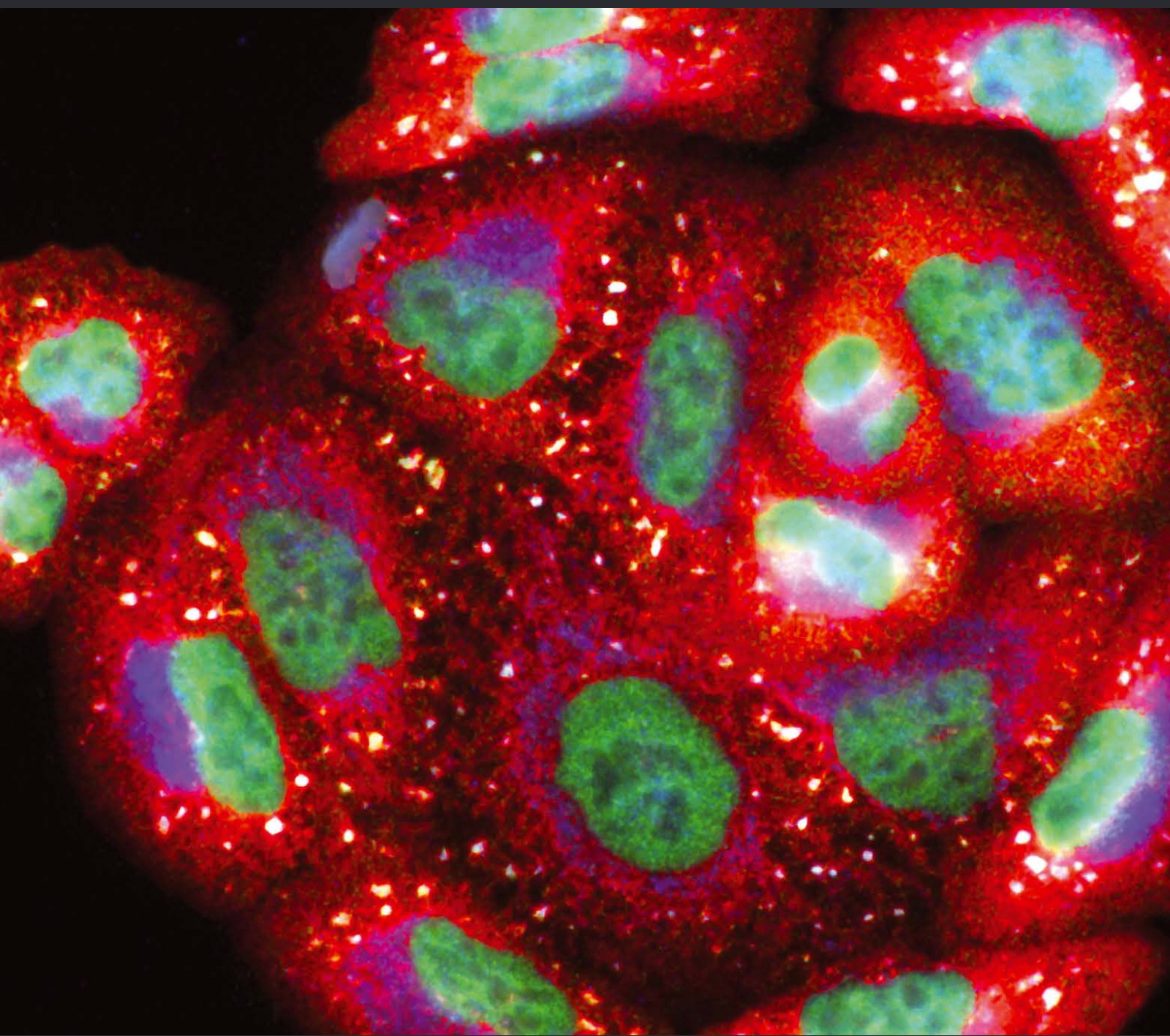
Oxidative Medicine and Cellular Longevity

Oxidative Stress in Infection and Consequent Disease

Guest Editors: Alexander V. Ivanov, Birke Bartosch, and Maria G. Isaguliants



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Editorial Board

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Editorial

Oxidative Stress in Infection and Consequent Disease

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Viral, bacterial, and parasitic infections comprise a vast group of etiological agents that cause acute or chronic diseases. According to WHO, they represent one of the major causes of human morbidity and mortality. AIDS, lower respiratory tract infections, and diarrheal diseases underlie up to 5 million deaths each year, especially in the middle- and low-income countries. Some of the infections causing chronic disease lead to the development of an array of deadly pathologies including cancer, autoimmune diseases, diabetes mellitus, and malfunctions of various organs. During the last two decades it has been clearly established that many of these infections trigger the production of reactive oxygen (ROS) and nitrogen (RNS) species. This is particularly true for infections caused by the blood-borne hepatitis viruses (B, C, and D), human immunodeficiency virus (HIV), influenza A, Epstein-Barr virus, respiratory syncytial virus, and other viruses. For acute respiratory viral infections, ROS/RNS have been implicated in lung tissue injury and epithelial barrier dysfunction which in turn increased the susceptibility to secondary infections. In case of chronic viral hepatitis, oxidative stress was shown to promote liver fibrosis, cirrhosis, and cancer, as well as metabolic dysfunction. HIV-induced oxidative stress was shown to contribute to neurodegenerative complications which are often observed in AIDS patients. Last but not least, a virus-induced oxidative burst has been recently associated with the development of the acute childhood lymphoblastic leukemia. In bacterial infections oxidative stress arises, at least in part, from altered metabolic pathways and has also been implicated in organ damage and the development of malignancies. *Helicobacter pylori*, for example, induces ROS-generating enzymes such as spermine oxidase and upregulates proinflammatory and procancerogenic redox-regulated genes like cyclooxygenase 2. Despite the overwhelming evidence of the role of oxidative stress in acute and chronic infection and the associated diseases, the impact of the majority of infectious agents on the host redox systems is not sufficiently characterized, with published data plagued by the controversies.

The current special issue aims to bring together both research papers and review articles on the mechanisms by which viral and bacterial infections trigger ROS production and induce and modulate the antioxidant defense systems and papers on the role of reactive oxygen species in viral propagation and infection-associated pathologies. We have also invited manuscripts from other fields of redox biology in order to bring new perspectives to the studies of oxidative stress in infections and consequent disease.

Contributed manuscripts emphasize the heavy impact of oxidative stress on the pathogenesis of viral infections. Two independent reviews discuss oxidative stress in chronic infection with hepatitis C virus (HCV). The review by R. Medvedev et al. scrutinizes multiple mechanisms by which HCV enhances ROS production providing at the same time an in-depth analysis of viral influence on the protective Nrf2 pathway. Special attention is paid to the redox-regulated events by which HCV triggers autophagy such as ER stress

and the unfolded protein response. A concise review article of K. Rebbani and K. Tsukiyama-Kohara focuses on ROS-sensitive mechanisms of HCV-induced hepatocarcinogenesis. In particular, this review discusses the cholesterol biosynthesis enzyme 3β-hydroxysterol Δ24-reductase (DHCR24), induced by HCV and recognized as a host marker of oxidative stress, as biomarker for the prognosis of hepatocellular carcinoma. These reviews are complemented by a research paper by O. A. Smirnova et al. on the mechanisms of induction of oxidative stress by a component of HCV replication complex nonstructural protein NS5A. Earlier NS5A was thought to induce oxidative stress by altering calcium homeostasis [1]. In contrast to this, O. A. Smirnova et al. found that NS5A contributes to ROS production by activating expression of NADPH oxidases 1 and 4 as well as cytochrome P450 2E1. NOX1 and NOX4 induction was mediated by enhanced production of transforming growth factor β1. Earlier similar results were obtained by us for HCV core protein [2]. This perfectly exemplified the capacity of the virus to induce oxidative stress by several multistep mechanisms.

One review and an experimental manuscript deal with oxidative stress in HIV infection. A review by the editors of this issue summarizes the data on the oxidative stress markers associated in HIV infection, analyzes mechanisms by which this virus triggers massive ROS production, and scrutinizes the scarce data that exist on the effect of ROS on HIV-1 replication. The review presents the current state of knowledge on the redox alterations as crucial factors of HIV-1 pathogenicity, namely, neurotoxicity, dementia, and T-cell exhaustion, as well as certain side effects of the antiretroviral therapy, in comparison to the pathologies associated with the nitrosative stress. A thorough experimental work by X. Huang et al. analyzes the status of oxidative stress in HIV/HCV coinfection delineating the contribution of HIV to HCV-induced liver damage. The study also discusses correlations between liver pathology and level of oxidative stress in HIV/HCV-coinfected patients, as well as the capacity of HIV infection to accelerate HCV-associated liver damage in HIV/HCV-coinfected individuals.

Not only RNA, but also a variety of DNA viruses are associated with the increased oxidative stress promoting DNA damage, high mutagenicity, and initiation and/or progression of neoplasia [3]. A review article by M. Mushtaq et al. summarizes current knowledge on the metabolic disturbances induced by DNA viruses, many of which are achieved through the redox-sensitive processes. This review focuses on the enhanced consumption of glucose, its conversion into pyruvate (glycolysis), and the switch to lactate formation, characteristic for cells transformed with tumor DNA viruses. Under physiological conditions, one glucose molecule is converted into two pyruvate molecules. Oxidation of pyruvate to CO_2 and O_2 results in synthesis of 38 ATP molecules. At low oxygen concentrations, pyruvate is not oxidized but instead converted to lactate; this process still produces ATP but is independent of O_2 and can occur under hypoxic conditions. Glucose is not only important for ATP production, but also used by the pentose phosphate pathway to produce nucleic acids and NADPH which in turn required to neutralize ROS. Cancer cells increase frequently glucose uptake and lactate production, which are features of the Warburg effect [4, 5]. The review by M. Mushtaq et al. summarizes evidence that shows that not only tumor DNA viruses, but also single viral proteins can enhance glucose uptake and control the tumor microenvironment, thus promoting tumor growth and metastasis.

While six papers of the special issue deal with the oxidative stress and consequent disease caused by viral infections, G. S. Krasnov et al. uncover the changes in transcriptome in the tumor tissues from patients with colorectal cancer associated with infection with *Bacteroides fragilis*. This study reveals a significant induction of a number of enzymes involved in the biosynthesis and degradation of biogenic polyamines including spermine oxidase (SMO). SMO converts spermine into spermidine with formation of H_2O_2 and acrolein as by-products [6]. Earlier studies associated this enzyme with the development of gastrointestinal tumors in patients infected with both *Bacteroides fragilis* [7] and *Helicobacter pylori* [8]. We believe that such a modulation of the polyamine metabolism is also highly relevant to viral infections. Indeed, we have recently shown similar induction of SMO by hepatitis C virus and its proteins [9]. However, the impact of changes to the polyamine metabolism on the physiopathology associated with viral infections remains to be studied.

Autoimmune arthritis can be induced experimentally; however, in human disease, both viral and bacterial infectious agents have been extensively discussed as triggers [10–12]. In this special issue, one paper deals with autoimmune arthritis, albeit induced by an experimental immunization. A. A. Andreev-Andrievskiy et al. present the evidence of promising therapeutic activity of the mitochondrially targeted antioxidant SkQ against rheumatoid arthritis experimentally induced by immunizing rats with collagen. In our view, a treatment approach based on the usage of antioxidants could be applied to prevent infection-associated pathologies and deserves experimental testing in the relevant animal models of human infections.

Finally, a very interesting paper of V. A. Mitkevich et al. discusses glutathionylation of Na,K-ATPase. The paper shows that this redox-sensitive posttranslational modification is promoted by hypoxia, an event accompanied by a severe oxidative stress. The authors provide evidence that the glutathione residues are shielding by the protein inside the protein globule. This paper, even though it does not deal with an infectious agent, may be mechanistically highly applicable to future studies in redox biology of human pathogens. To our knowledge, there is almost no published data on the redox-sensitive modifications of bacterial or viral proteins, with the exception of HIV protease.

We hope that this issue will help to deepen our understanding of the role of redox homeostasis in the pathologies associated with human infection by drawing attention to the novel aspects of redox control, such as the polyamine metabolism, glutathionylation, and novel therapies based on the use of antioxidants.

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Review Article

Oxidative Stress during HIV Infection: Mechanisms and Consequences

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It is generally acknowledged that reactive oxygen species (ROS) play crucial roles in a variety of natural processes in cells. If increased to levels which cannot be neutralized by the defense mechanisms, they damage biological molecules, alter their functions, and also act as signaling molecules thus generating a spectrum of pathologies. In this review, we summarize current data on oxidative stress markers associated with human immunodeficiency virus type-1 (HIV-1) infection, analyze mechanisms by which this virus triggers massive ROS production, and describe the status of various defense mechanisms of the infected host cell. In addition, we have scrutinized scarce data on the effect of ROS on HIV-1 replication. Finally, we present current state of knowledge on the redox alterations as crucial factors of HIV-1 pathogenicity, such as neurotoxicity and dementia, exhaustion of $CD4^+/CD8^+$ T-cells, predisposition to lung infections, and certain side effects of the antiretroviral therapy, and compare them to the pathologies associated with the nitrosative stress.

1. Introduction

Reactive oxygen species (ROS) is a general term of oxygen intermediates with high reactive capacity towards various biological molecules. They include hydroxyl radical ($HO^\bullet$), singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and other reactive species [1, 2]. ROS are produced in various cellular processes and organelles: electron leakage from the mitochondrial electron transport chain (ETC), degradation of lipids, amino acids, and biogenic polyamines, protein folding in the lumen of endoplasmic reticulum (ER), and so forth [3–7]. The most reactive type of ROS is the hydroxyl radical. It is produced from hydrogen peroxide that oxidizes divalent iron cations via the Fenton reaction

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^\bullet + HO^- \quad (1)$$

or as a result of the Haber-Weiss cycle that involves a reduction of ferric ions by superoxide anions into ferrous ions followed by the Fenton reaction:

$$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2 \quad (2)$$

Thus, the net reaction of the Haber-Weiss cycle can be described as

$$O_2^{\bullet-} + H_2O_2 \longrightarrow HO^\bullet + O_2 + HO^- \quad (3)$$

Superoxide anions have several sources in cells. First, they are generated in mitochondria. Electron transport through the ETC during oxidative phosphorylation is generally accompanied by escape of up to 1-2% of electrons that are trapped by molecular oxygen [7]. Alteration of mitochondrial bioenergetics by various factors usually gives rise to superoxide anion production. Secondly, superoxide anion is produced by a family of NADPH oxidases (NOX/DUOX), comprised of seven isoforms: NOX1–NOX5 and DUOX1-DUOX2 [6]. They transport electrons across the membranes and generate superoxide with the exception of NOX4 that produces hydrogen peroxide [8]. Activation of NOX-mediated ROS production can be achieved by various mechanisms. For example, NOX4 is controlled only at the level of transcription since this enzyme is constitutively active [6]. NOX1–NOX3 are generally induced on the transcriptional level and activated by a controlled assembly of the multisubunit complexes. Finally, several isoforms including NOX5 and DUOX1-DUOX2 possess calcium-binding domains that mediate additional level of ROS production. Third, superoxide anions are generated by cytochromes P450 (CYP) which catabolize various endogenous compounds and xenobiotics [9]. Hydrogen peroxide is mainly formed as a stoichiometric by-product in catabolic reactions and through formation of disulfide bonds during protein folding in the ER [5, 10]. Finally, reactive oxygen species can derive from the activity of xanthine oxidoreductase (XOR) [11, 12]. XOR is widely distributed throughout various organs including the liver, gut, lung, kidney, heart, and brain as well as the plasma. It is generally accepted that the enzyme is normally present *in vivo* as an NAD-dependent cytosolic dehydrogenase (XDH), incapable of ROS production. However, sulfhydryl oxidation or limited proteolysis converts the XDH into xanthine oxidase (XO) which produces superoxide and hydrogen peroxide, with the latter being the major product under physiological conditions [11]. Furthermore, both XO and XDH can oxidize NADH, with the concomitant formation of the reactive oxygen species [11, 12].

