deficiency, as seen in HO-2$^{(-/-)}$ animals, has already been shown to exacerbate renal histopathological abnormalities in streptozotocin- (STZ-) induced model of oxidative stress [28]. SOD3$^{(-/-)}$ mice also exhibit chronic oxidative stress with insufficient stimulation of the heme-HO system; as such, we examined renal morphology in these mice (and WT controls) with and without pharmacological induction of HO. Histological examination of renal sections revealed no significant abnormalities in WT mice treated or not with CoPP, showing no dilatation of glomeruli, proximal and distal tubules, and absence of inflammatory infiltration (Figures 2(a), 2(b), 2(e), and 2(f)). In contrast, EC-SOD$^{(-/-)}$ mice exhibited corticotubular damage, characterized by dilatation and loss of epithelial cells in the tubular structures with the presence of cellular infiltrate (Figure 2(c)). A considerable number of Bowman's capsules showed dilatation with hyperproliferation of capillary tufts and cellular infiltration (Figure 2(c)). Inner and outer medulla showed breakage of vasa recta (Figure 2(g)).

CoPP treatment abrogated this glomerular-tubulointerstitial and microvascular pathology in EC-SOD$^{(-/-)}$ mice (Figures 2(d), 2(h)).

Renal morphological abnormalities, in EC-SOD$^{(-/-)}$, were accompanied by alterations in apoptotic and antiapoptotic signals. ASK1 was significantly ($P < 0.05$) elevated in renal homogenates of SOD3 KO animals as compared to WT animals. Similarly, antiapoptotic protein Bcl-xl was attenuated ($P < 0.05$) in renal homogenates from SOD3 KO as compared to their WT counterparts. The expression levels of ASK1 and Bcl-xl proteins were restored in EC-SOD$^{(-/-)}$ mice treated with CoPP (Figures 2(i), 2(j)).

3.3. Effects of HO Induction, in SOD3 KO Animals, on Systolic Blood Pressure and Vascular Endothelial Function. EC-SOD$^{(-/-)}$ mice have been documented to have elevated blood pressure and exhibit vascular endothelial dysfunction [17]. In this regard, we examined the effect of HO induction on vascular endothelial function, and factors regulating it, in EC-SOD$^{(-/-)}$ mice. First, vascular redox status was assessed using lucigenin detectable chemiluminescence which showed that SOD3 gene deletion was associated with increased ($P < 0.05$) vascular O_2^- levels; attenuated by concurrent exposure to CoPP ($P < 0.05$) (Figure 3(a)). EC-SOD$^{(-/-)}$ mice demonstrated a significantly ($P < 0.05$) attenuated vasorelaxation response to increasing concentrations of acetylcholine. CoPP treatment enhanced acetylcholine-induced vasodilation ($P < 0.05$) in these mice (Figure 3(b)) and in complimentary experiments, CoPP rescued the attenuative effects ($P < 0.05$) of SOD3 gene knockdown on the expression of p-eNOS ($P < 0.05$) (Figure 3(d)). Blood pressure measurements, performed in WT and EC-SOD-deficient mice, revealed elevated baseline blood pressure in EC-SOD$^{(-/-)}$ mice (WT -112.4 ± 1.8 versus KO -120.4 ± 0.9 mm Hg, $P < 0.05$). This elevated blood pressure was successfully normalized in mice exposed to CoPP (KO + CoPP -109.8 ± 2.4) (Figure 3(c)).

3.4. Effects of HO Induction, in SOD3 KO Animals, on Epoxide and Serum Adiponectin Levels. In addition to endothelial dysfunction, vascular levels of epoxides were significantly reduced in EC-SOD$^{(-/-)}$ mice (WT -2.15 ± 0.2 versus KO -0.89 ± 0.3 ng/mg protein/hr, $P < 0.05$). This effect of SOD3 KO on EET levels was reversed in CoPP treated animals (2.35 ± 0.2 ng/mg/protein/hr, $P < 0.05$ versus KO) (Figure 4(a)).

Stimulation of the heme-HO system, along with enhancement of epoxides, has been shown to be associated with increased serum and tissue levels of adiponectin. Attenuation of HO activity, in EC-SOD$^{(-/-)}$ mice, was accompanied by significant ($P < 0.05$) attenuation of serum adiponectin levels (WT -6.10 ± 0.3 versus KO $-0.93 \pm 0.45\,\mu$g/mL). WT mice treated with CoPP exhibited serum adiponectin levels similar to those in controls; however, CoPP treatment restored serum adiponectin levels in SOD3 deficient mice (KO + CoPP $-8.05 \pm 0.9\,\mu$g/mL, $P < 0.05$) (Figure 4(b)), indicative of its association with HO activity. In addition, expression of p-AKT, an adiponectin-dependent regulatory pathway, was also attenuated ($P < 0.05$) in vascular tissues from EC-SOD$^{(-/-)}$ mice and was restored in animals concurrently exposed to CoPP ($P < 0.05$) (Figure 4(c)).

4. Discussion

The present study demonstrates that chronic oxidative stress, as seen in EC-SOD$^{(-/-)}$ mice, has reciprocal effects on HO expression and activity and that pharmacological induction of HO restores redox homeostasis and reverts pathophysiological abnormalities observed in these animals. These effects of HO induction are associated with concomitant increases in epoxide and adiponectin levels, which may contribute towards the HO-induced reversal of renovascular abnormalities in EC-SOD$^{(-/-)}$ mice.

The first key finding presented in this study is the observation that mice expressing reduced levels EC-SOD have increased renovascular oxidative stress which, although accompanied by induction of HO-1 expression, concurrently results in a significant ($P < 0.05$) attenuation in HO activity. This discordance in HO-1 expression and activity is in line with recent reports showing that oxidative stress, in *in vitro* systems, inhibits HO activity by a yet unidentified post-translational modification [21, 29, 30]. In addition, investigators have also shown an insufficient induction of HO-1 in conditions of severe prolonged oxidative stress, where levels of HO are unable to provide adequate cellular antioxidant protection [31]. Accordingly, irrespective of the underlying mechanisms involved, accumulating evidence now suggests attenuation of HO activity by chronic redox imbalances in face of an increased HO-1 expression. Such an effect of prolonged oxidative stress, though counterintuitive, may explain the need and protective role of therapeutic HO induction in various models of chronic oxidative stress [32], including the current model of SOD3 KO animals. How pharmacological HO-1 induction does overcome the inhibitory effect of chronic oxidative stress and restores HO activity cannot be fully explained at this time but may entail an excess of HO and HO-derived products overwhelming the suppressive effect of cellular redox.

The second key finding presented in this study is the modulatory effect of heme-HO system on vascular epoxide and serum adiponectin levels in EC-SOD$^{(-/-)}$ mice. In these mice, a chronic oxidative state is characterized by insufficient HO activity and where both epoxide and adiponectin levels are attenuated. Recovery of vascular epoxide and serum adiponectin levels with CoPP-induced HO stimulation implicates the role of HO in modulating these pathways. These results are in line with our earlier report [27] showing interplay between HO and epoxides in animal models of metabolic syndrome. Recent reports have also shown that induction of HO-1 expression and activity leads to enhancement of adiponectin levels along with activation of adiponectin directed signaling pathways [31, 33, 34]. HO-mediated stimulation of adiponectin release from adipose tissues [31, 35] appears to involve the HO-induced restoration of adipocyte function including reduced oxidative stress, inflammation, and increased release of protective adipokines

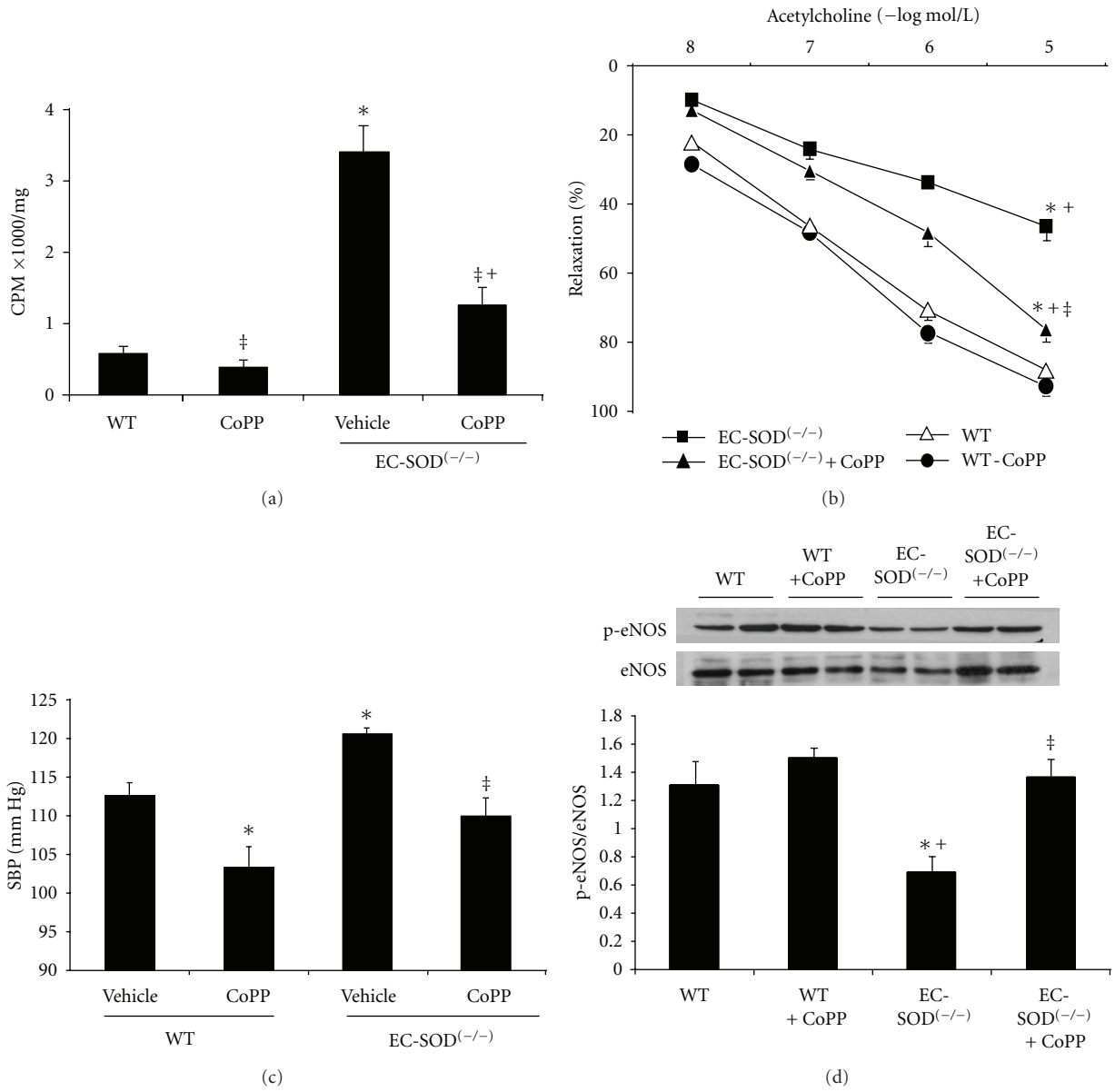

Figure 3: (a) Superoxide levels in femoral arteries of WT mice, EC-SOD$^{(-/-)}$ mice, and mice injected with CoPP. Superoxide levels were determined as described in methods. Results are mean ± SE, $n = 4$; $^{*}P < 0.01$ versus WT, $^{\ddagger}P < 0.01$ versus EC-SOD$^{(-/-)}$; $^{+}P < 0.05$ versus WT + CoPP. (b) Dose-response curves for acetylcholine-induced vascular relaxation after preconstriction with phenylephrine. Results are mean ± SE, $n = 4$; $^{*}P < 0.05$ versus WT, $^{\ddagger}P < 0.05$ versus EC-SOD$^{(-/-)}$, $^{+}P < 0.05$ versus WT + CoPP, and (c) systolic blood pressure measurements at the completion of the study ($n = 4$). Mice were injected with CoPP once a week as described in Methods. Blots are representative of four separate experiments. Results are expressed as mean ± SE, $^{*}P < 0.05$ versus WT, $^{\ddagger}P < 0.01$ versus EC-SOD$^{(-/-)}$, $^{+}P < 0.05$ versus WT + CoPP mice, and Western blot and densitometry analysis of (d) eNOS and p-eNOS.

[9]. In addition, epoxides, whose synthesis was enhanced by HO-induction in EC-SOD$^{(-/-)}$ mice, have been shown in the past to attenuate dysfunctional adipogenesis and enhance adiponectin release from adipocytes [36], in an HO-dependent manner. Thus, our results presented here support the existence of an interdependent physiological axis formed by the heme-HO, epoxide, and adiponectin systems, which regulates cardiovascular-renal function, and where redox-induced attenuation of HO activity negatively affects the other two components.