Different types of ROS are characterized by their varying ability to react with biological molecules. The most reactive ROS is the hydroxyl radical, $HO^{\bullet}$, the one-electron oxidized form of the hydroxide ion (HO^{-}) [1, 13]. It can oxidize almost any molecule in its proximity including DNA, phospholipids, and proteins [13, 14]. Oxidation results in the accumulation of 8-oxoguanine (8-oxoG) and other oxidized nucleic bases, malondialdehyde (MDA), and 4-hydroxynonenal (HNE) as typical lipid peroxidation products and in protein damage manifested in the increase of the protein carbonyl content [15]. Much less active is the superoxide anion: its reactivity is hampered by a negative charge of the species; however, its protonation generates the perhydroxyl radical ($HO_2^{\bullet}$) with a higher oxidizing potential [16]. The reaction potential of H_2O_2 is also very low; however, it is converted into the hydroxyl radical with a much higher oxidizing capacity [17]. H_2O_2 also possesses a unique (for ROS) capacity to cross biological membranes which turns it into a classical signaling molecule [17, 18].

Eukaryotic cells have developed multiple mechanisms of ROS neutralization ("scavenging") in order to protect themselves against oxidation of biological molecules. First, ROS can be neutralized directly by the low molecular weight compounds referred to as antioxidants, such as vitamins C and E and glutathione (GSH) [19], and a wide set of ROS-converting enzymes [20] including NAD(P)H:quinone oxidoreductase 1 (Nqo1) that scavenges superoxide anion [21] and superoxide dismutases (SODs) that convert $O_2^{\bullet-}$ into H_2O_2 [22]. SODs exist in three isoforms expressed in different cellular compartments: SOD1 (Cu/Zn-SOD) is mostly localized in the cytoplasm; SOD2, (MnSOD) in the mitochondrial matrix; and SOD3 (EC-SOD), at the cell surface. Neutralization of H_2O_2 is performed by multiple enzymes such as catalase (CAT), glutathione peroxidases (GPx, eight isoforms), and peroxiredoxins (Prdx, six isoforms) [23, 24]. Of these enzymes, GPx4 and 1-Cys peroxiredoxins are responsible for scavenging lipid peroxides thus protecting lipids from the oxidative damage [25–27]. Additional protection from ROS is mediated by heme oxygenase, the rate-limiting enzyme of heme catabolism which leads to the release of free iron, which in turn offers protection against oxidative stress [28]. Other antioxidant proteins include enzymes that mediate biosynthesis of glutathione and proteins that recycle oxidized glutathione, peroxiredoxins, and glutathione peroxidases (glutaredoxins and thioredoxins) [20, 29]. Noteworthily, expression of a wide set of antioxidant enzymes is controlled by NF-E2-related factor 2 (Nrf2), a transcription factor that recognizes a common short sequence, referred to as Antioxidant Response Elements (ARE), in the promoters of genes encoding ROS-converting enzymes [20]. Components of antioxidant defense systems differ in their capacity to neutralize ROS. Hydrogen peroxide is much more efficiently neutralized by peroxiredoxins and glutathione peroxidases, while classical antioxidants such as glutathione have a much lower potential [16, 30]. The actual levels of ROS are defined by the balance between the activities of ROS-generating and ROS-scavenging molecules, being different for different cellular compartments [31].

Several techniques are currently used to analyze the redox status of the cell and to determine the levels of ROS. Firstly, oxygen radicals can be detected by the electron paramagnetic resonance (EPR) using a spin-trapping technique; however, this method requires highly specialized equipment [32]. Secondly, ROS levels can be quantified indirectly using low molecular weight compounds (sensors) that are oxidized by ROS into fluorophores. They include 2′,7′-dichlorodihydrofluorescein ($DCFH_2DA$), dihydroethidium (DHE) and its mitochondrially targeted derivative MitoSOX, and boronate probes [33, 34]. Protein sensors such as HyPER or roGFP can be introduced as genes which makes them suitable for measuring ROS levels in almost any organelle [35–37]. Thirdly, the oxidative stress can be assessed indirectly by evaluating the levels of oxidative stress biomarkers, such as stable (by-)products generated under conditions of oxidative stress which enter the tissues, cells, or circulation, such as oxidized glutathione (GSSG), MDA, and HNE (for lipids) and 8-oxoG (DNA) and protein carbonyls [15, 38]. In addition, the cellular redox state can be quantified by estimating the capacity of blood/serum/tissue samples to oxidize/reduce

some standard compounds that mimic cellular targets of ROS (e.g., see [39, 40]).

Oxidative stress accompanies a wide variety of viral infections including those induced by hepatitis B (HBV) [41], C (HCV) [42], and delta (HDV) [43] viruses, herpes [44, 45], respiratory [46, 47], and other viruses. In this review, we will summarize data on the mechanisms by which HIV triggers massive ROS production in the host cell and deregulates the antioxidant defense system. We will also present current concepts on the role of HIV-induced oxidative stress in the development of HIV-associated pathologies.

2. HIV-1 Biology

The human immunodeficiency virus type-1 (HIV-1) is a lentivirus that infects and by various mechanisms kills vital cells of human immune system, such as T-helper cells, macrophages, and dendritic cells, thus causing immunodeficiency [48, 49]. The acquired immunodeficiency syndrome (AIDS) is a condition in humans in which the progressive failure of the immune system undermined by HIV-1 infection allows life-threatening opportunistic infections and cancers to thrive. Without treatment, the survival time of HIV-1 infected individuals is estimated to be 9 to 11 years. However, during the three decades since its discovery, 27 antiretroviral drugs have been approved for HIV therapy [50]. Current antiretroviral therapy (ART), based on combinations of 3-4 drugs, now allows us to efficiently suppress HIV viral load and to prolong life of HIV/AIDS patients almost to the one of the general population, at least in high-income countries [51].

HIV is a single-stranded, positive-sense, enveloped RNA virus. The genome carries nine genes (*gag*, *pol*, *env*, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*) that encode 19 proteins; the coding sequence is framed by the long terminal repeats (LTRs) [48, 49]. Three of these genes, *gag, pol,* and *env*, contain information needed to make new viral particles. Processing of *pol* gene results in formation of three enzymes: reverse transcriptase (RT), integrase, and protease. Translation of *env* gene produces glycoprotein 160 (Gp160) that further is processed to give Gp120 and Gp41. *Gag* gene ensures production of matrix (MA), capsid (CA), nucleocapsid (NC), and P6 proteins as well as spacer peptides 1 (SP1) and 2 (SP2). The six remaining genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (or *vpx* in the case of HIV-2), are regulatory genes for proteins that control the ability of HIV to infect cells, produce new copies of virus (replicate), or cause the disease [52]. Upon entry into the target cell, HIV reverse-transcribes the RNA genome into the double-stranded DNA, transports it into the cell nucleus, and integrates into the chromosomes, the activities mediated by virus-encoded enzymes reverse transcriptase and integrase, and cellular cofactors [48]. Once integrated, the virus may become latent, which allows the infected cells to avoid detection by the immune system. Alternatively, the virus may be transcribed and translated, producing new RNA genomes and viral proteins that are packaged and released from the cell as new virus particles to start the new infection cycle.

3. Oxidative Stress during HIV Infection

To date, numerous lines of evidence show that HIV infection triggers pronounced oxidative stress in both laboratory models and the context of *in vivo* infection. HIV-infected individuals exhibit enhanced ROS production in monocytes [53] and severely elevated levels of oxidized nucleic bases such as 8-oxoG and lipid peroxidation products, including MDA in plasma and alkanes in the breath output [54–63].

Compensation of the pathogenic effects of HIV-1 replication requires intact functions of ROS detoxifying enzymes. Parsons et al. showed that HIV-1 individuals with a null-allele polymorphism in *gstm1* gene, associated with a loss of function of the Phase II detoxifying enzyme glutathione S-transferase [64], exhibit lower count of CD4 T-cells, increased HIV viral load, and increased 8-oxoG in mitochondrial DNA [65]. However, HIV-infected individuals demonstrate a reduction of total antioxidant capacity [59], decreased GSH/GSSG ratio in epithelial lung fluid [3], and decreased GSH content in blood [56, 58, 61, 63, 66–69]. Marked elevation of ROS levels was also detected in the HIV-infected cell cultures [70, 71]. The most profound decrease of the total antioxidant capacity was detected in subsets of $CD4^+$ and $CD8^+$ T-lymphocytes [66], with low CD4 T-cell counts correlating with more severe oxidative stress [59, 60, 62, 72]. Corroborating these observations, the number of $CD4^+$ cells positively correlates with the total levels of ROS scavengers such as glutathione [56]. Noteworthily, these changes are more pronounced in treatment-naive patients than in patients under ART [58, 61] since ART restores the numbers of $CD4^+$ T-cells but at the same time augments the imbalance of the redox status [73]. In HIV/HCV-coinfected patients, the levels of oxidative stress markers are generally higher than in individuals with HIV monoinfection, as indicated by MDA and GSSG plasma levels [74, 75].

Elevated levels of the oxidative stress markers are also detected in other tissues and body fluids. Brain tissues (brain frontal cortex collected from autopsia) of HIV-infected individuals are characterized by the increased levels of 8-oxoG in the nuclear DNA [76] and increased HNE levels [69]. Elevated levels of superoxide radical and HNE are also detected in the cerebrospinal fluid [69, 76]. Interestingly, similar effects are observed in the NL4-3Δ transgenic rat model expressing HIV proteome devoid of the Gag-Pol polypeptide. In these animals, high levels of superoxide anion can be indirectly detected by electron spin resonance spectroscopy/ESR using CMH probe in the aortas [77] and by fluorescent microscopy with DHE dye in the lungs [78]. Altogether, this indicates that HIV-1 actively interferes with the development of oxidative stress response.

4. Mechanisms of ROS Production during HIV Infection

HIV-1 induces oxidative stress by deregulation of oxidative stress pathways with escalation of ROS production and by inducing mitochondrial dysfunction [70, 71]. The enhancement of ROS production is mediated by the envelope protein

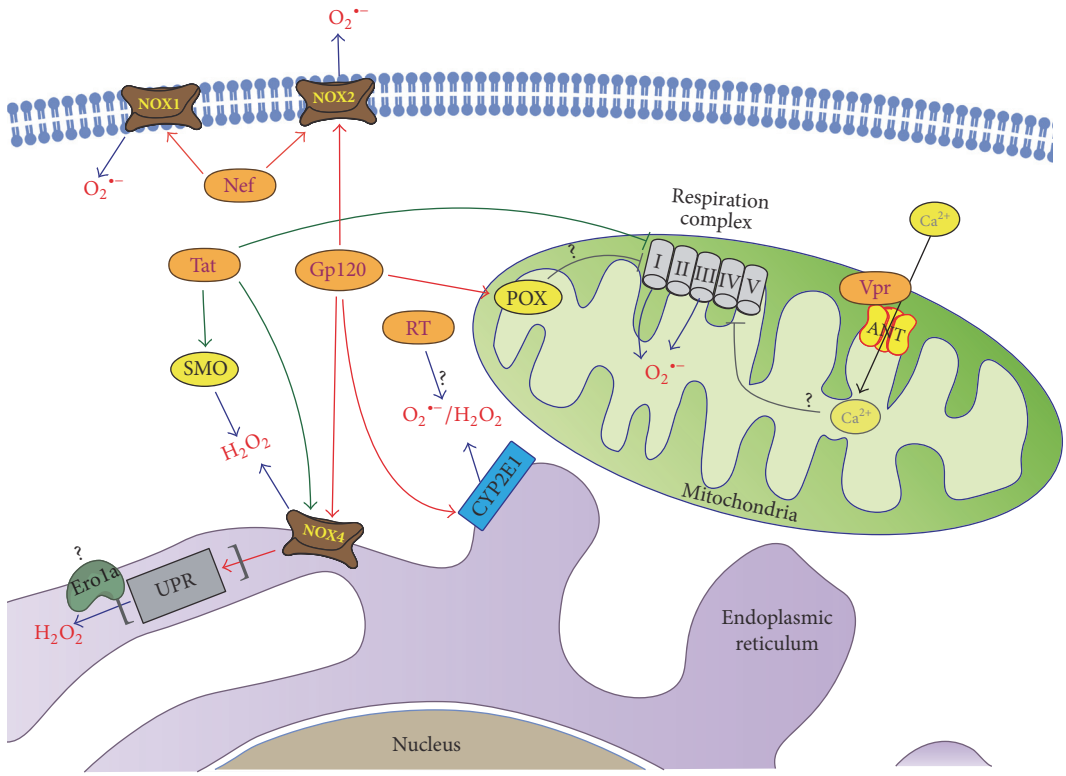

Figure 1: Cellular sources of reactive oxygen species in HIV infection. Several HIV proteins enhance ROS production by different mechanisms. These viral proteins include amongst others the envelope protein Gp120, Tat, Nef, Vpr, and RT. The envelope protein Gp120 enhances ROS production via upregulation of cytochrome P450 2E1 (CYP2E1), proline oxidase (POX), and activation of NOX2 and NOX4. Tat protein induces spermine oxidase (SMO), an enzyme involved in catabolism of biogenic polyamines, and may impact mitochondrial function. Tat also activates NADPH (but not xanthine) oxidases and in particular Nox4, which in turn may induce other peroxide-generating enzymes involved in unfolded protein response (UPR) such as ER oxidoreductin 1α (Ero1α). Vpr protein interacts with adenine nucleotide translocator (ANT, a component of mitochondrial permeability transition pore (PTP)) that is implicated in Ca^{2+} influx into mitochondria. Nef protein can directly interact with the p22phox subunit of NADPH oxidases without affecting NOX expression. Finally, RT triggers ROS production by yet undiscovered mechanism(s).

Gp120 [79–85], Tat [83, 84, 86–88], Nef [89–91], Vpr [71, 92, 93], and reverse transcriptase (RT) [94].

The envelope protein Gp120 enhances ROS production in various cell lines of lymphoid origin [82], in endothelial brain cells [83], microglia cells, neurons, and astrocytes [79, 80]. In astrocytes, it enhances ROS production by several parallel mechanisms: via cytochrome P450 2E1 (CYP2E1), NOX2 and NOX4, and the Fenton-Weiss-Haber reaction (Figure 1) [79, 95]. The effect of Gp120 on CYP2E1 is mediated through upregulation of CYP2E1 expression. Interestingly, however, EPR analysis of the HIV-1 infected monocyte-derived macrophages revealed no increase in the production of either hydroxyl or other oxygen radicals [96]. In neuroblastoma cells, Gp120 was shown to induce proline oxidase (POX) that produces pyrroline-5-carboxylate with a concomitant generation of ROS (Figure 1) [85].

The regulatory Tat protein triggers ROS production via several independent mechanisms (Figure 1). The first involves the NADPH (but not xanthine) oxidases [86]. The second implies the induction of spermine oxidase (SMO), an enzyme involved in the catabolism of biogenic polyamines [88, 97]. The third relies on mitochondrial dysfunction [98] but was questioned in a later study [86]. A detailed analysis of the levels of ROS in different subcellular compartments of the HIV-1 infected cells revealed no significant increase in the content of H_2O_2in either cytoplasm or mitochondria but a strong increase in the ER [99]. ER is the primary "residence" for NOX4 that produces hydrogen peroxide [8, 100]. An increase in H_2O_2 levels in ER of HIV-1 infected cells was demonstrated using a genetically encoded ratiometric HyPER sensor [99]. Moreover, in these cells, NOX4 mediated the induction of unfolded protein response (UPR). In concordance with these data, an elegant study demonstrated that the suppression of NOX4 by RNAi in Tat-expressing cells results in a significant reduction of H_2O_2 levels in the ER [99]. The results generated using HyPER sensor could be questioned. Such sensors have been used in a number of studies (such as [4, 99]) that demonstrated that the dynamic range of its signal is small [101] to negligible [102], with changes in the $HyPER_{ER}$ fluorescence reflecting not so much the changes in peroxide levels but rather the influence of other factors such as proline disulphide isomerases (PDI) [103]. Also, one cannot rule out that NOX4 contributes to the induction of oxidative stress indirectly, through the induction

of other peroxide-generating enzymes involved in UPR. If so, one could propose a component of the protein-folding machinery which could be involved, namely, ER oxidoreductin 1α (Ero1α) [104] which is upregulated within the PERK branch of UPR (Figure 1) [105]. Intriguingly, the elevation of hydrogen peroxide levels in the ER contradicts the existing concept on the efficient neutralization of hydrogen peroxide in the ER by scavenging enzymes, including peroxiredoxin 4 [106] and glutathione peroxidases 7/8 [101, 107]. This may not be widely accepted, which leaves open the actual mechanism of the Tat-mediated oxidative stress in the ER.