Physiological implications of attenuation of HO-adiponectin axis are evident as renal corticomedullary lesions in EC-SOD$^{(-/-)}$ mice, amenable to reversal with CoPP. Abrogation of these renal histopathological changes, in mice treated with CoPP, was accompanied by attenuation of apoptotic and enhancement of antiapoptotic pathways, including ASK1 and Bcl-Xl. ASK1 is a stress-response protein negatively regulated by cellular redox [37], where reduced thioredoxin binds and inhibits ASK1. This inhibitory effect is overcome under conditions of oxidative stress [38], thus

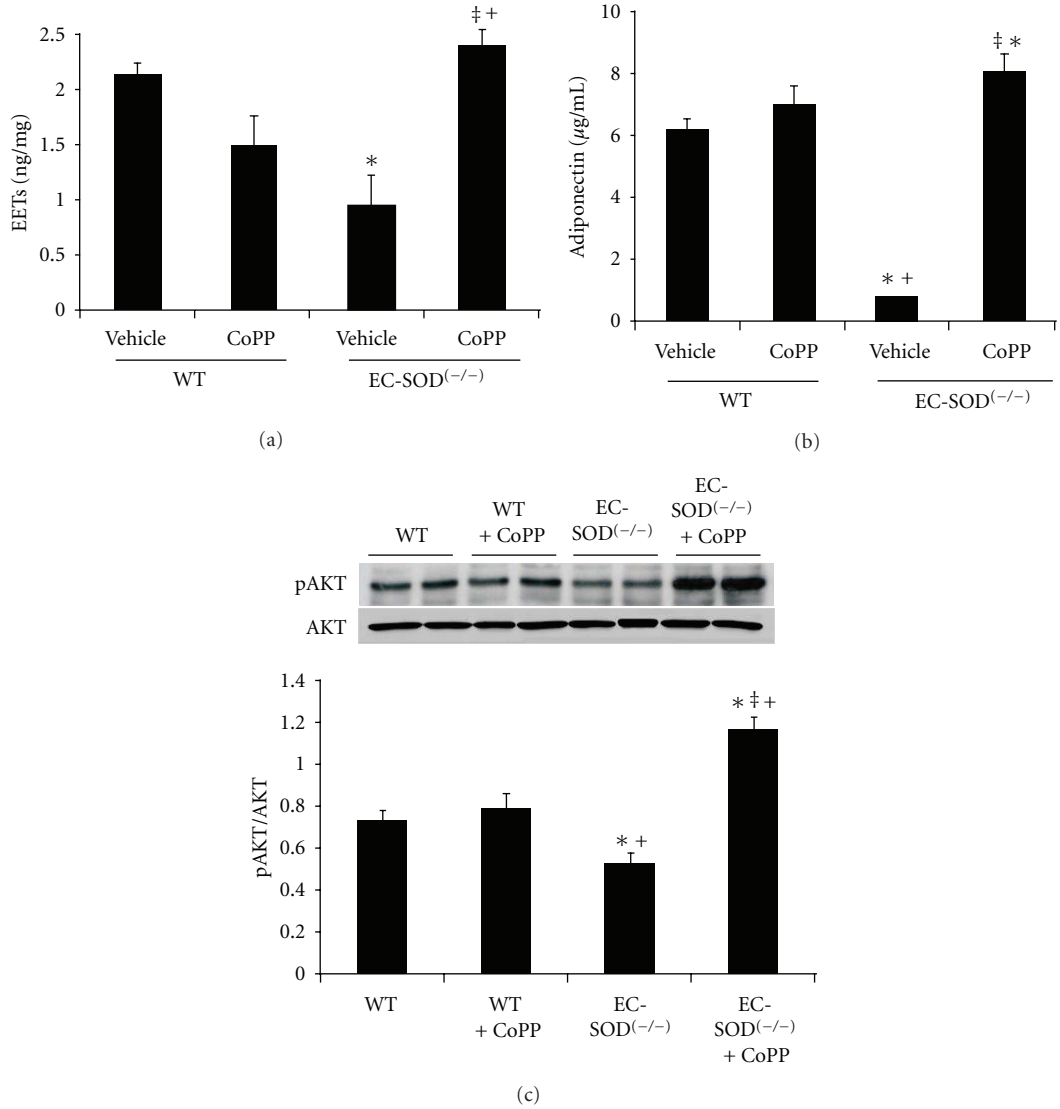

Figure 4: (a) EETs levels in WT and EC-SOD$^{(-/-)}$ mice and mice treated with CoPP. EETs levels were determined as described in Methods. Results are mean ± SE, $n = 4$; $^{*}P < 0.05$ versus WT mice, $^{\ddagger}P < 0.05$ versus EC-SOD$^{(-/-)}$ and $^{+}P < 0.05$ versus WT + CoPP mice. (b) Adiponectin levels in WT and EC-SOD$^{(-/-)}$ mice and mice treated with CoPP. Adiponectin levels were determined as described in methods. Results are mean ± SE, $n = 4$; $^{*}P < 0.05$ versus WT mice, $^{\ddagger}P < 0.05$ versus EC-SOD$^{(-/-)}$ mice, and $^{+}P < 0.05$ versus WT + CoPP mice. (c) AKT and pAKT proteins in arteries of WT and EC-SOD$^{(-/-)}$ mice. Mice were injected with CoPP once a week as described in Methods. Blots are representative of four separate experiments. Results are expressed as mean ± SE, $^{*}P < 0.05$ versus WT, $^{\ddagger}P < 0.01$ versus EC-SOD$^{(-/-)}$, $^{+}P < 0.05$ versus WT + CoPP mice.

increasing ASK1 expression. Increased cellular expression of ASK1 has been linked to recruitment of inflammatory components, tumorogenesis, and endothelial dysfunction [39]. Reduced HO activity, in EC-SOD$^{(-/-)}$ mice, could facilitate oxidative stress in turn increasing ASK1 expression, and contributing towards pathological alterations observed in these animals. Induction of HO-1 expression and activity, via CoPP administration, restores cellular redox, attenuates ASK1 expression and prevents renal damage. In addition, EC-SOD$^{(-/-)}$ animals also demonstrate reduced tissue expression of Bcl-XL and p-AKT/AKT proteins. AKT interacts with HSP-90 and is involved in inhibition of ASK1 and associated pathways [15]. Increased HO-1 and adiponectin levels enhance p-AKT/AKT expression with resultant cardio-reno-protective effects [36, 40] including suppression of ASK1. These results are also in line with earlier reports [11, 41] demonstrating renoprotective effects of adiponectin in patients with chronic kidney disease.

Finally, significant improvement of endothelial function and attenuation of elevated blood pressure, in EC-SOD$^{(-/-)}$ mice, by induction of heme-HO system underscores the role of the HO-epoxide-adiponectin system in regulating vascular homeostasis. Induction of HO via CoPP provides excess CO and BV, where BV acts as an endogenous chain breaking antioxidant [1] and increases NO bioavailability and CO has been shown to induce vasodilation via activation of vascular

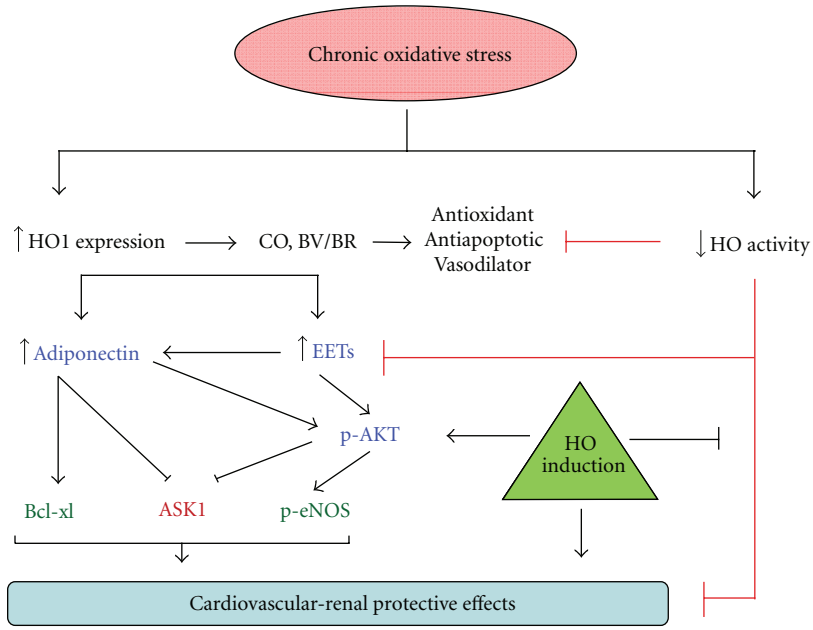

Figure 5: The schematic outlines the salient points discussed in the paper. Chronic oxidative stress upregulates HO-1 expression whose products, CO & BV have antioxidant properties and help restore redox homeostasis. In addition, HO induction stimulates epoxide synthesis and these pathways combined enhance adiponectin release. Antioxidant, antiapoptotic, vasomodulatory, and renoprotective properties of HO-EET-adiponectin axis restore cardiovascular-renal homeostasis often disrupted in conditions of oxidative stress. HO induction overcomes a concurrent inhibition of HO activity by redox-dependent mechanisms which offset the protective effects of enhanced HO expression thus contributing towards pathophysiological abnormalities observed in conditions of chronic oxidative stress.

K_{ca} channels [1, 42]. Another aspect of the HO-1 induction-mediated amelioration of vascular dysfunction may involve upregulation of eNOS and stimulation of epoxide synthesis. Epoxides are one of the candidates for endothelium derived hyperpolarizing factor (EDRF) and induce vasodilation along with inhibition of inflammatory response and stimulation of epithelial cell growth [43, 44]. In addition, HO stimulation is accompanied by increased adiponectin release and adiponectin has been shown to induce generation of NO in vascular endothelial cells [45] and has also been shown to mediate AKT-dependent vasodilation in the rat aorta [46]. Thus, current evidence, in light of previous reports, implicates the interaction between the HO, epoxide and adiponectin systems in governing vascular endothelial function. The antioxidant and vasomodulatory properties of HO products are complimented by upregulation of vascular p-eNOS, increase in vasodilatory epoxides and stimulation of adiponectin-AKT pathway. These vascular and hemodynamic effects EC-SOD gene deletion may also contribute towards renal histopathological alteration observed in these mice and hemodynamic improvement, observed in CoPP-treated SOD3 KO mice, could thus contribute towards attenuation of renal pathology in these animals.

In conclusion (Figure 5), this study puts in perspective two emerging concepts in cardiovascular pathophysiology. First, it demonstrates a reciprocal effect of chronic oxidative stress on HO-1 expression and activity and secondly, the present study suggests interdependence amongst three physiological systems, that is, the heme-HO, adiponectin and epoxide systems. Where chronic oxidative stress conditions, such as SOD3 deficiency potentiate HO-1 expression, a concurrent inhibitory effect on HO activity is observed. This effect is accompanied by renovascular abnormalities and suppression of epoxide and adiponectin synthesis. Restoration of HO activity, via pharmacological modulators of the heme-HO system, not only restores cellular redox, but also recovers vascular epoxide and serum adiponectin levels along with abrogation of renal pathology, improvement of vascular function, and attenuation of blood pressure.

Abbreviations

Akt:	Serine/threonine protein kinase
ASK-1:	Apoptotic signaling kinase-1
CO:	Carbon monoxide
CoPP:	Cobalt protoporphyrin
Cu/Zn SOD:	Copper-zinc superoxide dismutase
EC-SOD:	Extracellular superoxide dismutase
HO:	Heme oxygenase
ROS:	Reactive oxygen species
SE:	Standard error
SOD:	Superoxide dismutase.

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References

[1] N. G. Abraham and A. Kappas, "Pharmacological and clinical aspects of heme oxygenase," *Pharmacological Reviews*, vol. 60, no. 1, pp. 79–127, 2008.

[2] J. Balla, H. S. Jacob, G. Balla, K. Nath, J. W. Eaton, and G. M. Vercellotti, "Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 20, pp. 9285–9289, 1993.

[3] T. W. Sedlak and S. H. Snyder, "Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle," *Pediatrics*, vol. 113, no. 6 I, pp. 1776–1782, 2004.

[4] R. Stocker, Y. Yamamoto, and A. F. McDonagh, "Bilirubin is an antioxidant of possible physiological importance," *Science*, vol. 235, no. 4792, pp. 1043–1046, 1987.

[5] D. Shippen-Lentz and E. H. Blackburn, "Functional evidence for an RNA template in telomerase," *Science*, vol. 247, no. 4942, pp. 546–552, 1990.

[6] M. P. Soares, Y. Lin, J. Anrather et al., "Expression of heme oxygenase-1 can determine cardiac xenograft survival," *Nature Medicine*, vol. 4, no. 9, pp. 1073–1077, 1998.

[7] S. Brouard, L. E. Otterbein, J. Anrather et al., "Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis," *Journal of Experimental Medicine*, vol. 192, no. 7, pp. 1015–1025, 2000.

[8] N. G. Abraham and A. Kappas, "Heme oxygenase and the cardiovascular-renal system," *Free Radical Biology and Medicine*, vol. 39, no. 1, pp. 1–25, 2005.

[9] D. H. Kim, A. P. Burgess, M. Li et al., "Heme oxygenase-mediated increases in adiponectin decrease fat content and inflammatory cytokines tumor necrosis factor-α and interleukin-6 in Zucker rats and reduce adipogenesis in human mesenchymal stem cells," *Journal of Pharmacology and Experimental Therapeutics*, vol. 325, no. 3, pp. 833–840, 2008.

[10] L. Tao, E. Gao, X. Jiao et al., "Adiponectin cardioprotection after myocardial ischemia/reperfusion involves the reduction of oxidative/nitrative stress," *Circulation*, vol. 115, no. 11, pp. 1408–1416, 2007.

[11] K. Sharma, S. RamachandraRao, G. Qiu et al., "Adiponectin regulates albuminuria and podocyte function in mice," *Journal of Clinical Investigation*, vol. 118, no. 5, pp. 1645–1656, 2008.

[12] M. A. Di Noia, S. Van Driesche, F. Palmieri et al., "Heme oxygenase-1 enhances renal mitochondrial transport carriers and cytochrome c oxidase activity in experimental diabetes," *Journal of Biological Chemistry*, vol. 281, no. 23, pp. 15687–15693, 2006.

[13] T. F. Franke, D. R. Kaplan, and L. C. Cantley, "PI3K: downstream AKTion blocks apoptosis," *Cell*, vol. 88, no. 4, pp. 435–437, 1997.

[14] D. R. Alessi, M. Andjelkovic, B. Caudwell et al., "Mechanism of activation of protein kinase B by insulin and IGF-1," *The EMBO Journal*, vol. 15, no. 23, pp. 6541–6551, 1996.

[15] R. Zhang, D. Luo, R. Miao et al., "Hsp90-Akt phosphorylates ASK1 and inhibits ASK1-mediated apoptosis," *Oncogene*, vol. 24, no. 24, pp. 3954–3963, 2005.

[16] A. Mugge, J. H. Elwell, T. E. Peterson, and D. G. Harrison, "Release of intact endothelium-derived relaxing factor depends on endothelial superoxide dismutase activity," *American Journal of Physiology*, vol. 260, no. 2, pp. C219–C225, 1991.

[17] W. J. Welch, T. Chabrashvili, G. Solis et al., "Role of extracellular superoxide dismutase in the mouse angiotensin slow pressor response," *Hypertension*, vol. 48, no. 5, pp. 934–941, 2006.

[18] P. Stralin, K. Karlsson, B. O. Johansson, and S. L. Marklund, "The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 15, no. 11, pp. 2032–2036, 1995.

[19] Y. Chu, S. Iida, D. D. Lund et al., "Gene transfer of extracellular superoxide dismutase reduces arterial pressure in spontaneously hypertensive rats: role of heparin-binding domain," *Circulation Research*, vol. 92, no. 4, pp. 461–468, 2003.

[20] O. Jung, S. L. Marklund, H. Geiger, T. Pedrazzini, R. Busse, and R. P. Brandes, "Extracellular superoxide dismutase is a major determinant of nitric oxide bioavailability: in vivo and ex vivo evidence from ecSOD-deficient mice," *Circulation Research*, vol. 93, no. 7, pp. 622–629, 2003.

[21] R. Kinobe, Y. Ji, and K. Nakatsu, "Peroxynitrite-mediated inactivation of heme oxygenases," *BMC Pharmacology*, vol. 4, article 26, 2004.

[22] C. L. Fattman, L. Y. Chang, T. A. Termin, L. Petersen, J. J. Enghild, and T. D. Oury, "Enhanced bleomycin-induced pulmonary damage in mice lacking extracellular superoxide dismutase," *Free Radical Biology and Medicine*, vol. 35, no. 7, pp. 763–771, 2003.

[23] N. G. Abraham, R. Rezzani, L. Rodella et al., "Overexpression of human heme oxygenase-1 attenuates endothelial cell sloughing in experimental diabetes," *American Journal of Physiology*, vol. 287, no. 6, pp. H2468–H2477, 2004.

[24] K. M. Mohazzab-H, P. M. Kaminski, R. P. Fayngersh, and M. S. Wolin, "Oxygen-elicited responses in calf coronary arteries: role of H_2O_2 production via NADH-derived superoxide," *American Journal of Physiology*, vol. 270, no. 3, pp. H1044–H1053, 1996.

[25] N. G. Abraham, T. Kushida, J. McClung et al., "Heme oxygenase-1 attenuates glucose-mediated cell growth arrest and apoptosis in human microvessel endothelial cells," *Circulation Research*, vol. 93, no. 6, pp. 507–514, 2003.

[26] N. G. Abraham, G. Scapagnini, and A. Kappas, "Human heme oxygenase: cell cycle-dependent expression and DNA microarray identification of multiple gene responses after transduction of endothelial cells," *Journal of Cellular Biochemistry*, vol. 90, no. 6, pp. 1098–1111, 2003.

[27] K. Sodhi, K. Inoue, K. H. Gotlinger et al., "Epoxyeicosatrienoic acid agonist rescues the metabolic syndrome phenotype of HO-2-null mice," *Journal of Pharmacology and Experimental Therapeutics*, vol. 331, no. 3, pp. 906–916, 2009.

[28] A. I. Goodman, P. N. Chander, R. Rezzani et al., "Heme oxygenase-2 deficiency contributes to diabetes-mediated increase in superoxide anion and renal dysfunction," *Journal of the American Society of Nephrology*, vol. 17, no. 4, pp. 1073–1081, 2006.

[29] A. L. Kruger, S. J. Peterson, M. L. Schwartzman et al., "Up-regulation of heme oxygenase provides vascular protection in an animal model of diabetes through its antioxidant and anti-apoptotic effects," *Journal of Pharmacology and Experimental Therapeutics*, vol. 319, no. 3, pp. 1144–1152, 2006.

[30] S. Quan, P. M. Kaminski, L. Yang et al., "Heme oxygenase-1 prevents superoxide anion-associated endothelial cell sloughing in diabetic rats," *Biochemical and Biophysical Research Communications*, vol. 315, no. 2, pp. 509–516, 2004.

[31] S. J. Peterson, D. H. K. Kim, M. Li et al., "The L-4F mimetic peptide prevents insulin resistance through increased levels of

HO-1, pAMPK, and pAKT in obese mice," *Journal of Lipid Research*, vol. 50, no. 7, pp. 1293–1304, 2009.

[32] G. Sambuceti, S. Morbelli, L. Vanella et al., "Diabetes impairs the vascular recruitment of normal stem cells by oxidant damage, reversed by increases in pAMPK, heme oxygenase-1, and adiponectin," *Stem Cells*, vol. 27, no. 2, pp. 399–407, 2009.

[33] A. Nicolai, M. Li, D. H. Kim et al., "Heme oxygenase-1 induction remodels adipose tissue and improves insulin sensitivity in obesity-induced diabetic rats," *Hypertension*, vol. 53, no. 3, pp. 508–515, 2009.

[34] S. J. Peterson, G. Drummond, D. H. Kim et al., "L-4F treatment reduces adiposity, increases adiponectin levels, and improves insulin sensitivity in obese mice," *Journal of Lipid Research*, vol. 49, no. 8, pp. 1658–1669, 2008.

[35] A. Burgess, M. Li, L. Vanella et al., "Adipocyte heme oxygenase-1 induction attenuates metabolic syndrome in both male and female obese mice," *Hypertension*, vol. 56, no. 6, pp. 1124–1130, 2010.

[36] D. H. Kim, L. Vanella, K. Inoue et al., "Epoxyeicosatrienoic acid agonist regulates human mesenchymal stem cell-derived adipocytes through activation of HO-1-pAKT signaling and a decrease in PPARγ," *Stem Cells and Development*, vol. 19, no. 12, pp. 1863–1873, 2010.

[37] H. Ichijo, E. Nishida, K. Irie et al., "Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways," *Science*, vol. 275, no. 5296, pp. 90–94, 1997.

[38] M. Saitoh, H. Nishitoh, M. Fujii et al., "Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1," *The EMBO Journal*, vol. 17, no. 9, pp. 2596–2606, 1998.

[39] K. Hattori, I. Naguro, C. Runchel, and H. Ichijo, "The roles of ASK family proteins in stress responses and diseases," *Cell Communication and Signaling*, vol. 7, p. 9, 2009.

[40] R. Olszanecki, R. Rezzani, S. Omura et al., "Genetic suppression of HO-1 exacerbates renal damage: reversed by an increase in the antiapoptotic signaling pathway," *American Journal of Physiology*, vol. 292, no. 1, pp. F148–F157, 2007.

[41] J. H. Ix and K. Sharma, "Mechanisms linking obesity, chronic kidney disease, and fatty liver disease: the roles of fetuin-A, adiponectin, and AMPK," *Journal of the American Society of Nephrology*, vol. 21, no. 3, pp. 406–412, 2010.

[42] S. W. Ryter, J. Alam, and A. M. K. Choi, "Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications," *Physiological Reviews*, vol. 86, no. 2, pp. 583–650, 2006.

[43] J. D. Imig, "Epoxide hydrolase and epoxygenase metabolites as therapeutic targets for renal diseases," *American Journal of Physiology*, vol. 289, no. 3, pp. F496–F503, 2005.

[44] A. A. Spector and A. W. Norris, "Action of epoxyeicosatrienoic acids on cellular function," *American Journal of Physiology*, vol. 292, no. 3, pp. C996–C1012, 2007.

[45] H. Chen, M. Montagnani, T. Funahashi, I. Shimomura, and M. J. Quon, "Adiponectin stimulates production of nitric oxide in vascular endothelial cells," *Journal of Biological Chemistry*, vol. 278, no. 45, pp. 45021–45026, 2003.

[46] W. Xi, H. Satoh, H. Kase, K. Suzuki, and Y. Hattori, "Stimulated HSP90 binding to eNOS and activation of the PI3-Akt pathway contribute to globular adiponectin-induced NO production: vasorelaxation in response to globular adiponectin," *Biochemical and Biophysical Research Communications*, vol. 332, no. 1, pp. 200–205, 2005.

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Research Article

Heme Oxygenase-1 Attenuates Hypoxia-Induced sFlt-1 and Oxidative Stress in Placental Villi through Its Metabolic Products CO and Bilirubin

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One of the most prevalent complications of pregnancy is preeclampsia, a hypertensive disorder which is a leading cause of maternal and perinatal morbidity and premature birth with no effective pharmacological intervention. While the underlying cause is unclear, it is believed that placental ischemia/hypoxia induces the release of factors into the maternal vasculature and lead to widespread maternal endothelial dysfunction. Recently, HO-1 has been shown to downregulate two of these factors, reactive oxygen species and sFlt-1, and we have reported that HO-1 induction attenuates many of the pathological factors of placental ischemia experimentally. Here, we have examined the direct effect of HO-1 and its bioactive metabolites on hypoxia-induced changes in superoxide and sFlt-1 in placental vascular explants and showed that HO-1 and its metabolites attenuate the production of both factors in this system. These findings suggest that the HO-1 pathway may be a promising therapeutic approach for the treatment of preeclampsia.

1. Introduction

Preeclampsia is a pregnancy-specific hypertensive disorder classically characterized by proteinuria and edema after the twentieth week of gestation [1]. Preeclampsia is a common complication of pregnancy, with an incidence of ~5–10% of all pregnancies [2]. Though the underlying mechanisms of preeclampsia are not well understood, a central factor believed to be important in the development of the disease is placental ischemia and hypoxia which result from a failure of the maternal uterine vasculature to remodel into high-capacitance vessels, leading to placental hypoperfusion [3–5]. In response to hypoxia, the placenta begins to produce a number of soluble factors which are secreted into the maternal circulation. Once, in circulation, these factors induce widespread maternal endothelial dysfunction, one of the key hallmarks of this disorder [6, 7]. There are a number of molecular pathways which are involved in the cascade from placental ischemia to maternal endothelial dysfunction. Two pathways which have been the subject of intense research are the production of the soluble form of the vascular endothelial growth factor (VEGF) receptor-denominated soluble fms-like tyrosine kinase (sFlt-1) and the production of placental hypoxia-induced reactive oxygen species (ROS).

VEGF signaling is necessary for endothelial health and maintenance [8]. When, in circulation, sFlt-1 binds to free VEGF and placental growth factor (PlGF) sequestering them and making them unavailable for proper signaling [9]. sFlt-1 can be directly induced by hypoxia through the actions of HIF-1α and has been shown to be produced by human placental trophoblasts and villi in response to decreased oxygen [10–12]. It has also been shown to be produced in the placenta during preeclampsia and is found to be elevated in the circulation of preeclamptic women, often before the onset of maternal symptoms [13, 14]. In addition, numerous experimental models have demonstrated the importance of sFlt-1 overexpression in the development of preeclampsia [15–18]. A second factor implicated in the pathophysiology of preeclampsia is oxidative stress. Oxidative stress has been shown to be elevated in both the placenta and the

maternal vasculature of women with preeclampsia [19–21]. The symptoms associated with experimental rodent placental hypoperfusion have also shown to be attenuated by either the superoxide dismutase mimetic Tempol or the NADPH oxidase inhibitor apocynin [22, 23]. This suggests that oxidative stress, at least partially induced by NADPH oxidase, is a crucial factor in the symptomatic manifestation of preeclampsia.

One potential palliative agent suggested for the normalization of these two pathways is the enzyme heme oxygenase-1 (HO-1). HO-1 typically catalyzes the rate-limiting step in the heme salvage pathway, converting the prooxidant heme to biliverdin, which is then rapidly converted by biliverdin reductase to bilirubin, a known antioxidant [24, 25]. As a side product, HO-1 releases carbon monoxide, a vasodilator [26]. Recently, it has been reported that HO-1 could negatively regulate VEGF or interferon-γ-induced sFlt1- release in vitro. Furthermore, CO could directly act in a similar manner [27]. Also, we have recently demonstrated that induction of HO-1 in a rodent placental ischemia model attenuated the associated hypertension and angiogenic imbalance [28]. In the present work, we have extended this previous work to examine the direct effects of HO-1 and its byproducts CO and bilirubin on hypoxia-induced oxidative stress and sFlt-1 production.

2. Materials and Methods

2.1. Animals. Timed pregnant Sprague-Dawley rats were obtained from Harlan, Inc. (Indianapolis, Ind). All animal protocols were approved by The University of Mississippi Medical Center Institutional Animal Care and Use Committee and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animals were maintained at constant temperature (23°C) with a 12 : 12 h light : dark cycle. On day 19 of gestation, the animals were sacrificed by pneumothorax and cardiac excision followed by tissue harvest.

2.2. Placental Explants. Placental explants were cultured as described previously [29]. Briefly, after tissue excision, placentas were immediately placed into cold Dulbecco's Phosphate Buffered Saline (Sigma, St. Louis, Mo). The mesometrium and decidua were carefully removed, and villous bundles from the trophospongium and labyrinth were excised. The villous explants were plated in 24-well cell culture plates coated with 0.2 mL of Matrigel Matrix Basement Membrane from BD Bioscience (Bedford, Mass). Explants were grown in Dulbecco's Modified Eagle's Media-Ham's F-12 supplemented with 10% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, and 25 μg/mL ascorbic acid as previously described [30]. The explants were maintained at constant oxygen tensions of either 6% or 1% in double gas incubators purged with nitrogen.

2.3. Experimental Protocol. Explants were randomly assigned into control and experimental groups. Control explants were incubated in media with no supplementation. The HO-1 inducer cobalt (III) protoporphyrin IX chloride (CoPP, Frontier Scientific, Logan, Utah) was prepared in 0.1 M NaOH in saline, and the pH was adjusted to a final pH of 8.5 CoPP was utilized at a final concentration in culture of 20 μM. CORM-3 has been previously described [31] and was prepared in media immediately before use at a final concentration of 40 μM. Bilirubin was utilized at a final concentration of 100 μM. At 48 hours after treatment, the cell culture media was removed from the explants and both media and tissue were frozen for further analysis. A minimum of 5 samples were obtained for every experimental group.