HIV-1 Nef protein has shown a prooxidant activity in microglial cells and in neutrophils [89–91]. The activity is related to the ability of Nef to interact with Vav protein (Figure 1) [89]. Vav is a nucleotide exchange factor for Rac1 that is recruited to the NOX1–NOX3 complexes [6], with the $p22^{phox}$ subunit of NADPH oxidases, but without affecting NOX expression [91]. These interactions are in perfect concordance with the absence of changes in the expression of NOX1, NOX2, and NOX4 in NL4-3Δ *gag-pol* transgenic rats compared to the wild-type animals [77].

Viral protein R (Vpr) is another important regulator of ROS production [108]. In yeast, Vpr expression induces an oxidative stress leading first to the decreased levels of superoxide anion and hydroxyl radical as well as glutathione and significantly decreased activities of catalase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, and glutathione S-transferase and later on to elevated levels of superoxide anion and peroxides and increased activities of most of antioxidant enzymes [108]. It was shown that Vpr triggers oxidative stress by causing mitochondrial dysfunction [92, 109, 110] and ROS production in mitochondria (Figure 1) [71]. Mitochondrial dysfunction is promoted by binding of Vpr to the adenine nucleotide translocase (ANT) [110], a protein that forms an inner channel of the mitochondria permeability transition pore (PTP) [110]. This indicates the propensity of Vpr to unbalance the redox state of the cells contributing to the HIV-1 pathology.

Mitochondrial dysfunction is a general mechanism of ROS production common for most viral infections [111–113]. NADPH oxidases and CYP2E1 serve as the major sources of ROS in infections with human hepatitis C, influenza, and respiratory syncytial viruses [114–121]. The overview of the field demonstrates that sources of ROS operational in HIV-1 infection follow similar trends.

5. HIV and Antioxidant Defense Pathways

The effect of HIV-1/HIV-1 proteins on the cellular antioxidant defense system is debatable. Several groups reported a decrease in SOD (SOD3 in particular), CAT, and GPx activities in plasma of the HIV-infected individuals [61, 63, 75, 122]. The data on Gp120 is controversial; it was shown to either enhance [123] or not affect the expression of *sod2* gene [82]. However, the individual Tat protein causes an opposite effect: it suppresses the expression of MnSOD through inhibition of binding of Sp1 and Sp3 transcription factors to *sod2* gene promoter and binding to its mRNA [124, 125]. In addition, studies done in HIV-1 NL4-3Δ transgenic rats demonstrate a decrease in the Cu/Zn-SOD expression, whereas the expression of MnSOD remains unaltered [77].

Overall, both Gp120 and Tat suppress expression of the glutathione synthesizing and metabolizing enzymes. Both downregulate the expression of glutathione synthase (GSS), glutathione reductase (GR), and GPx, leading to a decrease in the total glutathione content and an increase of the GSSG/GSH ratio [83, 84, 123, 126]. Gp120 also shows a strong ROS-dependent inhibitory effect on the expression of glutamine synthase (GS) [127]. Interestingly, Tat exhibits a stronger inhibitory effect on glutathione than Gp120 [83]. In addition to the inhibition of GSH biosynthesis pathways, Tat induces the expression of glutathione peroxidase isoform GPx4 [126], which scavenges lipid peroxides. At the same time, Tat has no effect on the expression of thioredoxin reductase [126], an enzyme that reduces thioredoxin, which in turn reduces glutathione peroxidases and peroxiredoxins [29]. Vpr is yet another virus protein that triggers a decrease in the GSH levels [128]. The latter is caused by the suppression of ATP biosynthesis in mitochondria [128] (two molecules of ATP are required for biosynthesis of every glutathione moiety [129]).

A majority of glutathione metabolizing genes are controlled by the Nrf2 transcription factor [20]. *In vivo*, HIV-1 appears to suppress the Nrf2/ARE pathway. Indeed, brain cortex tissues of HIV-1 infected individuals demonstrate the decreased levels of heme oxygenase 1 [130]. This effect is not mediated by Tat, Nef, or Vpr proteins but is apparently due to the replication of the viral genome. HO-1 protein expression correlates negatively with HIV replication levels. *In vitro* analysis of HO-1 expression in HIV-infected macrophages, a primary central nervous system (CNS) HIV reservoir along with microglia, demonstrated a decrease in HO-1 as HIV replication increased; HO-1 repression was mediated by high levels of IFN-γ concomitant with virus replication in the CNS [131]. While HIV replication seems to (indirectly) suppress the Nrf2/ARE pathway, the effects of the individual viral proteins are the opposite. HIV reverse transcriptase activates Nrf2 and upregulates the transcription of both HO-1 and Nqo1, at least in the cell culture system [94]. An ability to activate the Nrf2/ARE pathway was recently reported also for Tat [88]. It is mediated through the induction of spermine oxidase and concomitant production of hydrogen peroxide. Gp120 induces yet another classical Nrf2-dependent gene, multidrug resistant protein 1 (Mrp1) [81]. Such discrepancy between the factual data from *in vitro* studies and the status of Nrf2/ARE signaling during HIV infection has been observed also for other viruses such as HCV [132–135]. The actual (also long-term) effects of HIV-1 on the Nrf2/ARE pathway and their outcomes for the pathogenesis of HIV-1 infection remain to be elucidated.

6. ROS in HIV's Life Cycle

Hypoxia induces oxidative stress via an overgeneration of ROS [136]. A crucial role in the mammalian response to

oxygen levels is played by the transcription factor Hypoxia-Inducible Factor-1 (HIF-1). Increased expression of HIF-1α contributes to the mitochondrial activity and ROS formation during the hypoxia [137]. HIV-1 protein Vpr induces HIF-1 resulting in the ROS-dependent activation of HIV LTR [71]. HIV-1 LTR is activated even by low concentrations of H_2O_2 [138] (whereas antioxidants inhibit viral transcription [139]). Further enhancement of the transcription is triggered by proinflammatory cytokines, including TNF-α [140, 141] induced through the redox-dependent NF-κB pathway [142]. Additional influence of elevated ROS levels on HIV life cycle is achieved through the redox-sensitive transcription factors AP-1 and p53 [143]. Interestingly, transcription activation by exogenous hydrogen peroxide takes place only after the prolonged treatment, allowing us to hypothesize that virus-induced oxidative stress can play a crucial role in activation of the latent viral infection [138]. Supporting this, activation of the latent infection was triggered by modest changes in the cell redox potential (25 mV) [144]. Such changes can be induced either directly by HIV proteins or indirectly through the induction of proinflammatory cytokines such as TNF-α [145].

Oxidative stress may be also beneficial for the late stages of the HIV life cycle, since glutathione treatment of chronically infected cells leads to the abrogation of virion budding and release [146] preventing the infection of new T-cells [147]. The addition of GSH or GSH analogues is able to block late steps of viral replication [148], possibly by inhibiting the proper folding of glycosylated surface viral proteins in the ER, as was demonstrated for influenza virus [149]. The inhibitory effects of antioxidant treatment could also be attributed to the ability of ROS to induce CXCR4 receptor [150] as well as the glucose transporter Glut1 [151]. Enhanced expression of Glut1 was observed in both the infected cell cultures [152] and the neuronal tissues of the patients [153]. It leads to the elevation of glucose influx into the lymphocytes, monocytes, and epithelial cells. Enhanced glucose flux is known to promote infection with oncogenic viruses [154, 155]. In case of HIV-1, it also leads to augmented ROS production and enhanced infection of target cells [147, 156, 157]. However, opposite data were also reported: overexpression of peroxide-scavenging enzyme, GPx1, enhances production of HIV virions, whereas treatment of such cells with buthionine sulfoximine (BSO) that inhibits glutathione biosynthesis inhibits such increase [158].

7. ROS in HIV-1 Related Pathologies

HIV-induced oxidative stress plays an important role in the development of a wide spectrum of virus-associated pathologies. Among them are neurotoxicity and dementia and immune imbalance with the exhaustion of the pool of CD4 T-lymphocytes, as well as lung and cardiovascular disorders.

7.1. CNS Toxicity. Neurotoxicity and dementia are believed to be the direct consequences of HIV-1 infection: a majority of the cases with these neurological symptoms below 60 years of age are AIDS patients. HIV-1 affects the microglial cells; progressive infection leads to damage to astrocytes and neurons [159]. Levels of oxidative stress markers such as mitochondrial 8-oxoG in serum inversely correlate with the volume of the grey substance from selected brain areas (hippocampus, pallidum, etc.) [160]. Moreover, an increase in 8-oxoG in the nuclear DNA is accompanied by a decrease in the mitochondrial DNA content observed in the frontal cortex of the patients, altogether pointing at a direct link between ROS and neurological pathologies in AIDS patients [160]. The accumulated data points at the neurotoxicity being triggered by Gp120, Tat, and Vpr proteins which can penetrate the blood-brain barrier (BBB) (Figure 2) [161, 162]. Penetration is likely due to the disruption of BBB through several redox-regulated processes, including the induction of matrix metalloproteinases (MMP) 2 and 9 that target BBB tight junction receptors ZO-1, laminin, claudin 5, and occludin ([84, 163, 164], see also a comprehensive review by Toborek et al. [165]).

Gp120, Tat, and Vpr proteins contribute to the CNS pathology by both direct and indirect mechanisms (Figure 2). The direct mechanism involves induction of ROS production, which leads to the exhaustion of the antioxidant defense system and decreased cell viability [79, 98, 128, 166, 167]. Elevated levels of ROS result in the enhanced oxidation of DNA nucleic bases in both the nucleus and mitochondria, while their removal and DNA reparation are inhibited through suppression of DNA glycosylase 1 (enzyme the function of which is the removal of 8-oxoG; OGG1) [76]. This scenario leads to DNA instability, particularly to the deletion of the D-loop in mitochondrial DNA. Significantly, contribution to neurotoxicity of Gp120 and Tat is made by an increased lipid peroxidation and accumulation of ceramide [69].

An indirect promotion of CNS pathology is believed to be mediated by the enhanced production of the inflammatory cytokines and chemokines in astrocytes and microglia [159]. Gp120 and Vpr induce TNF-α, IL-6, IL-8, and MCP-1 in the ROS-dependent fashion (Figure 2) [80, 87, 167, 168]. In addition, Gp120 stimulates A-type transient outward K^+ currents that contribute to the cell death [169]. Notably, this effect is also ROS-dependent [80]. An additional contribution to the pathogenic effects could come from the induction of spermine oxidase, an enzyme that mediates one of the two alternative pathways of polyamine metabolism [5]. It catalyses a reaction that yields H_2O_2 and acrolein as stoichiometric by-products. The latter compound is implicated in the brain pathology during ischemia-reperfusion [170, 171]. The induction of SMO may therefore represent an important mechanism of the HIV-induced brain damage. Finally, recent data of Pandhare et al. revealed that Gp120-mediated induction of proline oxidase leads to autophagy that at least partially alleviates neurotoxicity [85].

Interestingly, certain regions of AIDS patient brain are characterized by an increased expression of the opioid receptors [172]. In line with this, drugs such as morphine and amphetamine can per se trigger ROS production and dysregulate the antioxidant defense system, augmenting the pathogenic properties of Gp120 (Figure 2) [79, 123]. This may

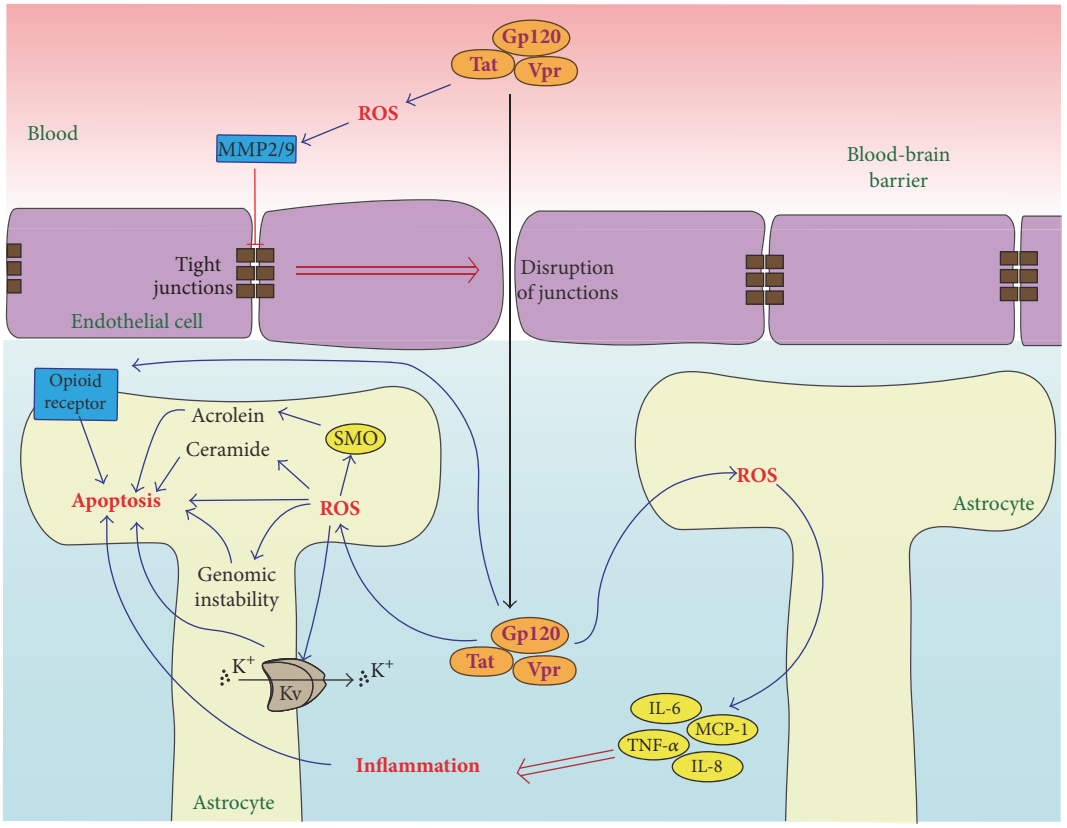

Figure 2: Mechanisms of HIV neurotoxicity. Enhanced ROS production, triggered by gp120, Tat, and Vpr proteins that circulate in the blood, results in alteration of blood-brain barrier (BBB) through matrix metalloproteinase 2/9- (MMP2/9-) mediated disruption of tight junction receptors ZO-1, laminin, claudin 5, and occludin. Gp120, Tat, and Vpr proteins activate a consequence of proapoptotic events. They include (i) oxidation of DNA and consequent genomic and mitochondrial DNA instability, (ii) increased lipid peroxidation and accumulation of ceramide that aggravates toxicity, (iii) induction of spermine oxidase (SMO) augmenting oxidative stress and producing toxic acrolein, (iv) stimulation of A-type transient outward K^+ currents by Kv channels, and (v) induction of proinflammatory cytokines. In addition, it upregulates expression of opioid receptors that contribute to neurotoxicity in HIV-infected drug addicts.

account for a more severe progression of the disease in the intravenous drug users.

7.2. Redox Associated Cardiovascular and Lung Pathologies. HIV-1 infection is accompanied by an increased risk of various cardiovascular diseases including arterial hypertension [173], atherosclerosis [174, 175], injury to coronary arteries [176], vasculitis [177], pericarditis, and myocarditis [173]. HIV-associated lung pathologies include increased susceptibility to infections, emphysema, and lung cancer [178, 179]. Their development is believed to be promoted by virus-induced oxidative stress. Oxidative stress in the lung leads to a decreased expression of the tight junction receptors, disrupting the epithelium and rendering lungs more susceptible to the microbes [180]. Moreover, treatment with lipopolysaccharide aggravates the redox imbalance in HIV-infected cells [78]. It may be speculated that these redox perturbations can trigger the inflammatory response, resulting in the tissue damage, as well as causing the genomic instability.