2.4. Western Blotting. Total intracellular protein was extracted by 5X repeated freeze-thaw lysis in FT buffer (600 mM KCl, 20 mM Tris-Cl, pH 7.8, 20% glycerol, 0.4 mg/mL Pefabloc, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 5 μg/mL aprotinin) [32]. Protein concentration was determined by Bradford assay (Bio-Rad). For western blots, 30 μg of protein was subjected to SDS-PAGE on 4–20% gradient SDS-polyacrylamide gels (Bio-Rad). Membranes were blocked with Odyssey blocking buffer (LI-COR, Lincoln, Neb) for two hours at room temperature. Primary incubation was undertaken overnight at 4°C with a rabbit anti-HO-1 polyclonal antibody (StressGen, Vancouver, Calif) at 1 : 2000 and a mouse anti-β-actin antibody (Gentest) at 1 : 5000. Secondary antibody incubation was done with Alexa Fluor 680 goat anti-rabbit (Molecular Probes) and IRDye 800 goat anti-mouse IgG (Rockland) for one hour at room temperature. Fluorescence was detected on an Odyssey infrared imager (LI-COR) for simultaneous detection of both species. Blot analysis was performed with the supplied Odyssey software, and HO-1 was normalized to β-actin, with $n = 6$ in each group.

2.5. Measurement of Superoxide by DHE. DHE fluorescence assays were carried out as previously described [33]. Individual wells of a black 96-well microtiter plate were filled with 200 uL of 5 μM DHE (Invitrogen) diluted into phosphate buffered saline. Fluorescence was monitored by excitation at 510 nm and emission at 610 nm. Fluorescence was monitored every 2 minutes for a total of one hour and the resulting measurements averaged over the life of the experiment. For each group, $n = 7$–13. Fluorescence was normalized to the average of normoxic controls. Statistical comparisons were performed by one-way ANOVA.

2.6. sFlt-1 Measurement. Total protein concentration of the cell culture supernatants was determined by the Bicinchoninic Acid (BCA) assay (Pierce, Rockford, Ill) using bovine serum albumin (BSA) as a standard. Measurement of s-Flt1 was performed by sandwich ELISAs (R&D Systems, Minneapolis, Minn) according to manufacturer protocols. The plates were read on a Tecan GENios microplate reader, and quantitation was performed with Megellan version 4.1 software. sFlt-1 levels were normalized to the total amount of media protein, and the results graphed with Origin Pro

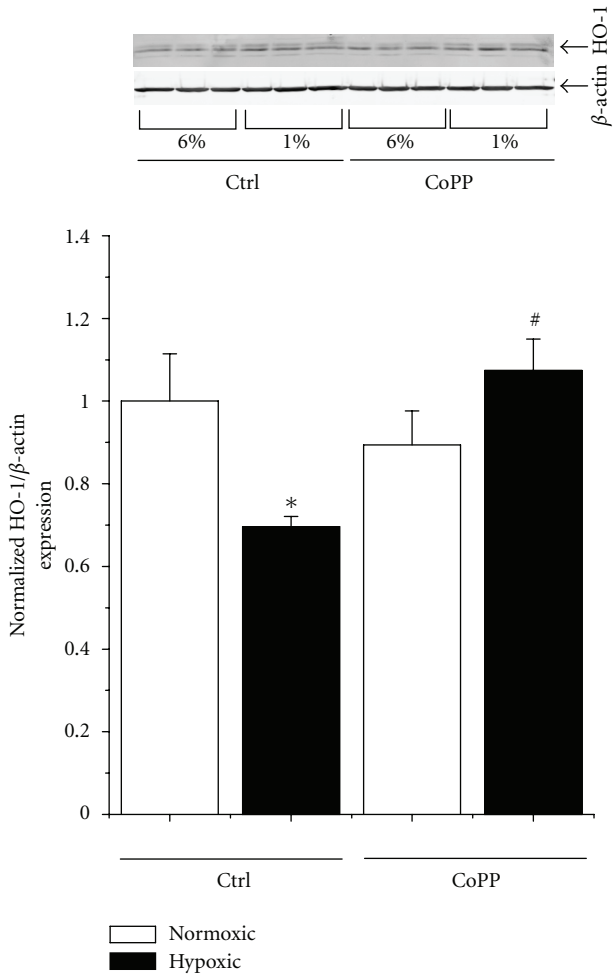

Figure 1: *Hypoxia decreases HO-1 expression in placental villous explants, which can be restored by CoPP.* Placental villous explants were incubated for 48 hours under 6% (normoxic) or 1% (hypoxic) oxygen to simulate the oxygen tension in a health and preeclamptic placenta. As assayed by western blot normalized to β-actin, in response to hypoxia treatment, there was an approximate 30% reduction in the tissue levels of HO-1. Cobalt protoporphyrin (CoPP), while having no significant effect under hypoxic conditions, fully restored HO-1 expression in the hypoxic villi. Statistical significance at the $P < 0.05$ level is indicated by "$*$" versus normoxic controls and "#" versus hypoxic controls.

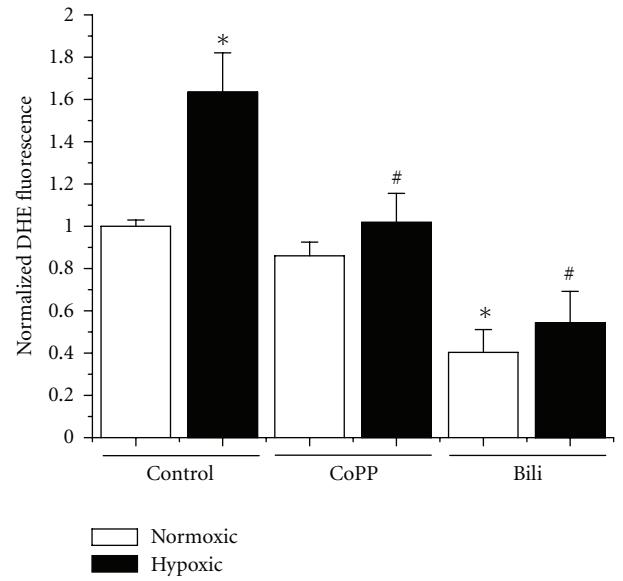

Figure 2: *HO-1 and bilirubin suppress hypoxia-induced increases in superoxide production.* In response to culture in hypoxic conditions, there is a significant ~60% increase in the level of superoxide generated by the placental explants as assayed by dihydroxyethidium (DHE) fluorescence. Administration of CoPP had no significant effect on normoxic explants but completely attenuated the hypoxia-induced increase seen in the control explants. Exogenous application of bilirubin (Bili) had a significant attenuation of superoxide in both the normoxic and hypoxic samples when compared to their respective controls. Statistical significance at the $P < 0.05$ level is indicated by "$*$" versus normoxic controls and "#" versus hypoxic controls.

8 (Microcal), which was also used for all statistical analyses. Statistical significance was determined by one-way ANOVA, with a significance threshold of $P < 0.05$.

3. Results

3.1. Hypoxia Suppresses HO-1 Expression in Placental Villi and CoPP Treatment Restores Normal HO-1 Levels. In order to assess the effect of hypoxia and CoPP on HO-1 expression in placental villi, villous explants were excised from the trophospongium and labyrinth of rodent placentas on day 19 of gestation. These tissues were cultured on synthetic basement membrane in either 6% (normoxic) or 1% (hypoxic) oxygen, reflecting the approximate oxygen tension of a normal and hypoxic placenta, respectively. As can be seen in Figure 1, at 48 hours, villous bundles in the hypoxic exhibited an approximate 30% reduction in their β-actin normalized HO-1 expression (6% = 1 ± 0.11 versus 1% = 0.69 ± 0.02, $P < 0.05$). In response to CoPP, there was no significant difference in the expression of HO-1 in the 6% treated group (0.89 ± 0.08). When CoPP was administered to villi exposed to 1% oxygen, however, there was a significant normalization of HO-1 expression when compared to hypoxic controls (1% = 0.69 ± 0.02 versus 1%+CoPP = 1.07 ± 0.08, $P < 0.05$), indicating that CoPP can restore HO-1 expression in hypoxic villi.

3.2. HO-1 Induction and the HO-1 Byproduct Bilirubin Attenuate Hypoxia-Induced Superoxide in Placental Villous Bundles. We next assessed the effects of both HO-1 levels and the direct effect of the HO-1 product bilirubin, a known antioxidant, on hypoxia-induced superoxide production. As seen in Figure 2, in response to hypoxic exposure, there is a significant increase in the tissue levels of superoxide of approximately 60% as determined by dihydroethidium fluorescence (6% = 1 ± 0.03 versus 1% = 1.63 ± 0.19, $P < 0.05$). Administration of CoPP had no significant difference under normoxic conditions (0.86 ± 0.06), but hypoxic tissues treated with CoPP exhibited a marked decrease in DHE

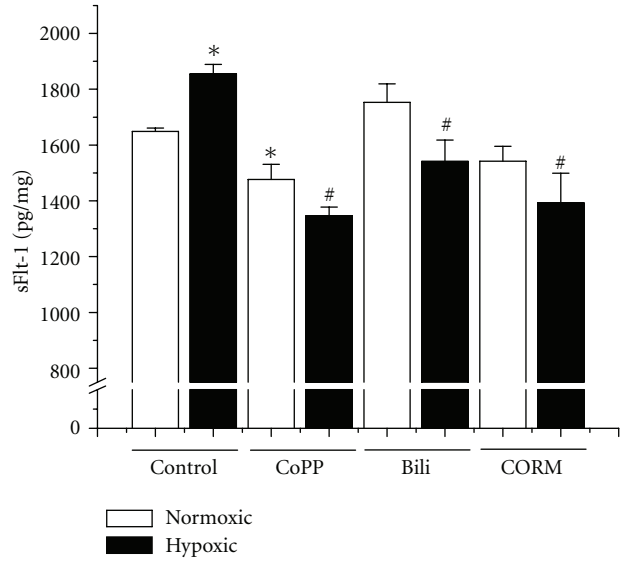

Figure 3: *HO-1 induction, CO, and Bilirubin attenuate hypoxia-induced sFlt-1 secretion.* In response to hypoxic incubation, placental explants demonstrate a significant increase in the production of sFlt-1 when compared to normoxic controls. Administration of CoPP significantly attenuated sFlt-1 released under both normoxic and hypoxic conditions. Bilirubin, while having no effect under normoxic conditions, slightly but significantly decreased sFlt-1 release when compared to hypoxic controls. Administration of a CO-releasing molecule (CORM) again had no effect under normoxic conditions but significantly attenuated sFlt-1 release in hypoxic explants. Statistical significance at the $P < 0.05$ level is indicated by "$*$" versus normoxic controls and "#" versus hypoxic controls.

fluorescence when compared to hypoxic controls (1% = 1.63 ± 0.19 versus 1% + CoPP = 1.01 ± 0.14, $P < 0.05$). Incubation with bilirubin had a marked effect on detected superoxide in both normoxic and hypoxic tissues. Under 6% oxygen, there was a significant decrease in DHE fluorescence when compared to normoxic controls (6% = 1 ± 0.03 versus 6% + Bili = 0.040 ± 0.11, $P < 0.05$). A similar level of DHE fluorescence was seen in hypoxic tissues, which were also significantly different from their hypoxic controls (1% = 1.63 ± 0.19 versus 1% + Bili = 0.54 ± 0.15, $P < 0.05$). These data suggest that both HO-1 and bilirubin significantly attenuate hypoxia-induced superoxide in placental villi.

3.3. HO-1 and Its Byproducts Attenuate Hypoxia-Induced sFlt-1 Production in Placental Villi. We also wished to determine what effect HO-1 and its byproducts would have on the hypoxia-induced production of sFlt-1. As seen in Figure 3, in response to hypoxia treatment, secreted sFlt-1 is significantly increased in response to hypoxia exposure (6% = 1649 ± 12.1 pg/mg versus 1% = 1854 ± 35 pg/mg, $P < 0.05$). Administration of CoPP decreased the amount of sFlt-1 secreted under both normoxic (6% = 1649 ± 12.1 pg/mg versus 6% + CoPP = 1477 ± 54 pg/mg, $P < 0.05$) and hypoxic (1% = 1854 ± 35 pg/mg versus 1349 ± 32 pg/mg, $P < 0.05$) conditions when compared to their respective controls. While bilirubin had no significant effect on sFlt-1 under normoxic conditions (1753 ± 56 pg/mg), in hypoxia-exposed explants, there was a significant decrease in the production of sFlt-1 when compared to hypoxic controls (1% = 1854 ± 35 pg/mg versus 1542 ± 76 pg/mg, $P < 0.05$). Similarly, administration of a CO donor molecule had no significant effect on the production of sFlt-1 under normoxic conditions (6% = 1649 ± 12.1 pg/mg versus 6% + CORM = 1542 ± 53 pg/mg) but exhibited a marked attenuation of sFlt-1 under hypoxic conditions (1% = 1854 ± 35 pg/mg versus 1392 ± 107 pg/mg, $P < 0.05$). Collectively, these data suggest that HO-1 through both CO and bilirubin suppresses hypoxia-induced sFlt-1 in placental villi.

4. Discussion

Preeclampsia remains a major health concern, affecting at least one out of every twenty pregnancies [34]. Besides the immediate risk for mother and fetus, there is increasing evidence that preeclampsia confers increased risk for cardiovascular complications to the offspring in later life [35]. One of the major roadblocks in the management of preeclampsia is the lack of an effective pharmacological intervention for its treatment. A potential therapy which has been proven effective in numerous experimental forms of hypertension is the manipulation of the HO-1 pathway [36–39].

HO-1 is hypothesized to attenuate hypertension through multiple pathways. HO-1 produces two bioactive compounds as products of heme metabolism: CO and bilirubin. Bilirubin has been shown in numerous systems to function as a powerful antioxidant [24, 25]. As it is recognized that production of reactive oxygen species is a major component of varied forms of hypertension, moderate increases in bilirubin could act to decrease overall oxidative stress. CO acts as a potent vasodilator, functioning in a manner similar to endothelium-derived relaxing factor [26]. Of particular interest to preeclampsia, HO-1 and CO have been shown to directly inhibit production of VEGF and interferon-γ-induced secretion of sFlt-1 [27]. Additionally, it has been reported that CO derived from HO-1 in vascular smooth muscle could inhibit production of endothelin-1 [40], a protein shown to be a final common pathological factor in several experimental models of preeclampsia.