7.3. Effects of the Oxidative Stress on the Immune System. Very recent vivid example of the effects of oxidative stress on retroviral infection was provided by the study of Brundu et al. in a murine model [181]. Infection with the murine leukemia virus LP-BM5 causes murine AIDS, a disease characterized by many dysfunctions of the immunocompetent cells. Mice infected with LP-BM5 murine leukemia have a marked redox imbalance reflected by GSH and/or cysteine depletion in multiple immune organs/tissues. Significant decrease in cysteine and GSH levels was measured also in pancreas and in the brain, respectively [181]. Mice demonstrated a predominance of T-helper 2 (Th2) responses manifested by the expression of Th2 cytokines. Their peritoneal macrophages expressed the genetic markers of the alternative M2 macrophage polarization as Fizz1, Ym1, and Arginase 1 [181]. Conversely, macrophages capable of expressing iNOS (a marker of classical activation of macrophages) produced predominantly T-helper 1 (Th1) cytokines [181]. Restoration of the GSH/cysteine levels in the infected mouse organs (done with a N-acetyl-cysteine supplier) reduced the expression of M2 macrophage markers and increased the production of

IFN-γ, while decreasing the production of Th2-cytokines as IL-4 and IL-5 [181]. Interestingly, this is not the first report of the association between the Th2 polarization and alteration of the redox status by retroviral infection and/or retroviral proteins. We have earlier shown that HIV-1 reverse transcriptase induces potent oxidative stress and, when expressed in mice as DNA immunogen, induces potent strongly Th2-polarized type of specific immune response [94]. Thus, HIV-1 infection and even expression of HIV-1 antigens induce an immune imbalance marked by M2-shift of the macrophage response and Th2-shift of the T-cell profiles which together promote the continuation of viral replication.

A separate set of immune abnormalities in HIV-1 infection is linked to the abnormalities in the tryptophan metabolism. In HIV-1 infected individuals, these abnormalities correlate with the enhanced oxidative kynurenine pathway of tryptophan catabolism [182, 183]. This pathway generates quinolinic acid, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid, all of which are known to have the ability to generate free radicals [184]. Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme involved in the first step of tryptophan catabolism [185]. Increased IDO expression occurs during human [186] and simian [187] retroviral infections. The data on the murine LP-BM5 immunodeficiency-causing retroviral infection is contradictory [188, 189]. In HIV-1 infection, increased IDO mRNA correlates with increased viral loads, while ART decreases IDO expression, which may be anticipated as a proof of the direct correlation between IDO and HIV virus propagation [190].

Catabolism of tryptophan by IDO leads to the reduction in tryptophan levels [191]. Th1 cell clones are more sensitive to changes in tryptophan levels than Th2 cell clones, resulting in a selective immunosuppression with the shift of the immune response towards the Th2-type [192]. Besides, IDO activity results in the increased levels of toxic downstream metabolites and generation of free radicals which contribute to Th1-cell suppression [191]. Furthermore, IDO activity causes even more imbalance in the T-cell subsets by increasing the proportion of T-regulatory [193] and decreasing the proportion of T-helper 17 cells [194]. In chronically infected hosts, the dysregulated activation/alterations in the immune regulatory mechanisms involving IDO lead to a compromised antiviral response and enhancement of viral replication [195–197]. In short, chronic IDO activation leads to the immune impairment, whereas IDO inhibition represses viral replication. Thus, the effects of IDO on the viral replication may in fact be indirect, being modulated by the disturbances in the virus-specific immune response.

These series of studies demonstrate that longitudinal (chronic) oxidative stress has detrimental consequences to the HIV-1 specific immune response, impairing the capacity of the body to control viral replication. On the contrary, suppression of the chronic oxidative stress with restoration of the antioxidant levels can reestablish the disturbed Th1/Th2 balance and open a possibility to control retroviral infection.

7.4. T-Cell Exhaustion. ROS levels correlate inversely with the $CD4^+$ cell counts [59, 60, 62, 72, 198]. This may relate to a decrease in the reduced glutathione pool and the exhaustion of ROS-scavenging systems of the host cells [68, 70, 199]. It could also be due to the accumulation of DNA damage in these cells due to both increased production of ROS and the suppression of the respective DNA repair enzymes [62]. The molecular interrelations between HIV-induced oxidative stress and $CD4^+/CD8^+$ cell exhaustion remain to be investigated.

7.5. Pathological Consequences of the Nitrosative Stress. HIV is capable of infiltrating the brain and infecting brain cells. In the years following HIV infection, patients show signs of various levels of neurocognitive problems termed HIV-associated neurocognitive disorders (HAND) which afflict about half of HIV-infected patients. In Section 7.1, we described multiple links between neurological pathologies in AIDS patients and ROS. It is important to note that the latter are attributed not only to the oxidative but also to nitrosative stress and overproduction of nitrosative species during neuroinflammation [200]. Both processes occur due to the early direct and indirect effects of the viral proteins and through the late effects on mitochondrial integrity during apoptosis. There is clear experimental and clinical evidence linking the CNS symptoms of HIV with the effects of reactive nitrogen species (RNS), specifically nitric oxide (NO).

Mammalian cells generate NO as a by-product of NO synthase (NOS) activity. Neurons express neuronal NOS (nNOS), a constitutive isoform that synthesizes moderate amounts of NO; glial cells express inducible NOS (iNOS), which generates major NO amounts [201]. The nitrosative species are involved in the posttranslational modification of the brain proteome. NO is required for regular neuronal function, is produced by neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) nitric oxide synthases, and is an important neurotransmitter in the brain.

At the same time, NO is the main mediator of mitochondrial dysfunction associated with HIV central nervous system symptoms, with an increased production of NO related to HIV-associated dementia. Nitrosative stress in microglia and astrocytes can be promoted by the individual viral proteins, such as Tat [202, 203]. Another viral protein Gp41 (its N-terminus) induces iNOS protein activity [204]. HIV-1 Gp120 is also involved in the induction of iNOS leading to the nitrosative stress [205]. Recent study by Mangino et al. in the murine model suggests a potential role in the promotion of the neuronal injury of the extracellular Nef which upregulates the expression of iNOS and production of NO [206].

The data on the effects of RNS outside of the brain is less "homogenous." The overproduction of NO and the reduction of mitochondrial transmembrane potential correlate with the level of apoptosis in PBMCs of HIV-1 patient [200, 207]. This may be explained by the inhibitory effects of NO on the electron transport chain in the mitochondria as well as by the amino acid modifications. Amino acid modifications ascribed to NO are associated with the S-nitrosylation of cysteine and nitration of tyrosine and tryptophan (resulting in 6-nitro tryptophan or nitrohydroxytryptophan). The latter may be, at least in part, responsible for the abnormalities in the

tryptophan pathway in HIV-1 infected individuals with neurological or psychiatric complications [182]. S-Nitrosylation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) has been demonstrated both in the brains of HIV-1 patients with HAND and in the HIV-Gp120 transgenic mouse model, leading to decreased Akt activity [208].

Another RNS-centered hypothesis for the mechanism of neuronal damage following HIV infection involves the downstream effects of nitrosative radicals produced during the immune response [209]. Proinflammatory factors (as iNOS) are released in the astrocytes by the HIV-infected macrophages [210, 211]. The severity of HIV-related dementia is correlated with the levels of iNOS expression [212]. This confirms a link between nitrosative stress and the neuroinflammatory environment in the HIV-1 infected brain [212–214].

HIV-1-infected children with high viral load exhibited higher NO blood levels than those with viral load below this threshold [215]. This and other studies in AIDS patients point at the involvement of NO in the apoptosis and functional impairment of the lymphocytes [215–217]. Pathophysiological significance of these findings was demonstrated by showing an enhanced effect of NO on HIV-1 replication *in vitro* [215]. This study has shown that the addition of NO donors together with TNF-alpha to mitogen-activated HIV-1-infected human peripheral blood mononuclear cell (PBMC) cultures produces a significant increase in viral replication, whereas the addition of iNOS specific inhibitors suppresses replication [215]. Altogether, these results suggest that NO promotes HIV-1 replication, especially in proinflammatory settings [215], which lines up with similar effects of ROS (as depicted in Section 6).

However, NO donor compounds present in the human circulatory system, such as S-nitrosothiols (RSNOs), can inhibit HIV-1 replication in acutely infected human PBMCs demonstrating an additive inhibitory effect on HIV-1 replication with 3′-azidothymidine (AZT) [218]. One of the explanatory mechanisms might be the inhibition of HIV-1 protease subjected to S-nitrosation [219, 220]. Thus, in acute HIV-1 infection, RNS may inhibit viral replication. Indeed, a study done with the fluorescent probes with an enhanced sensitivity to NO demonstrated that low NO and iNOS levels in PBMC from HIV-infected patients correlate with enhanced viral replication [221]. Interestingly, HIV-1 transgenic rats are also characterized by low NO-hemoglobin, serum nitrite, serum S-nitrosothiols, and the aortic tissue NO levels [77]. The latter indicates that the decreased levels of NO and its downstream products are linked to the direct effects of the viral proteins [77]. Their propensity to downregulate levels of RNS may create a microenvironment favouring (acute) viral infection. These considerations are in line with findings that HIV can be targeted by the compounds that affect oxidative status of the central and transitional memory T-cells: the major cellular reservoirs for HIV [222] (see Section 8 for an overview).

This set of somewhat contradictory data indicates that, in HIV-1 infection, the predominant tissue exposed to the effects of RNS is apparently the brain, while the effects of RNS on other tissues and organs of HIV-1 infected individuals may be positive and/or negative depending on RNS levels and duration of exposure.

8. Oxidative Stress and Antiretroviral Therapy

One of the milestone findings in the redox biology of HIV-1 was the induction of oxidative stress during antiretroviral therapy (ART). To date, numerous reports show that nucleoside and nonnucleoside RT inhibitors, as well as inhibitors of the viral protease, trigger massive ROS production in various cell types (e.g., [223–229]). Series of studies reported an increase in oxidative stress additional to the persistent redox imbalance associated with HIV-1 infection manifested by an increase in oxidants and a decrease in antioxidant serum levels [73, 230, 231]. Specifically, a study done in 84 HIV-infected patients during a 6-month period of ART demonstrated a significant increase in serum peroxidation potential, total hydroperoxide, MDA, and advanced oxidation protein product levels as well as a decrease in glutathione level, compared to their levels before the treatment and to healthy controls [232]. Ngondi et al. as well registered an aggravation of the oxidative stress by certain ART regimens in the form of a significant decrease in the levels of GSH (sulfhydryl group) [233]. Again, in patients receiving nonnucleoside reverse-transcriptase inhibitors, peroxide concentrations were significantly lower than in those treated with protease inhibitors [185]. This could be attributed to an enhancement in GSH utilization or/and to the limited intracellular reduction of its oxidized form [234]. It is generally acknowledged that the components of ART may contribute to the development of cardiovascular diseases and CNS pathologies. Some of the antiretroviral drugs, such as 2′,3′-dideoxycytidine (ddC), can penetrate the BBB and trigger oxidative stress also in the brain [235]. Experiments in the ART-exposed cell lines and laboratory animals demonstrated that the enhanced production of the oxidized metabolites occurs through the mitochondrial interference ([225]; reviewed in [73]). Mitochondrial dysfunction under ART arises from the altered replication of mitochondrial DNA and inhibited oxidative phosphorylation [236]. Some of the abovementioned dysfunctions correlate with the duration of antiretroviral therapy [237, 238]. The exact impact of oxidative stress on the efficacy of ART and HIV-1/AIDS progression and the molecular mechanisms of the redox imbalance in ART-treated HIV-infected individuals are still obscure and require further comprehensive studies.

Although ART is able to clear viremia and improve the immunological condition of HIV-infected individuals for prolonged time, the virus rebounds to levels comparable to those observed before treatment initiation shortly after treatment withdrawal due to intactness of the major cellular reservoirs for HIV, central and transitional memory T-cells (T_{CM} and T_{TM}, resp.) which harbour the transcriptionally silent form of viral DNA not affected by classical antiretroviral drug regimens. Interestingly, novel oxidative stress-based therapies are arising that target these major cellular HIV reservoirs that are inaccessible to classical ART. A candidate anti-HIV reservoir compound dubbed auranofin (AF) is a prooxidant gold-based drug that inhibits thioredoxin

reductases thus affecting cellular/protein redox status [239]. Auranofin was shown to exert a selective "antimemory" effect by exploiting the baseline oxidative status of lymphocytes [222]. A study by Chirullo et al. [240] explored the molecular bases of the effects of auranofin. T_{CM} and T_{TM} lymphocytes were shown to have lower baseline antioxidant defenses as compared with their naive counterparts. AF was able to exert a prodifferentiating and proapoptotic effect preferentially in these memory subsets. Namely, AF induced redox-sensitive cell death pathways initiated by an early activation of the p38 mitogen-activated protein kinase (p38 MAPK) followed by the mitochondrial depolarization and finalized by the burst in intracellular peroxides [240]. AF-induced apoptosis was inhibited by pyruvate, a well-known peroxide scavenger. Proapoptotic and prodifferentiating effects involved different pathways. Similar effect of AF was described for simian immunodeficiency virus (SIV) in monkey model [241, 242]. Additional effect on T-lymphocyte can be achieved by combining AF with drugs that inhibit glutathione biosynthesis and lower its level such as buthionine sulfoximine (BSO) [243]. Using a combination of AF, BSO, and standard ART drugs, Shytaj et al. achieved complete clearance of SIV viremia in macaques with a 100% AIDS-free survival for at least 2 years after discontinuation of the therapy [242, 244]. This data indicates that AF and other drugs inducing redox-sensitive cell death pathways can be recruited to restrict viral reservoirs *in vivo*, limit the "stem-cell-ness" of the T_{CM} and T_{TM} pools, and turn these cells into the short-lived lymphocytes [240].

9. Conclusions and Future Perspectives

In this review, we summarized current knowledge on the fact that HIV infection leads to a pronounced oxidative stress, described the mechanisms by which the virus triggers ROS production, and discussed the impact of HIV on antioxidant defense systems. In addition, we presented an analysis of HIV-driven oxidative stress on the associated pathology. All these data clearly show that reactive oxygen species underlie a wide spectrum of events in infected cells and tissues. At the same time, there are still notable gaps in the field that might become targets for future studies. As such, we can propose the following subjects. First, many of the multiple sources of ROS that are activated by HIV may undergo common regulation and, hence, common prohibitive or stimulative treatment. Second, the current data on the status of antioxidant defense systems are rather contradictory, and many efforts are still required to understand the actual effect of the virus in acute versus chronic infection, not only in *in vitro* and animal model systems. Third, HIV-induced oxidative stress might impact susceptibility towards other viral infections, and this question has not yet been properly addressed. Fourth, virus-triggered ROS production is a strong modulator of the immune system, a property which needs to be controlled and that can be targeted for immune suppression of viral replication. Continuation of these studies would contribute to the development of efficient antiretroviral treatments and HIV vaccines.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

The Dysregulation of Polyamine Metabolism in Colorectal Cancer Is Associated with Overexpression of c-Myc and C/EBPβ rather than Enterotoxigenic *Bacteroides fragilis* Infection

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Colorectal cancer is one of the most common cancers in the world. It is well known that the chronic inflammation can promote the progression of colorectal cancer (CRC). Recently, a number of studies revealed a potential association between colorectal inflammation, cancer progression, and infection caused by enterotoxigenic *Bacteroides fragilis* (ETBF). Bacterial enterotoxin activates spermine oxidase (SMO), which produces spermidine and H_2O_2 as byproducts of polyamine catabolism, which, in turn, enhances inflammation and tissue injury. Using qPCR analysis, we estimated the expression of *SMOX* gene and ETBF colonization in CRC patients. We found no statistically significant associations between them. Then we selected genes involved in polyamine metabolism, metabolic reprogramming, and inflammation regulation and estimated their expression in CRC. We observed overexpression of *SMOX*, *ODC1*, *SRM*, *SMS*, *MTAP*, *c-Myc*, *C/EBPβ* (*CREBP*), and other genes. We found that two mediators of metabolic reprogramming, inflammation, and cell proliferation c-Myc and C/EBPβ may serve as regulators of polyamine metabolism genes (*SMOX, AZIN1, MTAP, SRM, ODC1, AMD1*, and *AGMAT*) as they are overexpressed in tumors, have binding site according to ENCODE ChIP-Seq data, and demonstrate strong coexpression with their targets. Thus, increased polyamine metabolism in CRC could be driven by c-Myc and C/EBPβ rather than ETBF infection.