We have recently demonstrated that induction of the HO-1 pathway could significantly attenuate the preeclampsia-like pathological manifestations associated with experimental placental ischemia in the rodent. Specifically, HO-1 attenuated the associated hypertension, with concomitant decreases in placental sFlt-1 and oxidative stress, and increased circulating bioavailable VEGF [28]. However, in this in vivo model, it was not possible to determine the direct effects of HO-1 and the individual metabolites on the regulation of each of these factors within the placenta. In the present work, we have utilized a previously established model of placental vascular bundle culturing to determine the importance of each of these factors in the regulation of oxidative stress and sFlt-1 in response to hypoxia.

Western blotting analysis demonstrated that hypoxia exposure significantly decreased the expression of HO-1 in

the vascular bundles. This is surprising, as numerous reports have indicated hypoxia induces an increase in HO-1 [41–43]. However, this effect has never been examined in the placental vasculature. This could help explain the increase in oxidative stress in the preeclamptic placenta. CoPP administration had no effect under normoxic conditions but in hypoxia restored HO-1 to normoxic levels. This is consistent with our previous data which shows that CoPP induces HO-1 in the placenta only with the secondary insult of placental ischemia/hypoxia [28].

In response to hypoxia exposure, the placental explants exhibited increased production of sFlt-1. CoPP administration significantly decreased sFlt-1 secretion to levels which were below even normoxic controls. Though CO has been shown to negatively regulate VEGF and interferon-γ-induced sFlt-1 in placental explants [27], its effect on hypoxia-induced sFlt-1 release has not been reported. As suggested in the literature, CO did indeed significantly attenuate hypoxia-induced sFlt-1, returning to levels equivalent to CoPP induction. Perhaps most surprisingly, administration of bilirubin also significantly decreased the secretion of sFlt-1 during hypoxia. This may suggest that oxidative stress is playing a role in the induction of sFlt-1, though the s ameliorative effect of bilirubin was not as great as either CoPP or CO, suggesting that CO is the major factor in HO-1's effect on sFlt-1.

Superoxide was also increased in the explants exposed to hypoxia when compared to normoxic controls. Treatment with CoPP, which does not induce HO-1 expression under normoxic conditions, had no effect on superoxide levels. However, under hypoxic conditions where CoPP normalizes HO-1 levels, there was a significant reduction in the amount of superoxide produced by the explants. Tellingly, the exogenous application of bilirubin to the explants under either normoxic or hypoxic conditions led to a significant decrease in superoxide when compared to their respective controls. This is perhaps an intuitive result given the known antioxidant properties of bilirubin but strongly suggests that moderate elevations in bilirubin production as a result of increased HO-1 are driving the reduction in oxidative stress. This demonstrates a second pathway through which HO-1 induction is directly affecting hypoxia-induced changes in the placental vasculature. It seems evident from these data as a whole, that HO-1 induction, through both CO and bilirubin production, is having a direct effect on the placental vasculature during ischemia/hypoxia. Continued studies into the safety and efficacy of agents to increase HO-1 or deliver these metabolites directly is warranted.

5. Conclusions

We have demonstrated here that induction of HO-1, or delivery of its bioactive metabolites CO and bilirubin, is capable of significantly downregulating two major hypoxia-induced factors (oxidative stress and sFlt-1) implicated in the etiology of preeclampsia. This suggests a promising therapeutic approach for the treatment of preeclampsia. Future studies both in vitro and in vivo are necessary to fully elucidate the therapeutic potential of this approach.

Acknowledgments

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References

[1] J. M. Roberts and D. W. Cooper, "Pathogenesis and genetics of pre-eclampsia," *Lancet*, vol. 357, no. 9249, pp. 53–56, 2001.

[2] J. M. Roberts, G. Pearson, J. Cutler, and M. Lindheimer, "Summary of the NHLBI Working Group on research on hypertension during pregnancy," *Hypertension*, vol. 41, no. 3, pp. 437–445, 2003.

[3] K. H. Lim, Y. Zhou, M. Janatpour et al., "Human cytotrophoblast differentiation/invasion is abnormal in pre-eclampsia," *American Journal of Pathology*, vol. 151, no. 6, pp. 1809–1818, 1997.

[4] I. A. Brosens, W. B. Robertson, and H. G. Dixon, "The role of the spiral arteries in the pathogenesis of preeclampsia," *Obstetrics and Gynecology Annual*, vol. 1, pp. 177–191, 1972.

[5] Y. Khong and I. Brosens, "Defective deep placentation," *Best Practice and Research: Clinical Obstetrics and Gynaecology*, vol. 25, no. 3, pp. 301–311, 2011.

[6] J. M. Roberts, R. N. Taylor, T. J. Musci, G. M. Rodgers, C. A. Hubel, and M. K. McLaughlin, "Preeclampsia: an endothelial cell disorder," *American Journal of Obstetrics and Gynecology*, vol. 161, no. 5, pp. 1200–1204, 1989.

[7] J. S. Gilbert, M. J. Ryan, B. B. Lamarca, M. Sedeek, S. R. Murphy, and J. P. Granger, "Pathophysiology of hypertension during preeclampsia: linking placental ischemia with endothelial dysfunction," *American Journal of Physiology*, vol. 294, no. 2, pp. H541–H550, 2008.

[8] B. J. Ballermann, "Glomerular endothelial cell differentiation," *Kidney International*, vol. 67, no. 5, pp. 1668–1671, 2005.

[9] F. T. H. Wu, M. O. Stefanini, F. M. Gabhann, C. D. Kontos, B. H. Annex, and A. S. Popel, "A systems biology perspective on sVEGFR1: its biological function, pathogenic role and therapeutic use," *Journal of Cellular and Molecular Medicine*, vol. 14, no. 3, pp. 528–552, 2010.

[10] T. Nagamatsu, T. Fujii, M. Kusumi et al., "Cytotrophoblasts up-regulate soluble Fms-like tyrosine kinase-1 expression under reduced oxygen: an implication for the placental vascular development and the pathophysiology of preeclampsia," *Endocrinology*, vol. 145, no. 11, pp. 4838–4845, 2004.

[11] S. Ahmad and A. Ahmed, "Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia," *Circulation Research*, vol. 95, no. 9, pp. 884–891, 2004.

[12] O. Nevo, N. Soleymanlou, Y. Wu et al., "Increased expression of sFlt-1 in in vivo and in vitro models of human placental hypoxia is mediated by HIF-1," *American Journal of Physiology*, vol. 291, no. 4, pp. R1085–R1093, 2006.

[13] R. J. Levine, C. Lam, C. Qian et al., "Soluble endoglin and other circulating antiangiogenic factors in preeclampsia," *New England Journal of Medicine*, vol. 355, no. 10, pp. 992–1005, 2006.

[14] R. J. Levine, S. E. Maynard, C. Qian et al., "Circulating angiogenic factors and the risk of preeclampsia," *New England Journal of Medicine*, vol. 350, no. 7, pp. 672–683, 2004.

[15] S. E. Maynard, J. Y. Min, J. Merchan et al., "Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction hypertension, and proteinuria in preeclampsia," *Journal of Clinical Investigation*, vol. 111, no. 5, pp. 649–658, 2003.

[16] J. S. Gilbert, S. A. Babcock, and J. P. Granger, "Hypertension produced by reduced uterine perfusion in pregnant rats is associated with increased soluble fms-like tyrosine kinase-1 expression," *Hypertension*, vol. 50, no. 6, pp. 1142–1147, 2007.

[17] A. Makris, C. Thornton, J. Thompson et al., "Uteroplacental ischemia results in proteinuric hypertension and elevated sFLT-1," *Kidney International*, vol. 71, no. 10, pp. 977–984, 2007.

[18] S. R. Murphy, B. B. D. Lamarca, K. Cockrell, and J. P. Granger, "Role of endothelin in mediating soluble fms-like tyrosine kinase 1-induced hypertension in pregnant rats," *Hypertension*, vol. 55, no. 2, pp. 394–398, 2010.

[19] T. H. Hung, J. N. Skepper, D. S. Charnock-Jones, and G. J. Burton, "Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia," *Circulation Research*, vol. 90, no. 12, pp. 1274–1281, 2002.

[20] A. C. Staff, T. Ranheim, J. Khoury, and T. Henriksen, "Increased contents of phospholipids, cholesterol, and lipid peroxides in decidua basalis in women with preeclampsia," *American Journal of Obstetrics and Gynecology*, vol. 180, no. 3, pp. 587–592, 1999.

[21] A. M. Roggensack, Y. Zhang, and S. T. Davidge, "Evidence for peroxynitrite formation in the vasculature of women with preeclampsia," *Hypertension*, vol. 33, no. 1, pp. 83–89, 1999.

[22] M. Sedeek, J. S. Gilbert, B. B. Lamarca et al., "Role of reactive oxygen species in hypertension produced by reduced uterine perfusion in pregnant rats," *American Journal of Hypertension*, vol. 21, no. 10, pp. 1152–1156, 2008.

[23] M. H. Sedeek, Y. Wang, and J. P. Granger, "Increased oxidative stress in a rat model of preeclampsia," *American Journal of Hypertension*, vol. 17, no. Issue 5, supplement, p. S142, 2004.

[24] J. Neuzil and R. Stocker, "Bilirubin attenuates radical-mediated damage to serum albumin," *FEBS Letters*, vol. 331, no. 3, pp. 281–284, 1993.

[25] J. Neuzil and R. Stocker, "Free and albumin-bound bilirubin are efficient co-antioxidants for α- tocopherol, inhibiting plasma and low density lipoprotein lipid peroxidation," *Journal of Biological Chemistry*, vol. 269, no. 24, pp. 16712–16719, 1994.

[26] L. Wu and R. Wang, "Carbon monoxide: endogenous production, physiological functions, and pharmacological applications," *Pharmacological Reviews*, vol. 57, no. 4, pp. 585–630, 2005.

[27] M. Cudmore, S. Ahmad, B. Al-Ani et al., "Negative regulation of soluble Flt-1 and soluble endoglin release by heme oxygenase-1," *Circulation*, vol. 115, no. 13, pp. 1789–1797, 2007.

[28] E. M. George, K. Cockrell, M. Aranay, E. Csongradi, D. E. Stec, and J. P. Granger, "Induction of heme oxygenase 1 attenuates placental ischemia-induced hypertension," *Hypertension*, vol. 57, no. 5, pp. 941–948, 2011.

[29] E. M. George, K. Cockrell, T. H. Adair, and J. P. Granger, "Regulation of sFlt-1 and VEGF secretion by adenosine under hypoxic conditions in rat placental villous explants," *American Journal of Physiology*, vol. 299, no. 6, pp. R1629–R1633, 2010.

[30] I. Caniggia, C. V. Taylor, J. W. K. Ritchie, S. J. Lye, and M. Letarte, "Endoglin regulates trophoblast differentiation along the invasive pathway in human placental villous explants," *Endocrinology*, vol. 138, no. 11, pp. 4977–4988, 1997.

[31] J. E. Clark, P. Naughton, S. Shurey et al., "Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule," *Circulation research*, vol. 93, no. 2, pp. e2–e8, 2003.

[32] C. Rudolph, G. Adam, and A. Simm, "Determination of copy number of c-Myc protein per cell by quantitative Western blotting," *Analytical Biochemistry*, vol. 269, no. 1, pp. 66–71, 1999.

[33] H. M. Peshavariya, G. J. Dusting, and S. Selemidis, "Analysis of dihydroethidium fluorescence for the detection of intracellular and extracellular superoxide produced by NADPH oxidase," *Free Radical Research*, vol. 41, no. 6, pp. 699–712, 2007.

[34] J. J. Walker, "Pre-eclampsia," *Lancet*, vol. 356, no. 9237, pp. 1260–1265, 2000.

[35] K. Zandi-Nejad, V. A. Luyckx, and B. M. Brenner, "Adult hypertension and kidney disease: the role of fetal programming," *Hypertension*, vol. 47, no. 3, pp. 502–508, 2006.

[36] J. Cao, K. Inoue, X. Li, G. Drummond, and N. G. Abraham, "Physiological significance of heme oxygenase in hypertension," *International Journal of Biochemistry and Cell Biology*, vol. 41, no. 5, pp. 1025–1033, 2009.

[37] F. T. Botros, M. L. Schwartzman, C. T. Stier, A. I. Goodman, and N. G. Abraham, "Increase in heme oxygenase-1 levels ameliorates renovascular hypertension," *Kidney International*, vol. 68, no. 6, pp. 2745–2755, 2005.

[38] H. E. Sabaawy, F. Zhang, X. Nguyen et al., "Human heme oxygenase-1 gene transfer lowers blood pressure and promotes growth in spontaneously hypertensive rats," *Hypertension*, vol. 38, no. 2, pp. 210–215, 2001.

[39] L. Yang, S. Quan, A. Nasjletti, M. Laniado-Schwartzman, and N. G. Abraham, "Heme oxygenase-1 gene expression modulates angiotensin II-induced increase in blood pressure," *Hypertension*, vol. 43, no. 6, pp. 1221–1226, 2004.

[40] T. Morita and S. Kourembanas, "Endothelial cell expression of vasoconstrictors and growth factors is regulated by smooth muscle cell-derived carbon monoxide," *Journal of Clinical Investigation*, vol. 96, no. 6, pp. 2676–2682, 1995.

[41] M. V. Panchenko, H. W. Farber, and J. H. Korn, "Induction of heme oxygenase-1 by hypoxia and free radicals in human dermal fibroblasts," *American Journal of Physiology*, vol. 278, no. 1, pp. C92–C101, 2000.

[42] R. Motterlini, R. Foresti, R. Bassi, V. Calabrese, J. E. Clark, and C. J. Green, "Endothelial heme oxygenase-1 induction by hypoxia. Modulation by inducible nitric-oxide synthase and S-nitrosothiols," *Journal of Biological Chemistry*, vol. 275, no. 18, pp. 13613–13620, 2000.