1. Introduction

Colorectal cancer (CRC) is one of the most common cancers in the world. It has been estimated that in 2012 about 1.4 million people were diagnosed and more than 690 thousand died [1]. The lifetime risk of developing colorectal cancer is about 5% worldwide. Women have a higher risk for colon cancer that men [2]. The prognosis of colorectal cancer is closely related to the stage of disease at diagnosis [3]. CRC can have no symptoms in early stages, and mean 5-year survival rate for peoples detected at an early stage is about 90% compared to 10% for people diagnosed for cancer with distant metastases [4]. Certain factors increase a risk of developing the disease. These are age [5], polyps of the colon [6], history of cancer, heredity [7–9], smoking [10], diet and microbiota [11, 12], lack of physical activity [13], chronic inflammation (colitis and IBD) [14, 15], viruses [16, 17], and exogenous hormones [18]. The chronic inflammation caused by infection is one more risk factor for colorectal cancer [19, 20]. Some pathogenic strains of *Escherichia coli* (cyclomodulin-positive)

are able to induce chronic inflammation and can be involved in carcinogenesis. Cyclomodulin-positive *E. coli* strains were more prevalent in both the mucosa and tumors of patients with colorectal cancer (26% patients) versus diverticulosis control (6% patients) [19]. In addition, the number of colonic polyps was elevated in multiple intestinal neoplasia (Min) mice inoculated with a colon cancer-associated *E. coli* strain (11G5) [19].

Recent studies have demonstrated that the enterotoxigenic *Bacteroides fragilis* (ETBF) bacterium is an important cause of chronic inflammation in human and animal colon. It has been presented that the *bft* gene, which encodes *Bacteroides fragilis* toxin (BFT), is associated with colorectal neoplasia and may be a risk factor for developing CRC [21]. It was observed that BFT produced by bacteria upregulates both spermine oxidase (*SMOX*) gene expression at mRNA and protein levels in cultures of human normal colonic epithelial cells [20]. *SMOX* encodes SMO protein, which plays an important role in the regulation of polyamine metabolism. SMO catalyzes the oxidation of spermine to spermidine and produces hydrogen peroxide (H_2O_2) and aldehydes [22]. This results in apoptosis, DNA damage, and consequently the development of cancer. For example, cytotoxin produced by *Helicobacter pylori* strains causes an increase in spermine oxidase levels in human gastric epithelial cells. These pathogenic *H. pylori* strains contain cytotoxin-associated gene A (CagA) and represent a risk factor for gastric cancer. The strong association of *H. pylori* (Cag+) infection, SMO levels, apoptosis, and oxidative DNA damage has been observed [23, 24].

In recent years, we have seen a steady increase in the number of studies examining the role of intracellular polyamine metabolism in tumor development. Several important enzymes, spermidine/spermine N1-acetyltransferase (SSAT), N1-acetylpolyamine oxidase (APAO), and SMO, appear to play critical roles in many cancers. All such enzymes are highly inducible by multiple stress signals, including ones caused with bacterial pathogens, and have the potential to alter polyamine homeostasis. APAO and SMO enzymes produce reactive oxygen species (ROS), H_2O_2, and aldehydes, which are potentially harmful to cells. ROS are key signaling molecules, which play an important role in several pathways (e.g., NF-κB, ERK1/2, p38, PI3K, and others) and can contribute to the induction of inflammation and cancer [25].

Thus, the abundant pathogenic microbiota alters the host tissue microenvironment leading to chronic inflammation, immune dysregulation, and elevated levels of ROS. All these may result in activation of oncogenes, downregulation of tumor suppressor genes, DNA damage, and cell and tissue injury, thereby contributing to tumor growth. In the colon, the alteration of polyamine catabolism caused by infection with consequent H_2O_2 generation and DNA damage may be a common cause of inflammation and promotion of carcinogenesis. Moreover, an increase in polyamine catabolism rates and the production of H_2O_2 has been involved in the response to chemotherapeutic agents or specific antitumor polyamine analogues in several tumors, including colorectal cancer [26–30]. However, the clinical, molecular, and prognostic associations of infection and the expression of polyamine metabolism gene in colorectal cancer remain unclear.

2. Material and Methods

2.1. Tissue Specimens. A total of 50 paired specimens of stages I–IV colorectal cancer (CRC) and adjacent morphologically normal tissues were taken from patients with primary carcinoma of the colon and rectum, which had not been exposed to radiation or chemotherapy, during surgical resection. Each sample was frozen and placed in liquid nitrogen immediately after surgery. The specimens were characterized according to the American Joint Committee on Cancer (AJCC) staging system [31]. The diagnosis was verified by histopathology and only samples containing 70–80% or more tumor cells were used in the study. The tissue samples were collected in accordance with the guidelines issued by the Ethics Committee, National Medical Research Radiological Center, the Ministry of Health of the Russian Federation. All patients gave written informed consent, which is available upon request. The study was carried out in accordance with the principles outlined in the Declaration of Helsinki (1964).

2.2. RNA and DNA Isolation and cDNA Synthesis. Total RNA was isolated using Micro-Dismembrator S (Sartorius, Germany) and RNeasy Mini Kit (Qiagen, Germany) in accordance with the manufacturer's instructions. For the detection of bacteria, DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) and further treated with proteinase K in accordance with the manufacturer's protocol. Purified RNA and DNA were quantified using Qubit 2.0 fluorometer (Invitrogen, USA) and their quality was determined by Agilent Bioanalyzer 2100 (Agilent Technologies, USA). All RNA samples were treated with DNase I (Thermo Fisher Scientific, USA), and cDNA was synthesized using M-MLV Reverse Transcriptase (Thermo Fisher Scientific, USA) and random hexamers according to standard manufacturer's protocol.

2.3. qPCR. To detect and quantitate *Bacteroides fragilis* we used the primers targeting *Bft1* gene. These primers were taken from the work of Viljoen et al. [32]: forward 5′-GACGGTGTATGTGATTTGTCTGAGAGA-3′, reverse 5′-ATCCCTAAGATTTTATTATCCCAAGTA-3′. EvaGreen Dye (Biotium Inc., USA) was used as fluorescent DNA-binding dye for the detection and quantification of PCR products. Purified bacterial control DNA was obtained from Orekhovich Institute of Biomedical Chemistry, Russian Academy of Sciences (Moscow).

To evaluate gene expression, we used TaqMan Gene Expression Assays (Thermo Fisher Scientific, USA). This consists of a pair of specific unlabeled PCR primers and a TaqMan probe with a FAM dye label on the 5′ end and minor groove binder (MGB) nonfluorescent quencher (NFQ) on the 3′ end. All probes contained the dye FAM at 5′-end and RTQ1 at 3′-end. qRT-PCR was performed as described earlier using *RPN1* and *GUSB* reference genes [33–35].

All reactions were performed using AB 7500 Real-Time PCR System (Thermo Fisher Scientific, USA) with RQ (Relative Quantitation) software (Thermo Fisher Scientific, USA). PCR program was as follows: 10 min at 95°C and then 50 two-step cycles 15 s at 95°C and 60 s at 60°C. The total reaction volume was 20 μL in triplicate. PCR products were analyzed in 2% agarose gels, and nucleotide sequences of the amplicons were verified by Sanger sequencing with ABI Prism 3100 Genetic Analyzer (Thermo Fisher Scientific, USA).

2.4. Analysis of qRT-PCR Data. For the detection and quantification of bacterial DNA, absolute quantification method was used. A standard curve was constructed using serial 10-fold dilutions of control bacterial DNA. The genome size of *Bacteroides fragilis* (5.3 Mb) and the mass of DNA per genome were used to calculate the concentration of bacterial DNA [36]. Spearman's rank correlation analysis was used to check the dependence between target gene expression levels and the concentration of bacterial DNA within the same samples.

mRNA qRT-PCR data were analyzed using the relative quantification method ($\Delta\Delta C_t$) taking into account the efficiency of the PCR amplification using ATG (Analysis of Transcription of Genes) tool as described in [34, 37]. The relative inner variability between mRNA levels of reference genes (*RPN1* and *GUSB*) was not higher than twofold in tumor and normal tissues; therefore, twofold and higher mRNA level changes for the target genes were considered as significant. We used nonparametric Mann-Whitney *U* test to validate the significance of gene expression alterations ($p \leq 0.01$ was taken as the criterion of statistical significance). All statistical analyses were performed in the R environment. Pearson correlation coefficient was used to evaluate the coexpression of human genes ($p \leq 0.001$ was taken as the criterion of statistical significance). We supplemented the coexpression analysis with ENCODE ChIP-Seq data using previously developed CrossHub tool [38].

3. Results

3.1. Expression of SMOX Gene and Bacteroides fragilis Quantification. We analyzed 36 paired samples of primary colorectal carcinomas and adjacent normal tissues to quantify ETBF. Serial dilution of genomic DNA from ETBF was used as standard. At least one copy of ETBF DNA per 50 ng of total extracted DNA was detected in 8 paired tumor and adjacent normal samples, including three samples, which showed a significantly increased amount of the ETBF DNA (>1000 copies/1 ng of extracted DNA). Most of the tested samples (23/36) demonstrated less than a copy of bacterial DNA per 50 ng of total extracted DNA. Five samples were found to be ETBF-negative (Figure 1).

3.2. Expression Quantification of Genes Involved in Polyamine Metabolism and Regulation of Inflammatory Response. The set of 36 colorectal tumors and adjacent normal tissues was extended with an additional set of 14 paired samples in order to increase the statistical significance of the results. Thus, we used a set of 50 colorectal cancers to evaluate the expression of target genes, which encode enzymes of polyamine metabolism and genes participating in immune response and inflammation signaling.

Using RT-qPCR, we evaluated the relative expression level of 17 genes involved in polyamine metabolism and 4 genes mediating metabolic reprogramming, and the regulation of inflammatory response and cell proliferation: *c-Myc* (*MYC*), *n-Myc* (*MYCN*), *Max,* and *C/EBPβ* gene encoding CCAAT/enhancer binding protein (CEBPB). The results are given in Figure 2 (tumor/normal relative expression level) and Figure 3 (coexpression analysis coupled to ENCODE ChIP-Seq data).

Two genes, *c-Myc* and *SMOX*, demonstrated the highest upregulation in CRC. Both genes were overexpressed (at least 2 times) in 40 out of 50 samples (80%). *C/EBPβ*, *eIF5A2,* and *SRM* revealed upregulation in 50% samples. Several pairs of genes that demonstrated statistically significant coexpression were also found to have ChIP-Seq associations: *c-Myc-AGMAT, c-Myc-ODC1, c-Myc-SRM, c-Myc-AMD1, C/EBPβ-SRM, C/EBPβ-AGMAT, C/EBPβ-SMOX,* and *C/EBPβ-eIF5A2*. Moreover, *C/EBPβ* and *c-Myc* demonstrated strong coexpression ($r_s = 0.37$) and extremely high ChIP-Seq signal intensity according to ENCODE ChIP-Seq data (C/EBPβ binding to c-Myc). This suggests C/EBPβ to be a possible upstream regulator of *c-Myc*. In contrast to *c-Myc*, *n-Myc* showed statistically significant coexpression only with *AZIN1*.

4. Discussion

Bacteroides fragilis contains up to 1%-2% of the normal colonic microbial flora in humans [39, 40]. Pathogenic strains of *B. fragilis* that produce enterotoxin are associated with the development of inflammatory diarrheal disease in both children and adults [41, 42], IBD [43], and colitis [44], thereby contributing to chronic inflammation and onset of colon tumors. It was supposed that *B. fragilis* toxin (BFT), produced only by enterotoxigenic strains (ETBF), binds to a specific intestinal epithelial cell receptor and induces several signal transduction pathways [45, 46]. BFT cleaves E-cadherin [47, 48] and activates Wnt/β-catenin pathway signaling [49]. BFT was also shown to be involved in activation of tyrosine kinases, MAPKs, and NF-κB signaling pathways and to be able to increase cellular proliferation mediated by the elevated expression of c-Myc oncogene [50–53]. Moreover, as mentioned above, BFT may be implicated in polyamine catabolism through upregulation of SMO protein levels and enzyme activity [20]. Recent studies suggested SMO enzyme as a potential source of an inflammation-associated ROS produced during polyamine catabolism [54]. Thus, the association between ETBF colonization rates of CRC patients and the expression of *SMOX* gene appears to be an important link between chronic inflammation caused by infection, tumor onset, and progression.

In this study, we quantitated ETBF strain colonization rates and evaluated the expression of SMOX gene at mRNA level in CRC patients using qPCR. We found that the majority of CRC patients were colonized with toxin-producing strains of *B. fragilis*, but only small amounts of bacterial DNA were identified. A significantly increased content of bacterial DNA

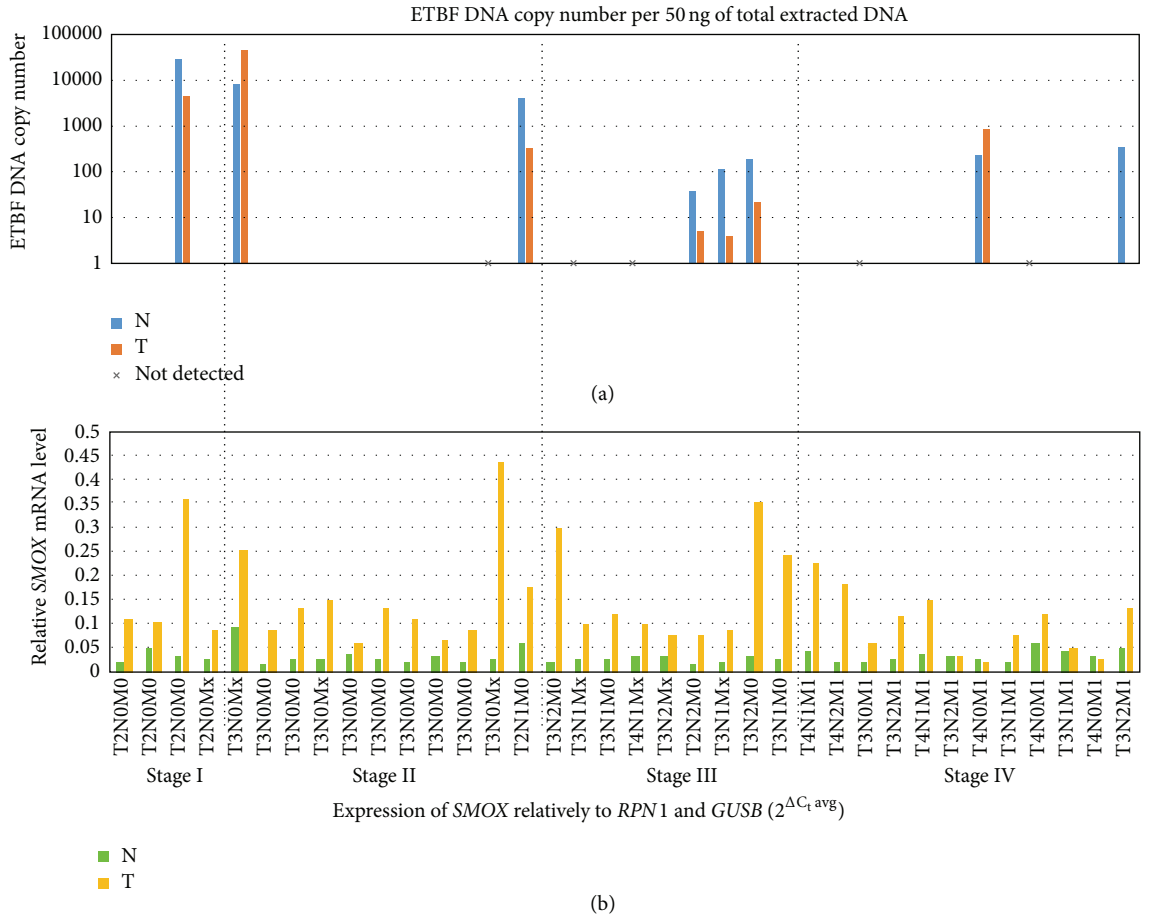

Figure 1: (a) Enterotoxigenic *B. fragilis* (ETBF) DNA copy number per 50 ng of total extracted DNA in paired samples of colorectal cancer (logarithmic scale). (b) *SMOX* expression level relatively to two reference genes: *RPN1* and *GUSB*. The samples with high rates of ETBF colonization tend to have higher expression of SMOX, especially in normal tissue (compared to the other norms). However, no statistically significant correlation between *SMOX* expression and ETBF colonization was observed.

was detected in three patients with I and II stages of CRC (in both tumor and normal tissues). In contrast, pathogenic cyclomodulin-positive *E. coli* strains, a possible cofactor of colorectal carcinogenesis, were predominantly found on mucosa of patients with stages III/IV [19]. Additional studies and extended sampling are needed to reveal possible associations between ETBF colonization and disease stage.