[43] M. S. Carraway, A. J. Ghio, J. D. Carter, and C. A. Piantadosi, "Expression of heme oxygenase-1 in the lung in chronic hypoxia," *American Journal of Physiology*, vol. 278, no. 4, pp. L806–L812, 2000.

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Research Article

Renal Inhibition of Heme Oxygenase-1 Increases Blood Pressure in Angiotensin II-Dependent Hypertension

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The goal of this study was to test the hypothesis that renal medullary heme oxygenase (HO) acts as a buffer against Ang-II dependent hypertension. To test this hypothesis, renal medullary HO activity was blocked using QC-13, an imidazole-dioxolane HO-1 inhibitor, or SnMP, a classical porphyrin based HO inhibitor. HO inhibitors were infused via IRMI catheters throughout the study starting 3 days prior to implantation of an osmotic minipump which delivered Ang II or saline vehicle. MAP was increased by Ang II infusion and further increased by IRMI infusion of QC-13 or SnMP. MAP averaged 113 ± 3, 120 ± 7, 141 ± 2, 153 ± 2, and 154 ± 3 mmHg in vehicle, vehicle + IRMI QC-13, Ang II, Ang II + IRMI QC-13, and Ang II + IRMI SnMP treated mice, respectively ($n = 6$). Inhibition of renal medullary HO activity with QC-13 in Ang II infused mice was also associated with a significant increase in superoxide production as well as significant decreases in antioxidant enzymes catalase and MnSOD. These results demonstrate that renal inhibition of HO exacerbates Ang II dependent hypertension through a mechanism which is associated with increases in superoxide production and decreases in antioxidant enzymes.

1. Introduction

Heme oxygenases (HO) are the critical enzymes responsible for the breakdown of endogenous heme to biliverdin, carbon monoxide (CO), and free iron. Biliverdin is subsequently reduced to bilirubin and free iron is sequestered in the cell via induction of ferritin [1]. The metabolites of HO have a multitude of actions in the cardiovascular system. Two major isoforms of HO exist, the inducible form, HO-1, and the constitutively expressed isoform HO-2.

Several studies have demonstrated that systemic induction of HO-1 can prevent the development of hypertension in both experimental and genetic models of hypertension [2–5]. One study has even reported that HO-1 induction for 3 weeks resulted in a chronic 9-month lowering of blood pressure long after the levels of HO-1 had returned to normal [6]. Despite the experimental evidence for the antihypertensive effect of systemic HO-1 induction, the mechanism by which HO-1 induction lowers blood pressure is not known. One reason for the dependence of systemic induction of HO-1 is the lack of appropriate models which allow for organ or cell-type specific induction of HO-1.

Given the importance of the kidneys in the long-term control of blood pressure, renal induction of HO-1 could play a significant role in the antihypertensive effect of systemic HO-1 inducers [7]. Previous studies have demonstrated an important role for HO enzymes and their metabolites in the regulation of renal blood flow [8]. Moreover, intrarenal medullary interstitial (IRMI) infusion of a metalloporphyrin-based HO inhibitor, chromium mesoporphyrin (CrMP), attenuated renal pressure-natriuresis and resulted in the development of salt-sensitive hypertension [9]. We recently reported that kidney-specific induction of HO-1 via direct intrarenal medullary interstitial infusion of cobalt protoporphyrin (CoPP) attenuated the development of Ang II-dependent hypertension in the mouse [10]. The results of these studies highlight the importance of intrarenal HO in the regulation of blood pressure.

Traditional HO inhibitors are built upon metalloporphyrins which contain central metal atoms and serve as

competitive inhibitors of HO enzymes [11, 12]. While these compounds are effective HO inhibitors, they also result in significant induction of HO-1 *in vivo* due to the metals that are utilized [13, 14]. Recently, a new class of imidazole-dioxolane HO inhibitors was described [15, 16]. These inhibitors are molecules which are similar in structure to heme, the natural HO substrate, but they do not contain metals and do not induce HO-1 when used *in vitro* [17]. We have previously demonstrated that the imidazole-dioxolane HO-1 inhibitor, QC-13, can effectively inhibit HO activity when administered *in vivo* either by *intraperitoneal* injection or IRMI infusion [18].

The role of renal medullary HO in the regulation of blood pressure in Ang II-dependent hypertension is not known. Previous studies have demonstrated that HO-1 is induced in the rat but not mouse kidney by Ang II [10, 19–22]. The goal of the present study was to determine the specific role of renal medullary HO-1 in the development of Ang II-dependent hypertension in mouse model by IRMI infusion of either a classical metalloporphyrin-based HO inhibitor, stannous mesoporphyrin (SnMP), or the imidazole-dioxolane HO-1 inhibitor, QC-13.

2. Methods

2.1. Animals. Experiments were performed on 12- to 16-week-old male C57BL/6J mice obtained from Jackson Labs (Bar Harbor, ME). The mice were fed a standard diet containing 0.29% NaCl and were provided water ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. Studies were performed on 16–20-week old male C57BL/6J mice (Jackson Labs, Bar Harbor, ME). All studies were performed in accordance with the approval of the University of Mississippi Medical Center Institutional Animal Care and Use Committee (IACUC) and in line with NIH guidelines. All mice underwent unilateral nephrectomy of the right kidney to remove potential contributions of the noninfused kidney to the blood pressure response to experimental manipulations. After seven days, intramedullary interstitial catheters were implanted 1.5–2 mm into the left kidney as previously described [10, 18]. Saline was then infused through the catheter for a period of 3 days after which the infusion was switched to QC-13, (2*R*,4*R*)-2-[2-(4-chlorophenyl)ethyl]-2-[(1*H*-imidazol-1-yl)methyl]-4-methyl-1,3-dioxolane hydrochloride (25 μM, in saline), or stannous mesoporphyrin (SnMP, 400 μM, in saline) in some mice ($n = 6$/group). Infusions were continued throughout the entire experimental protocol. Two days after the switch to QC-13 or SnMP mice were implanted with osmotic minipumps delivering either vehicle (saline) or Ang II at a rate of 1 μg/kg/min. Five days after implantation of the osmotic minipumps, carotid artery catheters were implanted into the mice as previously described [10, 22]. After a 2-day recovery period, blood pressures were measured in 3-hour periods over the next 3 days in conscious, freely moving mice. Blood pressure data were continuously collected by a PowerLab 8/sp polygraph (AD Instruments, Denver, Colorado) and acquired on a computer running Chart 4 software provided by the manufacturer. Mice were euthanized after the last blood pressure recording session and organs harvested.

2.2. Heme Oxygenase Assay. Heme oxygenase assays were performed on lysates prepared from the renal medulla. Medullary protein lysates were prepared previously described [10, 22]. Briefly, tissue was homogenized in 250 mM sucrose, 10 mM KPO_4, 1 mM EDTA, and 0.1 mM PMSF (pH 7.7) in the presence of protease inhibitors (2 μg/mL aprotinin, leupeptin, and pepstatin). The homogenate was then centrifuged at 3,000 g for 15 min at 4°C and the supernatant was collected. Protein concentration was measured using a Bio-Rad protein assay with BSA standards. Reactions were carried out in a 1.2 mls containing: 2 mM glucose-6-phosphate, 0.2 unites glucose-6-phosphate dehydrogenase, 0.8 mM NADPH, 20 μM hemin, and 0.5 mg of lysates as previously described [23]. The reactions were incubated for 1 hour at 37°C in the dark. The formed bilirubin was extracted with chloroform, and the change in optical density (ΔOD) at 464–530 nm was measured using an extinction coefficient of 40 mM/cm for bilirubin. HO activity was expressed as picomoles of bilirubin formed per hour per milligram of protein.

2.3. Western Blots. Western blots for HO-1 protein were performed on lysates prepared from the renal medulla as described above. Samples of 50 μg of protein were boiled in Laemmli sample buffer (Bio-Rad, Hercules, CA) for 5 min and electrophoresed on 7.5% SDS-polyacrylamide gels and blotted onto nitrocellulose membrane. Membranes were blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) for 2 hours at room temperature, then incubated with the following antibodies: mouse anti-HO-1 monoclonal antibody (StressGen, Vancouver, Canada 1 : 2000), rabbit anti-HO-2 polyclonal antibody (StressGen), extracellular (EC), copper-zinc (CuZN), and manganese (Mn) superoxide dismutase (StressGen, Vancouver, Canada, 1 : 1000), and mouse antimonoclonal antibody (Sigma, St. Louis, MO, 1 : 10,000).

Primary antibodies were used in conjunction with a mouse or rabbit anti-β-actin antibody (Gentest, 1 : 5,000) and incubated overnight at 4°C. The membranes were then incubated with Alex 680 goat anti-mouse or anti-rabbit IgG (Molecular Probes) and IRDye 800 goat anti-mouse or anti-rabbit IgG (Rockland, Gilbertsville, PA) for 1 hour at room temperature. The membranes were then visualized using an Odyssey infrared imager (Li-COR, Lincoln, NE) which allows for the simultaneous detection of two proteins. Densitometry analysis was performed using Odyssey software (LI-COR, Lincoln, NE). Levels of each specific protein are expressed as the ratio to β-actin for each sample.

2.4. Measurement of Renal Medullary Superoxide. Superoxide production in the renal medulla was measured using the lucigenin technique as previously described [22]. Briefly, kidneys were removed and separated into renal cortex and medulla. The medulla was then homogenized (1 : 8 wt/vol) in RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium

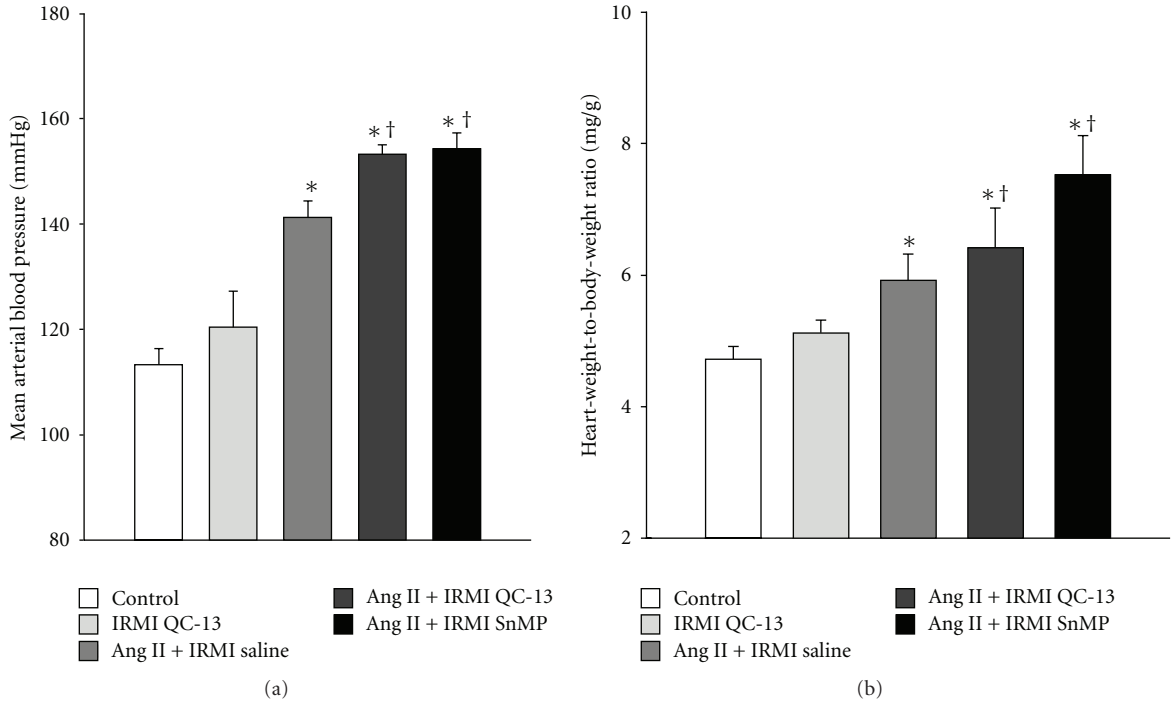

Figure 1: Mean arterial blood pressure (a) and cardiac mass index (b) in control, IRMI QC-13, Ang II + IRMI saline, Ang II + IRMI QC-13, and Ang II + IRMI SnMP-treated mice, $n = 6$/group. $^{*}P < 0.05$ as compared to value in control mice. $^{\dagger}P < 0.05$ as compared to Ang II-treated mice. IRMI, intrarenal medullary interstitial infusion.

deoxycholate, 0.1% SDS, and a protease inhibitor cocktail; Sigma Chemical). The samples were centrifuged at 12,000 *g* for 20 min at 4°C. The supernatant was incubated with lucigenin at a final concentration of 5 μM and NADPH at a final concentration of 100 μM. The samples were allowed to equilibrate for 3 min in the dark, and luminescence was measured every second for 5–15 min with a luminometer (Berthold, Oak Ridge, TN). Luminescence was recorded as relative light units (RLU) per min. An assay blank with no homogenate but containing lucigenin was subtracted from the reading before transformation of the data. The protein concentration was measured using a Bio-rad protein assay with BSA standards. The data are expressed as RLU per min per milligram protein.

2.5. Statistics. Mean values ± SE are presented. Significant differences between mean values were determined by ANOVA followed by a post hoc test (Dunnett's). A $P < 0.05$ was considered to be significant.