The expression of *SMOX* gene revealed no statistically significant correlation with the amount of bacterial DNA, but the samples with a high concentration of bacterial DNA (>1000 copies per 50 ng) demonstrated high SMOX expression levels in both normal tissue and tumor (relatively to *GUSB* and *RPN1* reference genes, Figure 1). These results do not contradict the hypothesis that ETBF strains can be proinflammatory and oncogenic bacteria, but additional studies are needed to understand the possible role of ETBF in colorectal carcinogenesis [21]. These results are consistent with a mechanism of *SMOX* induction independent of ETBF infection.

Next, we tested differential expression of 17 genes participating in polyamine metabolism and 4 genes involved in the mediation of metabolic preprogramming, cell proliferation (*c-Myc*, *n-Myc*, and *Max*) and inflammation (C/EBPβ). c-Myc and n-Myc form dimers with Max, translocate to the nucleus, and then activate the transcription of many genes participating in cell cycle regulation, glycolysis, energy metabolism, hypoxic adaptation, DNA replication, and other processes [55–58]. C/EBPβ is a transcription factor, which can form either homodimers or heterodimers with other CCAAT/enhancer binding proteins (alpha, delta, and gamma). C/EBPβ is known to be a mediator of inflammation and immunity [59, 60]. The most important C/EBPβ targets are interleukins IL-6, IL-4, IL-5, and TNF-α [61–64]. We found upregulation of *c-Myc*, *n-Myc*, and *C/EBPβ* in the analyzed samples. However, we did not find overexpression of *Max*. This is in agreement with a previous finding: overexpression of *c-Myc* and *n-Myc* but not *Max* is observed in many tumors [55, 58, 65, 66]. Our data showed increased expression of *SMOX* gene at all stages of colorectal cancer, this tendency being more pronounced at the early stages (Figure 2). Spearman correlation coefficient between disease stage and tumor/normal fold change of *SMOX* mRNA level is $r_s = -0.19$: *SMOX* expression tends to be lower with disease stage. However, this tendency is not statistically

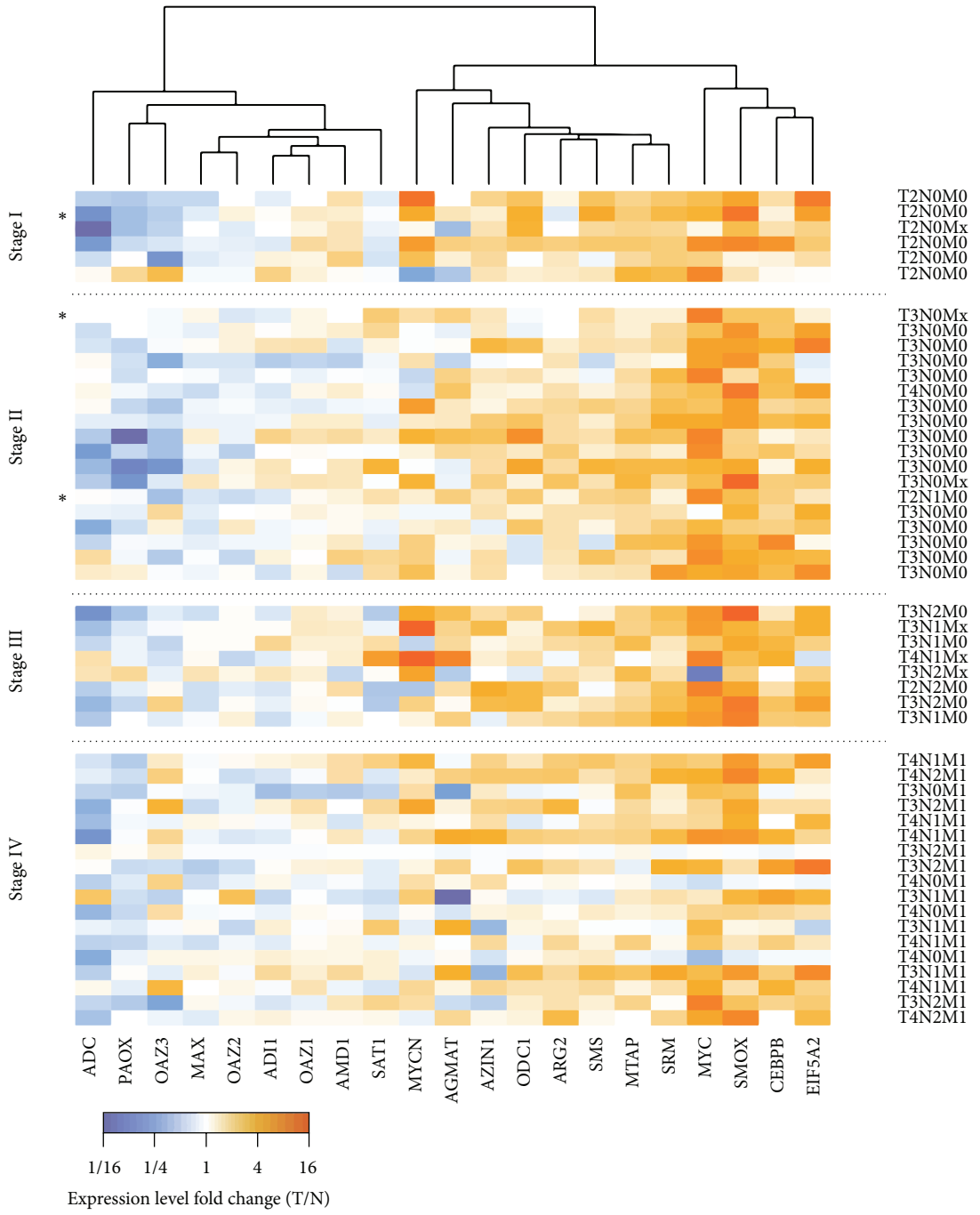

Figure 2: Results of the qPCR expression analysis of genes involved in polyamine metabolism and inflammation regulation in paired colorectal cancer samples. Cell color indicates expression level change in tumor compared to normal: increase (orange) and decrease (blue). Genes are rearranged according to the similarity of expression profiles. Samples with a high concentration of enterotoxigenic *B. fragilis* DNA (>1000 copies per 1 ng of total DNA) are marked with an asterisk.

significant ($p > 0.05$). Different tumors demonstrate dramatically distinct *SMOX* expression profiles. SMO was found to be elevated in both prostate adenocarcinoma and prostatic intraepithelial neoplasia [67] whereas breast cancer showed *SMOX* underexpression at both mRNA and protein levels [68]. Other genes that showed statistically significant correlation with disease stage were *MTAP* ($r_s = -0.30$, $p = 0.03$), and *SRM* ($r_s = -0.27$, $p = 0.05$), which tended to decrease the expression level with tumor stage, and *OAZ3* ($r_s = 0.42$, $p = 0.003$), which increased its expression with disease progression.

Polyamines are organic cations, which are essential for cell proliferation and growth, and their levels are frequently altered in many human tumors [69, 70]. Polyamines have

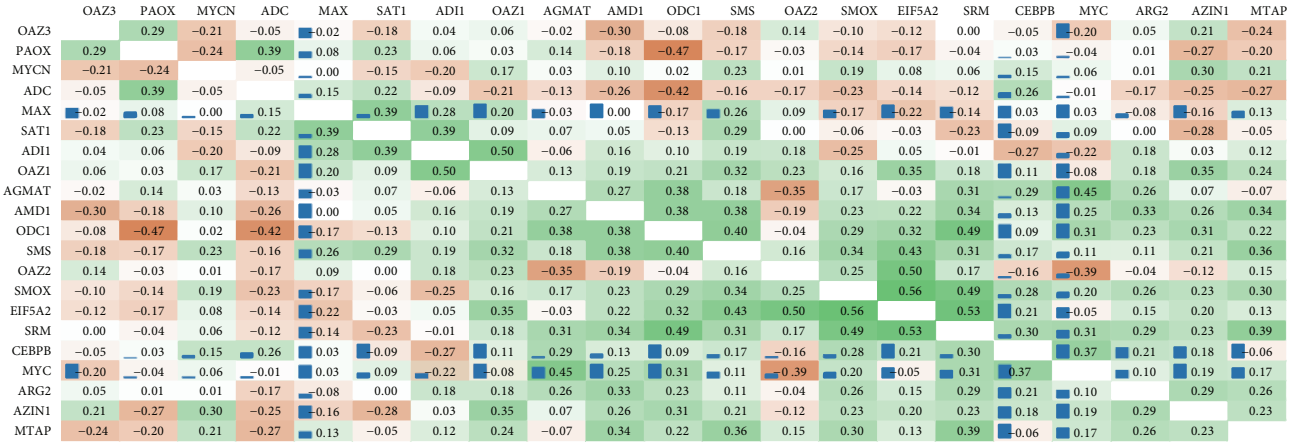

FIGURE 3: Results of coexpression analysis for genes participating in polyamine metabolism. Pearson correlation coefficients between the expression levels changes of genes participating in polyamine metabolism and inflammation across 50 colorectal cancer samples are presented. Cell color reflects these values (green: positive, brown: negative). Normalized ChIP-Seq score (according to ENCODE data) is indicated with blue bars.

also been shown to play an important role in inflammation-induced carcinogenesis [71]. Intracellular polyamine metabolism occurs via two pathways: classical and alternate (Figure 4). Polyamines (putrescine, spermidine, and spermine) are synthesized by mammalian cells, while agmatine is produced only by plants and bacteria, including intestinal microbial flora [72–74]. L-arginine carboxylase (ADC), found only in nonmammals, catalyzes a reaction of decarboxylation of L-arginine to agmatine [72, 75]. The latter is further hydrolyzed to putrescine and urea by agmatinase, which is encoded by *AGMAT* gene. Recent reports also supposed that different pathogens, such as viruses and bacteria, could upregulate agmatinase at mRNA and protein levels, thereby inducing polyamine synthesis [76, 77].

In the present study, we revealed that the expression of *ADC* gene decreased on the average by half in CRC samples compared to adjacent normal tissues. These findings indicate that, in case of colorectal cancer, the enhanced polyamine synthesis is not associated with alternate path and the intestinal microbiota does not significantly contribute to these processes.

The three major enzymes that are involved in polyamine metabolism (SSAT, APAO, and SMO) are encoded by *SAT1, PAOX*, and *SMOX* genes, respectively. One of the products of both SMO and APAO-mediated reactions are H_2O_2 and highly toxic aldehydes, which may also cause oxidative damage. SSAT cellular toxicity is thought to be caused by produced acetylated polyamines, which are utilized by APAO. APAO is constitutively expressed, while SSAT is an inducible enzyme [78]. SSAT expression is induced by different stimuli, such as toxins, hormones, cytokines, nonsteroidal anti-inflammatory agents, natural products, and pathogens. It is regulated via several pathways including TNF-α and NF-κB [79–82]. SSAT expression can be also mediated by Nrf2 in response to the addition of H_2O_2 in human hepatoma HUH7 cells [83]. We found that *SAT1* gene was differentially expressed in many CRC samples (either up- or downregulated), while a decreased expression of *PAOX* gene was observed. This indicates that the SSAT/APAO pathway is not hyperactive and does not seem to be a cause of oxidative damage in colorectal cancer. Inflammatory response agents and stress pathways are not the result of the induction of *SSAT* expression at mRNA level. Thus, it is possible that the increased *SMOX* gene expression and enzyme activity make the greatest contribution to the oxidative stress damage caused by polyamine catabolism in colorectal cancer [20].

It is known that specific protein products of oncogenes and tumor suppressor genes can regulate polyamine metabolism [84–86]. The results of coexpression analysis coupled to ENCODE ChIP-Seq data strongly suggest c-Myc and C/EBPβ as regulators of the expression of key enzymes of polyamine metabolism that are upregulated in colorectal cancer: *SMOX, AZIN1, MTAP, SRM, AMD1, ODC1*, and *AGMAT*. It should be mentioned that genes encoding polyamine metabolic enzymes are regulated transcriptionally/post-transcriptionally by changes in the levels of intracellular polyamines. Additional studies (*c-Myc* and *C/EBPβ* knockdown assays) are needed to prove our finding. Not surprisingly, three of these genes, *ODC1, AMD1*, and *SRM*, are already known c-Myc targets [87–89]. Besides these genes, c-Myc is also known to regulate *eIF5A2*. However, this gene did not demonstrate coexpression with *c-Myc* but did show ChIP-Seq association (c-Myc binding site). It is known that c-Myc induces the expression of ornithine decarboxylase (ODC), which catalyzes the first rate-limiting step in polyamine synthesis [89]. It has been also shown that bacterial infections can stimulate polyamine synthesis through ODC [90], whereas suppression of ODC leads to the depletion of cellular polyamine levels in human colorectal cancer cells [82, 91]. ODC inhibition with difluoromethylornithine (DFMO) is a possible anticancer therapy. It was reported that the treatment of normal intestinal epithelial cells of rats (IEC-6) with the DFMO led to the depletion of polyamines and subsequently inhibited cell growth and induced cell cycle arrest. A combination of drugs including DFMO could reduce recurrent adenomatous

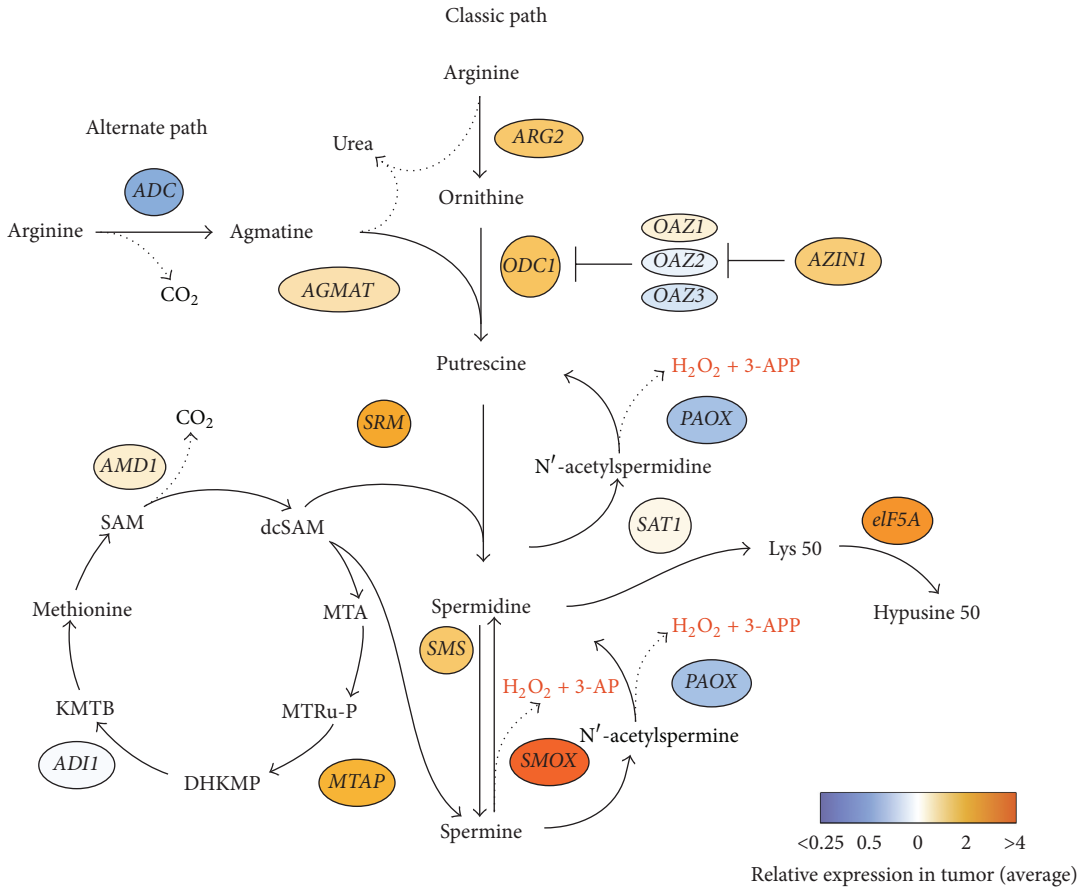

Figure 4: Classic path of polyamine metabolism consists of the following: (1) arginine is converted to ornithine through the action of ARG (arginase) in the urea cycle; (2) putrescine is formed from the reaction of ornithine decarboxylation catalyzed by ODC1 (ornithine decarboxylase-1). OAZ can bind to ODC1 to form OAZ-ODC1 complex and subsequently reduce polyamine synthesis. AZIN1 (antizyme inhibitor-1) brakes the ODC1-OAZ complex and liberates ODC1; (3) AMD1 (S-adenosylmethionine decarboxylase) decarboxylates S-adenosylmethionine (SAM) to decarboxylated SAM (dcSAM); (4) dcSAM provides aminopropyl groups to putrescine to produce spermidine by spermidine synthase (SRM) and spermine by spermine synthase (SMS). MTA (methylthioadenosine) is generated as a byproduct. Spermine can be recycled back to spermidine directly by spermine oxidase (SMOX). Spermine and spermidine can be recycled to spermidine and putrescine by spermidine/spermine-N1-acetyltransferase (SAT1) followed by oxidation by polyamine oxidase (PAOX) [101]. MTA can be processed to the methionine: MTA phosphorylase (MTAP) catalyzes the cleavage of MTA yielding 5-methylthioribose-1-phosphate (MTRu-P), which is further metabolized to DHKMP (1,2-dihydro-3-keto-5-methylthiopentene). ADI (acireductone dioxygenase) catalyzes DHKMP to 2-oxo-4-methylthiobutyrate (KMTB) and transamination of KMTB results in formation of methionine [102–104].

polyps in patients with history of resected sporadic colorectal adenomas [92].

n-Myc (*MYCN*) is oncogenic transcription factor, which can directly upregulate ODC expression in neuroblastomas [93]. Moreover, it has been shown that the reduction of n-Myc protein levels through inhibition of glycolysis may decrease ODC expression and potentiate polyamine levels in human neuroblastoma cell lines [84]. We showed that expression of *ODC1* gene was increased in a number of CRC samples, as well as mRNA level of *c-Myc* and *n-Myc* genes. However, *n-Myc* showed no expression correlations with *ODC1* and no ENCODE ChIP-Seq data are available for n-Myc.

It is worthy of note that the expression of *OAZ1, OAZ2,* and *OAZ3* genes, which encode major regulators of ornithine decarboxylation to putrescine through inhibition of ODC catalytic activity, and the expression of *AZIN1* gene, which is involved in inhibition of antizyme (OAZ) family, did not change in the majority of CRC samples compared with normal samples. This indicates that the production of putrescine, catalyzed by ODC during polyamine catabolism, is not suppressed in colorectal cancer.

We have observed a significant increase in *eIF5A2* mRNA levels in colorectal tumors. Cytosolic protein encoded by *eIF5A2* gene undergoes posttranslational modification of Lys 50 to hypusine [94]. Spermidine as a substrate is involved in the first step of this process. The eIF5A2 protein is essential for eukaryotic cell proliferation, but the molecular function of eIF5A remains incompletely clear. It was shown that c-Myc can possibly transactivate the *eIF5A2* gene [95, 96]. eIF5A2, in turn, was demonstrated to regulate MTA1 (metastasis-associated 1) via c-Myc in gastric cancer and colorectal carcinoma [97, 98]. Our data suggests that eIF5A2

can play an important oncogenic role in CRC and enhance the involvement of polyamines in this pathological process.

We elevated the expression of *C/EBPβ* gene, an important transcription factor, which controls the expression of genes involved in inflammatory response [60, 99, 100]. We observed a positive correlation between *C/EBPβ* gene expression and many key genes such as *ADC, SMOX, AGMAT,* and *SRM* involved in polyamine metabolism.

5. Conclusions

In summary, our results show that dysregulation of polyamine metabolism in all stages of CRC can be associated with chronic inflammation mediators rather than with the infection caused by ETBF. This is the first report that presents changed levels of expression of the key components of polyamine metabolism. We characterized some important aspects of the expression of *SMOX* and *PAOX* genes, which are responsible for cellular ROS generation. Two transcription factors, oncogenic c-Myc (responsible for metabolic reprogramming and cell proliferation) and C/EBP*β* (mediator of inflammation and immune response), were found to be the most likely regulators of several key enzymes of polyamine metabolic pathway.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Anastasiya V. Snezhkina and George S. Krasnov contributed equally to this work.

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Research Article

Efficacy of Mitochondrial Antioxidant Plastoquinonyl-decyl-triphenylphosphonium Bromide (SkQ1) in the Rat Model of Autoimmune Arthritis

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Rheumatoid arthritis is one of the most common autoimmune diseases. Many antioxidants have been tested in arthritis, but their efficacy was, at best, marginal. In this study, a novel mitochondria-targeted antioxidant, plastoquinonyl-decyl-triphenylphosphonium bromide (SkQ1), was tested *in vivo* to prevent and cure experimental autoimmune arthritis. In conventional Wistar rats, SkQ1 completely prevented the development of clinical signs of arthritis if administered with food before induction. Further, SkQ1 significantly reduced the fraction of animals that developed clinical signs of arthritis and severity of pathological lesions if administration began immediately after induction of arthritis or at the onset of first symptoms (day 14 after induction). In specific pathogen-free Wistar rats, SkQ1 administered via gavage after induction of arthritis did not reduce the fraction of animals with arthritis but decreased the severity of lesions upon pathology examination in a dose-dependent manner. Efficacious doses of SkQ1 were in the range of 0.25–1.25 nmol/kg/day (0.13–0.7 μg/kg/day), which is much lower than doses commonly used for conventional antioxidants. SkQ1 promoted apoptosis of neutrophils *in vitro*, which may be one of the mechanisms underlying its pharmacological activity. Considering its low toxicity and the wide therapeutic window, SkQ1 may be a valuable additional therapy for rheumatoid arthritis.

1. Introduction

Rheumatoid arthritis (RA) is one of the most prevalent autoimmune diseases. Infection by Epstein-Barr virus, human herpesvirus 6, and polyetiological periodontitis has been named among factors that could promote disease development and progression [1–3]. Commonly used clinical regimens can slow the disease progression; however, they have significant side effects and cannot be administered indefinitely. Immune therapies have a huge potential [4], but they are currently expensive due to high manufacturing costs of biopharmaceuticals. In addition, their side effects and potential for prolonged use are not entirely clear. Development of drugs to treat arthritis has mainly been aimed at protein interactions that are relatively well understood. Another component of inflammation signaling, the reactive oxygen species (ROS), has largely been neglected because it is very impractical to study molecules that have a short

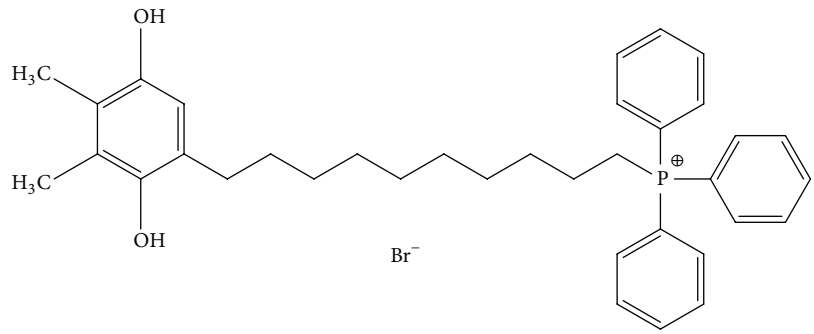

Figure 1: Structural formula of SkQ1 (plastoquinonyl-decyl-triphenylphosphonium bromide).

decay time and are not detectable by immunological assays, although there was a solid theoretical background for such therapies (reviewed in [5]). Empirical trials of antioxidants, such as hydrogen [6], melatonin [7], resveratrol [8], vitamins, and herbal extracts [9, 10], provided evidence of efficacy in animal models of RA. However, these results were hard to convert into clinical practice because the high doses of conventional antioxidants that are required to achieve efficacy can produce side effects.

Plastoquinonyl-decyl-triphenylphosphonium (SkQ1; Figure 1) is a fusion of plastoquinone, a plant analog of ubiquinone, and triphenylphosphonium, a lipophilic cation that has a positive charge smeared over three aromatic rings. The latter part of the molecule acts as a transmembrane transporter to the negatively charged inner lumen of the mitochondria [11], where the plastoquinone part provides rechargeable ROS-scavenging activity. This antioxidant activity was demonstrated in isolated mitochondria, in cell culture [12], and *in vivo* in laboratory animals [13]. Later studies showed that SkQ1 is especially efficient in protection of mitochondrial lipid cardiolipin from oxidation [14, 15]. The concentration of SkQ1 molecules in the mitochondria is estimated to be up to 8 logs higher than that in the cell culture medium [12]. Pharmacological efficacy of SkQ1 was demonstrated in a number of animal models that are associated with ROS-mediated damage, such as ischemia-reperfusion, autoimmune inflammation, and senile disorders (reviewed in [16]).

Previous studies suggested that SkQ1 potentially has a very wide therapeutic window (difference between beneficial antioxidant and toxic prooxidant concentrations) and might exhibit therapeutic efficacy at much lower doses than conventional antioxidants [12]. This study was aimed to test efficacy of a mitochondria-targeted antioxidant SkQ1 in the rat model of autoimmune arthritis.

2. Materials and Methods

2.1. Compound. Plastoquinonyl-decyl-triphenylphosphonium bromide (SkQ1) was synthesized as described previously [12]. In Series I (see below), an oxidized form of the compound (Figure 1) was used. SkQ1 was dissolved in ethanol to 10 mg/mL and then in water to a working concentration. In Series II, a reduced form of SkQ1 (a candidate drug formulation) was used. Reduction of SkQ1 was provided by addition of ascorbic acid (SkQ1 to ascorbic acid ratio 2.5 : 97.5).

2.2. In Vivo Experiments. Animal experiments were carried out at two laboratories. As experimental conditions differed significantly between these experiments, they are referred to as Series I (Institute of Cytology and Genetics (ICG), Siberian Division of the Russian Academy of Sciences, Novosibirsk) and Series II (Institute of Mitoengineering, Moscow State University, Moscow).

Series I was carried out using conventional in-house bred Wistar rats, which were obtained from the Shared Center for Genetic Resources of Laboratory Animals of the ICG. At the age of 4 weeks, the pups were weaned, housed in groups of five animals per cage, and kept under standard laboratory conditions (22°C ± 2°C, 60% relative humidity, and natural lighting). The animals were provided with standard rodent diet (PK-120-1, Laboratorsnab Ltd., Russia) and water *ad libitum*. Experimental protocols were approved by the ethical committee of the Institute of Cytology and Genetics.

Arthritis was induces by subcutaneous (s.c.) injection of 250 μg of type 2 chicken collagen (Sigma-Aldrich, St. Louis, USA) dissolved in a 1 : 1 mixture of incomplete and complete Freund's adjuvant (Sigma-Aldrich) to a total volume of 250 μL. In experiment I-1 (preventive administration), SkQ1 (250 nmol/kg/day) was applied with a dispenser on pieces of cottage cheese that were individually fed to experimental animals starting 55 days before induction of arthritis and until the end of experiment. Control rats received untreated cottage cheese. In this experiment, there were groups of rats aged 4 months ("young animals") and 20 months ("old animals"). The number of rats in the mock-immunized, immunized-untreated, and immunized-treated groups was 10, 10, and 10 (young rats) and 10, 8, and 10 (old rats), respectively. Severity of arthritis was evaluated by clinical scoring [17] at days 15, 18, 20, 25, 28, and 30. Rats were sacrificed at day 32 after arthritis induction.

In experiment I-2 (treatment), ten 3-month-old Wistar rats were mock-immunized (negative control). An untreated control group ($n = 15$) and two experimental groups ($n = 15$ in each) were immunized with chicken collagen as described above. SkQ1 was administered into the oral cavity of rats with a dispenser (250 nmol/kg/day) beginning at day

1 or day 14 after arthritis induction. Vehicle solution (2% ethanol in water) was administered to control rats. Animals were examined at days 14, 17, 19, 21, 24, 26, 28, and 30 after arthritis induction. Rats were sacrificed at day 32 after arthritis induction.

Series II was carried out in the specific pathogen-free (SPF) animal house of the Institute of Mitoengineering, Moscow. The experiment was approved by the Bioethics committee of the Institute of Mitoengineering (Protocol number 19, May 10, 2011). SPF Wistar rats aged 5-6 weeks were obtained from the Pushchino Breeding Facility (Moscow region, Russia). Autoimmune arthritis was induced by immunization with 250 μg type 2 porcine collagen (Chondrex, USA) in complete Freund's adjuvant according to the published protocol [18]. SkQ1 was administered via oral gavage at 50, 250, and 1250 nmol/kg/day. Control rats received vehicle (ascorbic acid solution without SkQ1). In a preliminary experiment the vehicle solution did not affect clinical course of RA (data not shown). Rats were sacrificed at day 30 after arthritis induction.

Joint specimens were fixed in 10% buffered formalin solution (pH = 7,4), decalcified in 14% ethylenediaminetetraacetate solution, and paraffin-embedded. Horizontal (paw) and vertical (knee) microtome sections (4 μm) were performed. Histopathological examination of the knee (Series I) and hind paw (Series II) joints was performed upon staining with hematoxylin and eosin. Acidic glycosaminoglycans were visualized with alcian blue staining. Distribution of collagen was visualized by Mallory's staining. All examinations and evaluations were done on blinded slides by professional animal pathologist. Five high-power magnification fields (HMF) were scored for each animal based on the criteria published in [19, 20]. Synovial inflammation was scored based on the amount of infiltrating mononuclear cells as follows: 0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%). Synovial hyperplasia was scored as 0, absent; 1, mild (3-4 layers for knee and 2 layers for paw); 2, moderate (5-6 layers for knee and 3 and more layers for paw); 3, severe (more than 6 layers for knee and 3 layers for paw). Cartilage erosion was evaluated based on the fraction of the cartilage surface that was eroded: 0, absent; 1, mild (1–10%); 2, moderate (10–30%); 3, severe (more than 30%). Bone erosion was scored as 0, none; 1, minor erosion(s) observed only at HMF; 2, moderate erosion(s) observed at low magnification; 3, severe transcortical or subtranscortical erosion(s).

2.3. Neutrophil Isolation. All experimental procedures were reviewed and approved by the Institutional Ethics Committee of the A. N. Belozersky Institute of Lomonosov Moscow State University before the study began. Written informed consent was obtained from blood donors. Peripheral blood was collected from healthy human donors into heparin-containing tubes. Neutrophils were isolated by dextran sedimentation and centrifugation on a Ficoll-Paque gradient. Residual erythrocytes were lysed by treating the cell pellets with distilled water for 45 seconds. The collected neutrophils were resuspended in RPMI-1640 medium (Paneco, Russia) containing 10% low-endotoxin fetal calf serum (PAA Laboratories, Germany). Isolated neutrophils were consistently >98% pure by modified Wright-Giemsa staining and >98% viable as determined by trypan blue dye exclusion.

2.4. Assessment of Neutrophil Apoptosis. Purified neutrophils (1×10^6/mL) were treated with SkQ1 and/or with mitochondrial debris and incubated in a final volume of 0.5 mL for 22 hours at 37°C in a 5% CO_2 humidified incubator. Neutrophil survival was assessed after 22 h using annexin V staining. Briefly, cells were washed twice with PBS, incubated for 20 min in the dark at 37°C with annexin-V-fluorescein isothiocyanate and propidium iodide, and analyzed with a Beckman Coulter FC500 system as described in [21]. Neutrophil apoptosis is expressed as the percentage of annexin-V-positive and propidium-iodide-negative cells.

2.5. Preparation of Mitochondrial Debris (MTD). Mitochondria were isolated from rat liver or from the human endothelial cell line Ea.hy926. All procedures were performed at 4°C. Cells were disrupted using a Potter homogenizer in buffer containing 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 50 mM NaCl, and 0.2 M sucrose. Cell debris was discarded by centrifugation at 1000 ×g for 10 min. The supernatant was centrifuged at 10,000 ×g for 20 min and the mitochondrial pellet was obtained. Mitochondria were resuspended in PBS and disrupted by ultrasound sonication (eight times for 20 s with 40 s intervals). Mitochondrial membranes were removed by centrifugation at 100,000 ×g for 50 min, and supernatant containing soluble MTD was obtained and stored in aliquots at −70°C. Protein concentration of the MTD solution was determined by the Bradford method using the Bio-Rad protein assay. For the neutrophil survival studies, the final protein concentration of MTD was 2.5 mg/mL.

2.6. Statistical Analysis. Statistical analysis was performed using GraphPad Prizm 5.0. Survival was analyzed with Fisher's exact test and log-rank test. Histopathological scores were analyzed with Kruskal-Wallis test with Dunn's postcorrection. Neutrophil survival was assayed using ANOVA with Dunn's postcorrection.