3. Results

3.1. IRMI Infusion of HO Inhibitors Exacerbates Ang II-Dependent Hypertension and Increases Cardiac Hypertrophy. Mean arterial pressure (MAP) averaged 113 ± 3 mmHg in control mice and was slightly higher (120 ± 7 mmHg) in mice which received IRMI infusion of QC-13 alone. MAP was significantly increased to 141 ± 2 mmHg in Ang II-treated mice which received IRMI infusion of saline (Figure 1(a)). MAP was significantly augmented in Ang II-treated mice which received IRMI infusion of either QC-13 or SnMP and averaged 153 ± 2 and 154 ± 3 mmHg in each group, respectively (Figure 1(a)). Cardiac hypertrophy as indexed by the ratio of the heart weight to body weight was significantly increased in mice infused with Ang II as compared to control and IRMI QC-13-infused mice averaging 5.8 ± 0.3, versus 4.8 ± 0.2, versus 5 ± 0.2 mg/g in each group, respectively (Figure 1(b)). IRMI infusion of QC-13 or SnMP in Ang II treated mice resulted in further increases in cardiac hypertrophy as compared to Ang II treated mice alone with heart weight to body weight ratios averaging 6.4 ± 0.6 and 7.6 ± 0.6 mg/g in each group, respectively (Figure 1(b)). No differences in body weights were apparent between the groups with body weights averaging 26 ± 0.7, versus 27 ± 0.7, versus 25 ± 0.4, versus 25 ± 0.9, versus 25 ± 0.8 grams in control, QC-13, Ang II, Ang II + QC-13, and Ang II + SnMP-treated mice, respectively.

3.2. HO Activity and Protein Levels in the Medulla of Mice Receiving IRMI Infusion of QC-13 and SnMP. The effectiveness of IRMI infusion of the HO inhibitors, QC-13 and SnMP, to inhibit renal medullary HO was determined in each of the experimental groups. In agreement with our previous studies, HO activity was not affected by Ang II infusion (Figure 2(a)) [10, 22]. However, IRMI infusion of QC-13 either alone or in combination with Ang II treatment resulted in a 38% decrease in medullary HO activity as compared to levels in both control and Ang II-treated mice

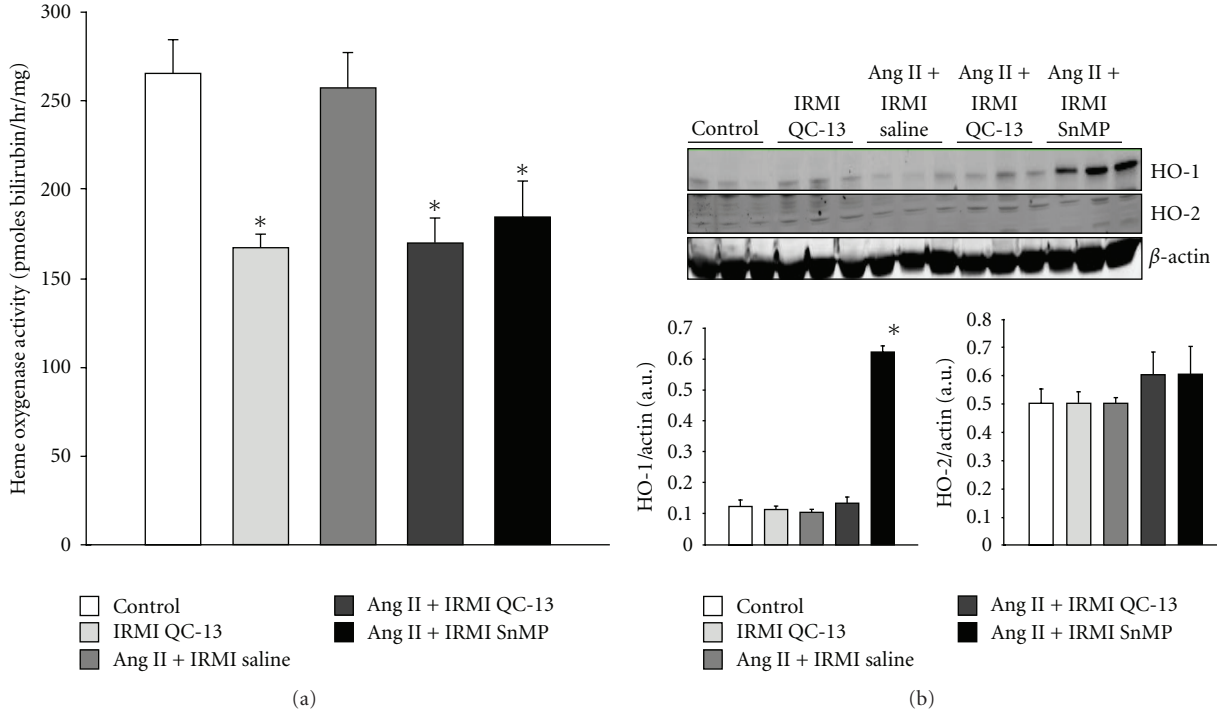

Figure 2: (a) Renal medullary heme oxygenase activity in control, IRMI QC-13, Ang II + IRMI saline, Ang II + IRMI QC-13, and Ang II + IRMI SnMP-treated mice, $n = 6$/group. (b) Representative western blots of HO-1 and HO-2 proteins from the renal medulla of control, IRMI QC-13, Ang II + vehicle, Ang II + IRMI QC-13, and Ang II + IRMI SnMP-treated mice, $n = 3$/group. $^*P < 0.05$ as compared to value in control mice.

(Figure 2(a)). Likewise, IRMI infusion of SnMP also resulted in a similar level of HO inhibition in the renal medulla (Figure 2(a)). IRMI infusion of QC-13 had no effect on the levels of HO-1 protein in the medulla when administered alone or in combination with Ang II (Figure 2(b)). However, IRMI infusion of SnMP in Ang II-treated mice resulted in a significant induction of HO-1 in the renal medulla (Figure 2(b)). In agreement with previous results, Ang II infusion had no effect on the levels of HO-1 protein in the renal medulla (Figure 2(b)) [10, 22]. None of the different treatments had any significant effect on the levels of HO-2 protein in the medulla (Figure 2(b)).

3.3. Inhibition of HO Activity Increases Superoxide Production in the Renal Medulla. We determined the effect of renal HO inhibition with QC-13 on superoxide production in the renal medulla of control and Ang II-infused mice. Superoxide production was significantly increased by more than 2.5 times in the medulla of Ang II-treated mice as compared to control mice (Figure 3). IRMI infusion of QC-13 alone resulted in a significant increase in superoxide production in the medulla as compared to control mice (Figure 3). IRMI infusion of QC-13 in mice treated with Ang II resulted in an even greater increase in renal medullary superoxide production with levels 3.5-fold greater than those observed in control mice and 35% greater than those observed in Ang II-treated mice (Figure 3).

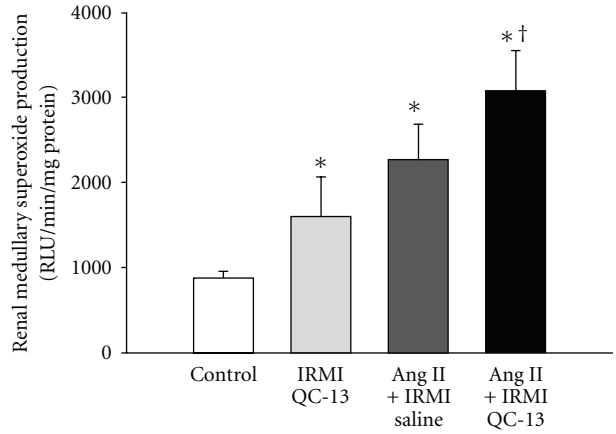

Figure 3: Renal medullary superoxide production in control, IRMI QC-13, Ang II + IRMI saline, and Ang II + IRMI QC-13-treated mice, $n = 6$/group. Renal medullary superoxide production was measured using the lucigenin technique as described in Section 2. $^*P < 0.05$ as compared to value in control mice. $^\dagger P < 0.05$ as compared to Ang II-treated mice.

3.4. Inhibition of HO Activity and Antioxidant Proteins in the Renal Medulla. We determined the effect of renal HO inhibition with QC-13 on the levels of antioxidant proteins catalase, CuZn, EC, and Mn SOD in control and Ang II-infused mice. IRMI infusion of QC-13 resulted in a

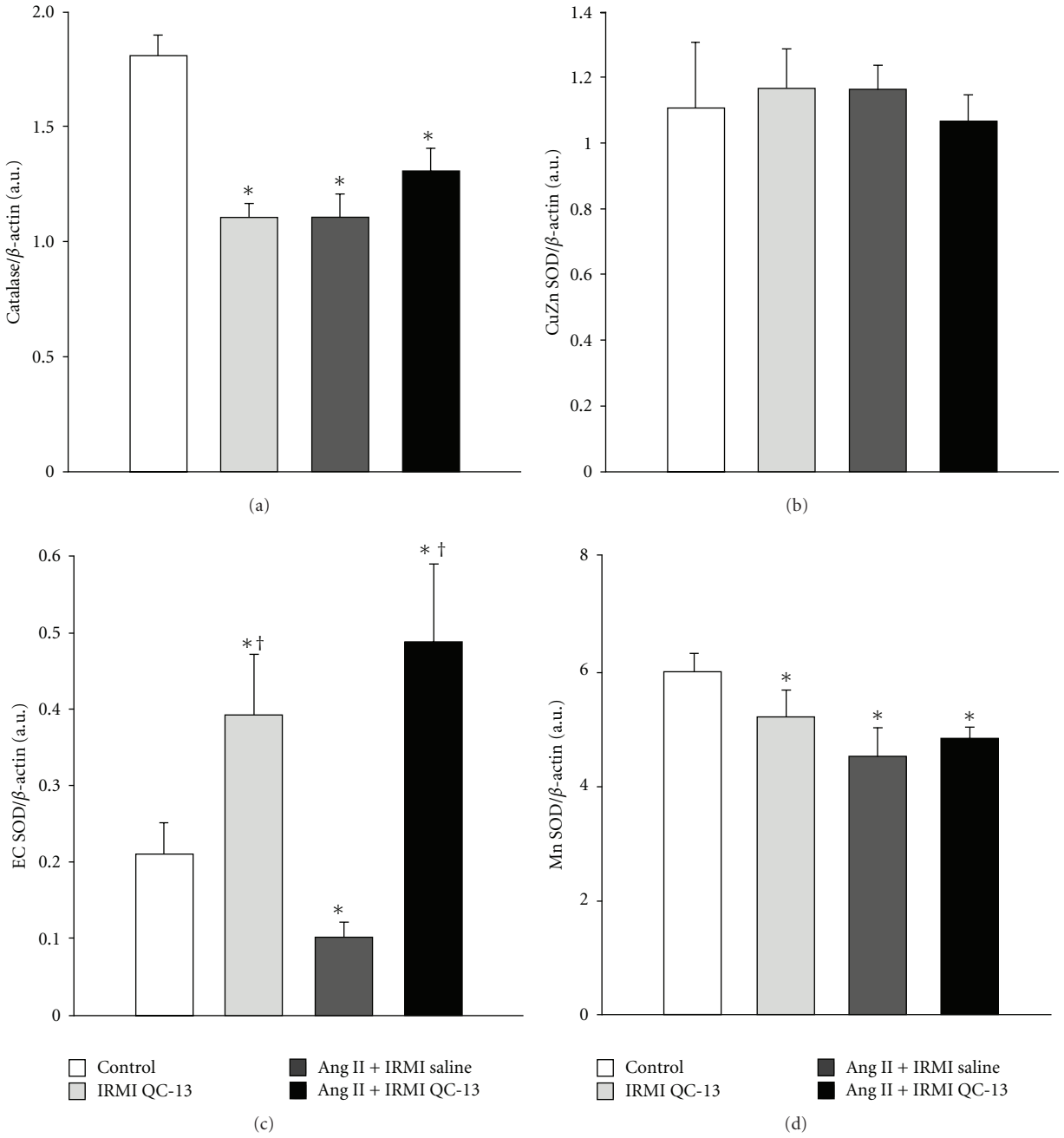

Figure 4: Representative western blots of catalase (a), copper-zinc (CuZn) superoxide dismutase (SOD) (b), extracellular (EC) SOD (c), and manganese (Mn) SOD (d) from the renal medulla of control, IRMI QC-13, Ang II + IRMI saline, and Ang II + IRMI QC-13-treated mice, $n = 6$/group. $^{*}P < 0.05$ as compared to value in control mice. $^{\dagger}P < 0.05$ as compared to Ang II-treated mice.

significant decrease in renal medullary catalase protein which was equivalent to the decrease observed in Ang II-treated mice (Figure 4(a)). However, inhibition of HO activity during Ang II infusion did not result in further decreases in renal medullary catalase protein levels (Figure 4(a)). No differences in renal medullary CuZn SOD protein levels were observed between any of the experimental groups (Figure 4(b)). Ang II infusion resulted in a significant decrease in EC SOD levels as compared to control mice (Figure 4(c)). Interestingly, IRMI infusion of QC-13 either alone or in combination with Ang II treatment resulted in a significant increase in the levels of renal medullary EC SOD protein (Figure 4(c)). IRMI infusion of QC-13 resulted in a significant decrease in renal medullary Mn SOD protein which was equivalent to the decrease observed in Ang II-treated mice (Figure 4(d)). Inhibition of HO activity during Ang II infusion did not result in further decreases in renal medullary Mn SOD protein levels (Figure 4(d)).

4. Discussion

The present study was designed to determine the importance of renal medullary HO in the progression of Ang II-dependent hypertension. Renal medullary HO activity was inhibited by IRMI infusion of two separate HO inhibitors, QC-13, an imidazole-dioxolane HO inhibitor or SnMP, a classical metalloporphyrin-based HO inhibitor. IRMI infusion of either QC-13 or SnMP resulted in significant inhibition of HO activity and enhancement of Ang II-dependent hypertension as compared to mice infused with vehicle. Our present results confirm our previous observations that infusion of Ang II does not induce either HO-1 protein or increase HO activity in the mouse kidney [10, 22]. These results are opposite to the effect that Ang II infusion has on HO protein and activity in the rat kidney where several studies have demonstrated that Ang II treatment is an inducer of renal HO-1 [19–21]. The differences in the renal response to Ang II between the rat and mouse are not known and could be due to species differences in the promoters of the HO-1 gene or in the renal response to Ang II infusion.

Our finding that inhibition of HO activity in Ang II-infused mice exacerbates the blood pressure response and increases cardiac hypertrophy despite the lack of effect of Ang II on HO activity and protein in the kidney being similar to that recently described in a model of low Ang II infusion in the rat [24]. In this study, inhibition of HO activity resulted in increased renal vascular resistance and decreased renal blood flow in Ang II-infused rats despite no measureable changes in HO activity or protein in the kidney with the dose of Ang II-infused in the study. Similar effects on renal vascular resistance and blood flow were not observed in control rats in which HO was blocked. Interestingly, inhibition of HO activity resulted in a lowering of blood pressure in the Ang II-infused rats in this study as measured acutely under anesthesia [24]. While the differences in blood pressure responses between the studies could be due to species differences or differences in the way in which blood pressures were measured, the results from these two studies lend support for the hypothesis that HO-1 plays an important role in the stress response of the kidney even in conditions in which its activity is not significantly increased. One limitation in the current study is the lack of peripheral measurement of HO activity in the mice receiving IRMI QC-13 or SnMP. Thus, it is possible that systemic spillover of the inhibitors could have contributed to the increase in blood pressure in the mice receiving IRMI infusions of QC-13 or SnMP. However, in a previous study by our group, we could not detected induction of HO-1 in organs such as the liver and heart following IRMI infusion of CoPP [10].

The effect of renal medullary inhibition of HO in the present study is consistent with our previous results which demonstrated that renal-specific induction of HO-1 can prevent the development of Ang II-dependent hypertension [10]. They are also in agreement with previous studies in the rat demonstrating that renal medullary HO inhibition results in salt-sensitive hypertension [9]. One potential mechanism for the increase in blood pressure in mice treated with QC-13 is the effects of HO inhibition on regional renal blood flow. HO and its metabolites play an important role in the regulation of renal medullary blood flow which is important in the pressure-natriuretic response [8, 25, 26]. However, the effect of HO inhibition on renal hemodynamics in Ang II hypertensive mice has yet to be examined.

The current results demonstrate that inhibition of renal medullary HO activity was associated with an increase in superoxide production in the renal medulla. This observation is consistent with our previous study in which induction of HO-1 in the renal medulla was associated with a decrease in superoxide production [10]. Moreover, specific adenoviral overexpression of HO-1 in TALH decreased Ang II-dependent oxidative damage [27]. The link between HO-1 and its metabolites has also been strengthened in additional studies from cultured TALH cells which have demonstrated that increases in HO-1 protein, bilirubin, or CO can attenuate the development of Ang II-dependent superoxide production [28]. Superoxide anion is a major regulator of sodium reabsorption in the TALH through direct actions and through interaction with NO [29–31]. Previous studies in cultured mouse TALH cells have demonstrated that blockade of bilirubin formation by inhibition of the conversion of biliverdin to bilirubin resulted in increased Ang II-mediated superoxide production and enhancement of sodium transport [32]. Thus, it is possible that the increase in renal medullary superoxide production observed after HO inhibition results in increased sodium reabsorption in the TALH and enhancement of Ang II hypertension.

Intramedullary interstitial infusion of QC-13 alone resulted in a significant increase in superoxide production; yet it only resulted in a slight but not statistically significant increase in blood pressure. It is not clear why blood pressure did not increase further in the mice receiving QC-13 alone but it is possible that in the absence of a physiological stressor such as Ang II or high salt that increases in superoxide production are not able to increase blood pressure.

Inhibition of renal medullary HO activity with QC-13 resulted in significant alterations in important antioxidant enzymes control mice. In agreement with our previous studies, Ang II infusion also resulted in a significant decrease in renal medullary catalase levels which was not affected by blockade of HO-1 with QC-13 [10, 22]. Ang II infusion also resulted in a significant decrease in renal medullary Mn SOD levels which again were not altered by intramedullary inhibition of HO-1 with QC-13. The decrease in renal medullary catalase and Mn SOD observed in the medulla of mice infused with QC-13 alone suggests an important role for HO and its metabolites in regulating antioxidant enzymes in the kidney. Interestingly, inhibition of HO activity with QC-13 resulted in a significant increase in the levels of EC-SOD which could be a compensatory response to the increase in superoxide production observed in mice in which QC-13 was infused.

QC-13 is an imidazole-dioxolane HO inhibitor that has been reported to selectively inhibit the HO-1 versus HO-2 isoform [15, 17]. In agreement with our previous *in vivo* study, we did not detect any measureable induction of HO-1 in the renal medulla of mice in which QC-13 was administered via intrarenal medullary interstitial infusion [18]. This

was in contrast to infusion of the classic porphyrin-based HO inhibitor, SnMP, which resulted in significant induction of HO-1 in the renal medulla. While a difference in the induction of HO-1 by these inhibitors was observed, they both resulted in similar degree of *in vitro* HO inhibition and an increase in blood pressure and cardiac hypertrophy in Ang II-infused mice. However, QC-13 has several technical advantages over porphyrin-based HO inhibitors like SnMP including water solubility and light insensitivity which make its use more appealing for chronic *in vivo* studies.

In summary, our results demonstrate that inhibition of renal medullary HO activity exacerbates Ang II-dependent hypertension and cardiac hypertrophy. The increase in blood pressure following HO inhibition in Ang II hypertension is also associated with an increase in renal medullary superoxide production and a decrease in antioxidant enzymes in the renal medulla. These results would suggest that renal HO-1 plays an important basal role to guard against further increases in blood pressure in Ang II hypertension through its antioxidant effects even in states in which it is not induced. Potential systemic effects of inhibitor spillover could also play a contribution to the increases in blood pressure. Further studies are needed to determine the precise roles of vascular versus tubular effects of HO inhibition as well as to determine the relative contributions of CO and bilirubin generation.

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References

[1] N. G. Abraham and A. Kappas, "Heme oxygenase and the cardiovascular-renal system," *Free Radical Biology and Medicine*, vol. 39, no. 1, pp. 1–25, 2005.

[2] F. T. Botros, M. L. Schwartzman, C. T. Stier, A. I. Goodman, and N. G. Abraham, "Increase in heme oxygenase-1 levels ameliorates renovascular hypertension," *Kidney International*, vol. 68, no. 6, pp. 2745–2755, 2005.

[3] P. Martasek, M. L. Schwartzman, A. I. Goodman, K. B. Solangi, R. D. Levere, and N. G. Abraham, "Hemin and L-arginine regulation of blood pressure in spontaneous hypertensive rats," *Journal of the American Society of Nephrology*, vol. 2, no. 6, pp. 1078–1084, 1991.

[4] H. E. Sabaawy, F. Zhang, X. Nguyen et al., "Human heme oxygenase-1 gene transfer lowers blood pressure and promotes growth in spontaneously hypertensive rats," *Hypertension*, vol. 38, no. 2, pp. 210–215, 2001.

[5] D. Sacerdoti, B. Escalante, N. G. Abraham, J. C. McGiff, R. D. Levere, and M. L. Schwartzman, "Treatment with tin prevents the development of hypertension in spontaneously hypertensive rats," *Science*, vol. 243, no. 4889, pp. 388–390, 1989.

[6] R. Wang, R. Shamloul, X. Wang, Q. Meng, and L. Wu, "Sustained normalization of high blood pressure in spontaneously hypertensive rats by implanted hemin pump," *Hypertension*, vol. 48, no. 4, pp. 685–692, 2006.

[7] A. C. Guyton, "The surprising kidney-fluid mechanism for pressure control—its infinite gain!," *Hypertension*, vol. 16, no. 6, pp. 725–730, 1990.

[8] A. P. Zou, H. Billington, N. Su, and A. W. Cowley Jr., "Expression and actions of heme oxygenase in the renal medulla of rats," *Hypertension*, vol. 35, no. 1, pp. 342–347, 2000.

[9] N. Li, F. Yi, E. A. Dos Santos, D. K. Donley, and P. L. Li, "Role of renal medullary heme oxygenase in the regulation of pressure natriuresis and arterial blood pressure," *Hypertension*, vol. 49, no. 1, pp. 148–154, 2007.

[10] T. Vera, S. Kelsen, and D. E. Stec, "Kidney-specific induction of heme oxygenase-1 prevents angiotensin II hypertension," *Hypertension*, vol. 52, no. 4, pp. 660–665, 2008.

[11] G. S. Drummond and A. Kappas, "Prevention of neonatal hyperbilirubinemia by tin protoporphyrin IX, a potent competitive inhibitor of heme oxidation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 78, no. 10, pp. 6466–6470, 1981.

[12] A. Kappas, G. S. Drummond, C. S. Simionatto, and K. E. Anderson, "Control of heme oxygenase and plasma levels of bilirubin by a synthetic heme analogue, tin-protoporphyrin," *Hepatology*, vol. 4, no. 2, pp. 336–341, 1984.

[13] A. Kappas and M. D. Maines, "Tin: a potent inducer of heme oxygenase in kidney," *Science*, vol. 192, no. 4234, pp. 60–62, 1976.

[14] Y. Shan, J. Pepe, T. H. Lu, K. K. Elbirt, R. W. Lambrecht, and H. L. Bonkovsky, "Induction of the heme oxygenase-1 gene by metalloporphyrins," *Archives of Biochemistry and Biophysics*, vol. 380, no. 2, pp. 219–227, 2000.

[15] R. T. Kinobe, J. Z. Vlahakis, H. J. Vreman, J. F. Brien, W. A. Szarek, and K. Nakatsu, "Selectivity of imidazole-dioxolane compounds for in vitro inhibition of microsomal haem oxygenase isoforms," *British Journal of Pharmacology*, vol. 147, no. 3, pp. 307–315, 2006.

[16] J. Z. Vlahakis, R. T. Kinobe, R. J. Bowers, J. F. Brien, K. Nakatsu, and W. A. Szarek, "Imidazole-dioxolane compounds as isozyme-selective heme oxygenase inhibitors," *Journal of Medicinal Chemistry*, vol. 49, no. 14, pp. 4437–4441, 2006.

[17] R. T. Kinobe, Y. Ji, J. Z. Vlahakis et al., "Effectiveness of novel imidazole-dioxolane heme oxygenase inhibitors in renal proximal tubule epithelial cells," *Journal of Pharmacology and Experimental Therapeutics*, vol. 323, no. 3, pp. 763–770, 2007.

[18] E. Csongradi, T. Vera, J. M. Rimoldi, R. S. V. Gadepalli, and D. E. Stec, "In vivo inhibition of renal heme oxygenase with an imidazole-dioxolane inhibitor," *Pharmacological Research*, vol. 61, no. 6, pp. 525–530, 2010.

[19] T. Aizawa, N. Ishizaka, J. I. Taguchi et al., "Heme oxygenase-1 is upregulated in the kidney of angiotensin II-induced hypertensive rats: possible role in renoprotection," *Hypertension*, vol. 35, no. 3, pp. 800–806, 2000.

[20] P. K. Datta, J. E. Moulder, B. L. Fish, E. E. Cohen, and E. A. Lianos, "Induction of heme oxygenase 1 in radiation nephropathy: role of angiotensin II," *Radiation Research*, vol. 155, no. 5, pp. 734–739, 2001.

[21] P. Li, H. Jiang, L. Yang et al., "Angiotensin II induces carbon monoxide production in the perfused kidney: relationship to protein kinase C activation," *American Journal of Physiology*, vol. 287, no. 5, pp. F914–F920, 2004.

[22] T. Vera, S. Kelsen, L. L. Yanes, J. F. Reckelhoff, and D. E. Stec, "HO-1 induction lowers blood pressure and superoxide production in the renal medulla of angiotensin II hypertensive mice," *American Journal of Physiology*, vol. 292, no. 4, pp. R1472–R1478, 2007.

[23] N. G. Abraham, J. H. C. Lin, M. W. Dunn, and M. L. Schwartzman, "Presence of heme oxygenase and NADPH

cytochrome P-450 (c) reductase in human corneal epithelium," *Investigative Ophthalmology and Visual Science*, vol. 28, no. 9, pp. 1464–1472, 1987.

[24] K. A. Nath, M. C. Hernandez, A. J. Croatt, Z. S. Katusic, and L. A. Juncos, "Heme oxygenase activity as a determinant of the renal hemodynamic response to low-dose ANG II," *American Journal of Physiology*, vol. 299, no. 5, pp. R1183–R1191, 2010.

[25] A. W. Cowley Jr., "Role of the renal medulla in volume and arterial pressure regulation," *American Journal of Physiology*, vol. 273, no. 1, pp. R1–R15, 1997.

[26] R. J. Roman, A. W. Cowley Jr., J. Garcia-Estan, and J. H. Lombard, "Pressure-diuresis in volume-expanded rats. Cortical and medullary hemodynamics," *Hypertension*, vol. 12, no. 2, pp. 168–176, 1988.

[27] S. Quan, L. Yang, S. Shnouda et al., "Expression of human heme oxygenase-1 in the thick ascending limb attenuates angiotensin II-mediated increase in oxidative injury," *Kidney International*, vol. 65, no. 5, pp. 1628–1639, 2004.

[28] S. Kelsen, B. J. Patel, L. B. Parker et al., "Heme oxygenase attenuates angiotensin II-mediated superoxide production in cultured mouse thick ascending loop of Henle cells," *American Journal of Physiology*, vol. 295, no. 4, pp. F1158–F1165, 2008.

[29] R. Juncos and J. L. Garvin, "Superoxide enhances Na-K-2Cl cotransporter activity in the thick ascending limb," *American Journal of Physiology*, vol. 288, no. 5, pp. F982–F987, 2005.

[30] P. A. Ortiz and J. L. Garvin, "Interaction of O2- and NO in the thick ascending limb," *Hypertension*, vol. 39, no. 2, pp. 591–596, 2002.

[31] P. A. Ortiz and J. L. Garvin, "Superoxide stimulates NaCl absorption by the thick ascending limb," *American Journal of Physiology*, vol. 283, no. 5, pp. F957–F962, 2002.

[32] S. C. Young, M. V. Storm, J. S. Speed et al., "Inhibition of biliverdin reductase increases ANG II-dependent superoxide levels in cultured renal tubular epithelial cells," *American Journal of Physiology*, vol. 297, no. 5, pp. R1546–R1553, 2009.