3. Results

3.1. Experiment I-1. Immunization of young Wistar rats with collagen resulted in development of arthritis in 7/10 animals. The disease was progradient (without spontaneous remissions) and progressed to clinical grade 4 in six animals. Preventive administration of SkQ1 with food completely averted development of arthritis in young Wistar rats ($p < 0.01$, Fisher's exact test; $p < 0.01$, log-rank test). In old animals, 6/8 rats developed clinical signs of arthritis by day 18. However, the disease resolved in three animals by day 25, and, therefore, it was not appropriate to analyze disease-free progression in this experiment. Pretreatment with SkQ1 resulted in a decrease in the proportion of animals (3/10) that had clinical signs of arthritis at one or more observation points and a decrease in the proportion of animals (2/20) that had arthritis at the experiment endpoint (day 25). However, these results did not differ significantly from the untreated control group ($p > 0.05$, Fisher's exact test).

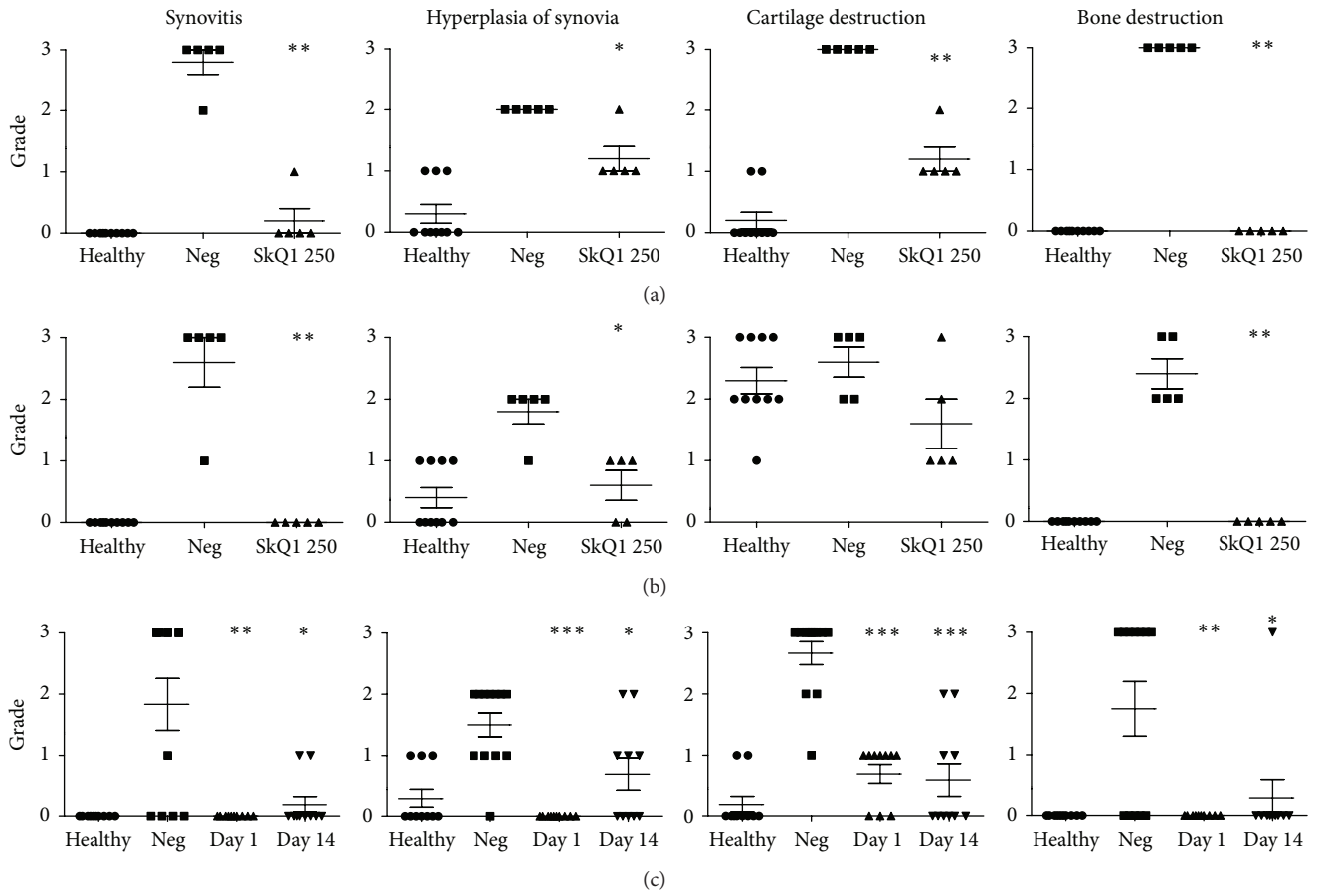

Figure 2: Pathological findings in 4-month-old (a) and 20-month-old (b) Wistar rats that were pretreated for 55 days with SkQ1 (250 nmol/kg/day) prior to induction of autoimmune arthritis and in 4-week-old rats treated with the same dose of SkQ1 daily from day 1 or day 14 after disease induction (c). Animals were sacrificed 32 days after arthritis induction. Severity of indicated pathological signs of arthritis was assayed upon H&E staining. Acidic glycosaminoglycans (cartilage) were visualized by alcian blue staining. Distribution of collagen was visualized by Mallory's staining. All evaluations were done on blinded slides. Five high magnification fields were assayed per sample. Severity of pathological manifestations was scored by 3-point scale, from 0 for normal tissue to 3 for maximum damage. Statistical significance of difference between the negative control and pretreated groups was tested with the Kruskal-Wallis test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Pathological investigation of the knee joint of five randomly selected rats per group showed that all untreated rats developed clear signs of arthritis (Figure 2(a)), which were significantly less pronounced or completely absent (bone destruction) in rats pretreated with SkQ1. Similar observations could be seen in old rats (Figure 2(b)); however, as the animals at 20 months already had age-related cartilage degeneration, autoimmune arthritis did not produce additional damage to the cartilage. It is of note that the degree of cartilage degeneration in old rats was lower in the SkQ1-treated group than in the untreated animals (not statistically significant).

3.2. Experiment I-2. Treatment of experimental autoimmune arthritis in young rats resulted in delayed progress of arthritis and a reduction in the number of animals that developed clinical signs of arthritis (Figure 3). The effect of treatment that commenced at day 1 after arthritis induction on disease-free survival was statistically significant ($p < 0.05$, log-rank test). Moreover, only one animal in each treated group progressed to severe arthritis (grade 4 by clinical scale [22]) compared to three animals in the control group; however, this effect was not statistically significant (data not shown). Pathological examination (Figure 2(c)) revealed a significant reduction or abrogation of inflammatory and degenerative lesions, which was more pronounced in the group treated from day 1 after arthritis induction.

3.3. Experiment II. Experiment II aimed to study if administration of SkQ1 into the stomach via gavage was better than administration with food. This series was carried out in a different animal facility on SPF rats obtained from another supplier and upon arthritis induction with different collagen. In this experimental series, SkQ1 administered from day 1 after arthritis induction at three doses (50, 250, and 1250 nmol/kg/day) did not have any effect on the proportion of rats that developed arthritis and only increased the mean time of disease onset from 13 to 14 days ($p < 0.05$, the

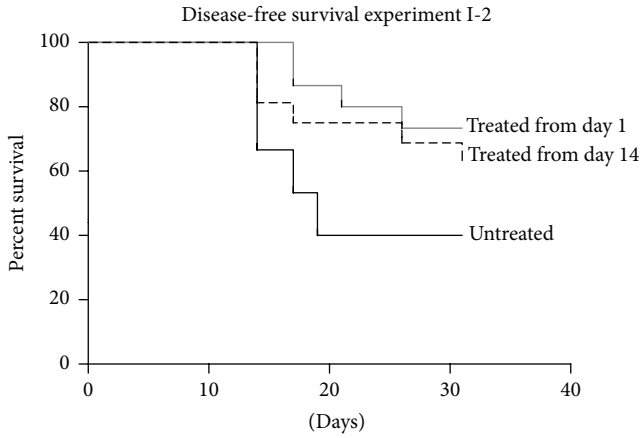

Figure 3: Disease-free survival of 3-month-old Wistar rats upon induction of autoimmune arthritis and treatment with 250 nmol/kg/day of SkQ1 initiated at day 1 or day 14 after arthritis induction. Day 0: induction of arthritis.

Kruskal-Wallis test with Dunn's postcorrection; not significant if compared as disease-free survival in log-rank test). Pathological examination revealed a dose-dependent effect of SkQ1 on all signs of arthritis (Figure 4); however, blind scoring and statistical analysis (Figure 5) revealed that only the effect of SkQ1 on inflammatory infiltration in the group that received 1250 nm/kg/day of SkQ1 was statistically significant (ANOVA/the Kruskal-Wallis test, Dunn's postcorrection, $p < 0.01$). The effect of SkQ1 was much less pronounced than the effect of dexamethasone that was used as a positive control, but it must be noted that at this dose (1.5 mg/kg intramuscular once a week) dexamethasone produced severe weight loss in some animals.

3.4. Effect of SkQ1 on Neutrophil Apoptosis. SkQ1 decreased morphological signs of arthritis even when administration was commenced late, just before the onset of overt disease. At this stage of disease, the pathogenesis is largely driven by infiltration of neutrophils that recognize cellular damage-associated molecular patterns (DAMPs). Multiple regulatory cascades acting on different levels make it hard to investigate distinct molecular pathogenesis pathways. To gain insight into the possible mechanism of SkQ1 action during the late stages of inflammation, we studied its effect on neutrophil apoptosis *in vitro*. Neutrophil apoptosis was delayed by mitochondrial DAMPs (mitochondrial debris, MTD) and promoted by SkQ1 treatment (Figure 6).

4. Discussion

There have been multiple lines of evidence supporting a beneficial role of antioxidants in arthritis and other autoimmune disorders. ROS have been commonly implicated in inflammation signaling (reviewed in [5]). In particular, in the model of autoimmune arthritis, overexpression of extracellular superoxide dismutase (SOD) in mice decreased disease manifestations [23], while a proteomics study implicated downregulation of redox-related proteins during pathogenesis of RA [24]. Despite this background for clinical use of antioxidants, some common antioxidants, such as vitamin E or N-acetylcysteine (NAC), were not effective in RA [10, 25]. Therefore, altering the general antioxidant status might not be sufficient for amelioration of arthritis symptoms. Interestingly, recent *in vitro* data obtained on normal human synoviocytes show that mitochondrial dysfunction induced ROS generation and inflammatory responses that could be prevented by mitochondria-targeted antioxidants [26]. In line with the theoretical predictions, SkQ1 demonstrated efficacy in reducing the key pathological signs of arthritis (inflammatory infiltration, damage to cartilage and bone) in two independent laboratories using different experimental techniques. The results of the two experimental series differed significantly. In Series I, the efficacy was very good, while in Series II the substance had an effect on infiltration by inflammatory cells but had no effect on clinical manifestations of arthritis. Importantly, the accepted clinical scoring scale takes into account mainly the paw swelling and is subject to operator mistakes, which is why the similarities in histopathology data from two series should be emphasized. Of particular importance is the fact that SkQ1 ameliorated the disruption of cartilage and bone in joints of both the paw and the knee. The absence of an effect of SkQ1 on clinical signs of inflammation in Series II is even less surprising given that similar observations (no clinical effect, but a reduction of the cartilage destruction) have been reported for another antioxidant, vitamin E [27].

Several factors could explain the difference between results in Series I and II. Models of autoimmune diseases are very sensitive to many factors, ranging from the animal food supplier to the season, and animal microbiological status constitutes a major factor. In particular, disease is more readily induced in those animals that have had less contact with diverse microflora of a conventional (non-SPF) animal facility. Therefore, we suggest that the difference in the immunological status of the rats used in Series I and Series II experiments could be one explanation for the different efficacy of SkQ1. Another major difference between the two experimental series was the administration technique. While in both series SkQ1 was given *per os*, in Series I it was given with food or into the oral cavity and in Series II it was administered into the stomach via gavage. Bioavailability of SkQ1 is significantly higher upon parenteral administration (data not shown), and, therefore, it is possible that administration of the drug into the oral cavity bypassed the limitations of intragastric administration. Therefore, a more prudent administration technique in Series II could have resulted in a lower amount of the substance being absorbed, explaining the lower apparent efficacy of SkQ1.

SkQ1 efficiently ameliorated pathological signs of experimental autoimmune arthritis in rats in both experimental series. ROS are involved in many steps of inflammation signaling, and so the potential targets of antioxidant therapy are numerous. One obvious target of mitochondrial antioxidants is the NLRP3 inflammasome [28], and, indeed, another mitochondrial rechargeable antioxidant, MitoQ, was

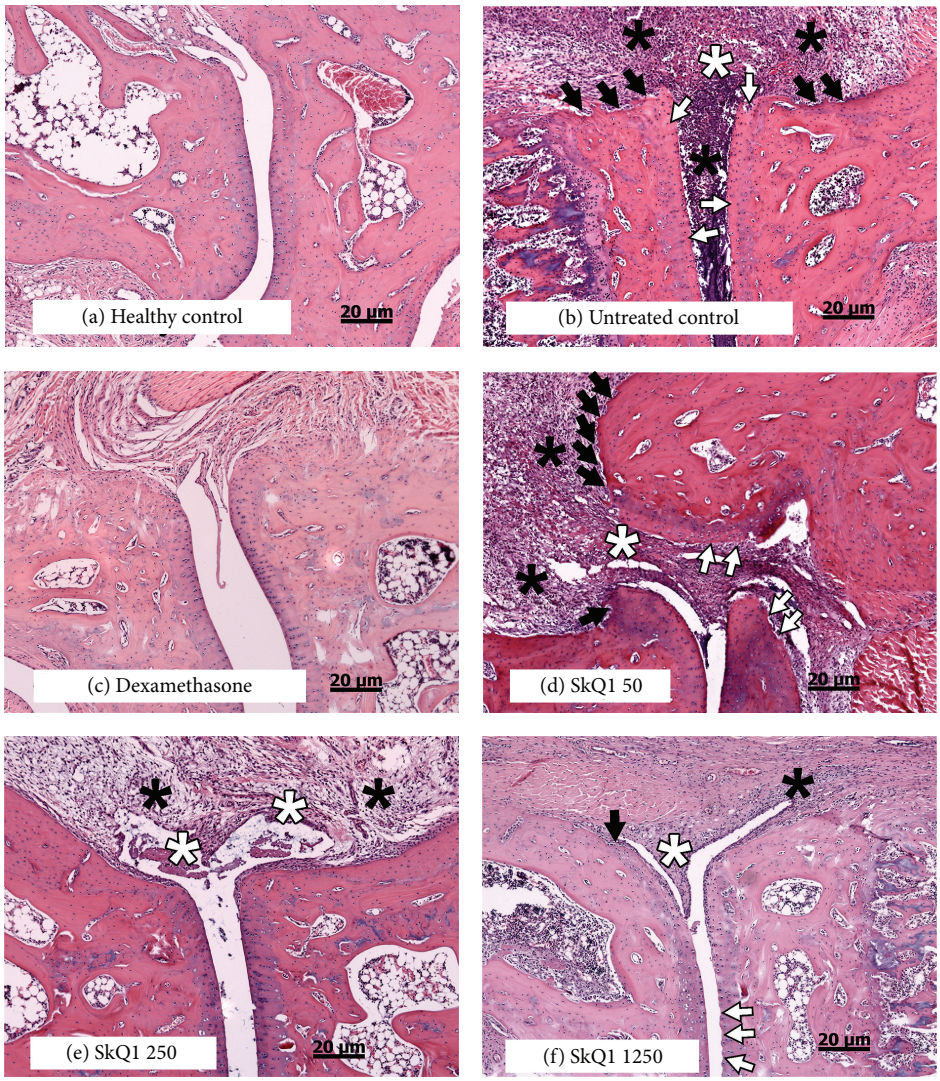

Figure 4: Microscopy of typical pathological lesions of paw joints of treated and untreated rats with experimental rheumatoid arthritis in experiment II. Animals were sacrificed 30 days after arthritis induction. H&E staining, 100x. (a) Normal paw joint, scores: inflammatory infiltration: 0, synovial hyperplasia: 0, cartilage destruction: 0, and bone destruction: 0; (b) untreated control: inflammatory infiltration: 3 (severe inflammatory infiltration), synovial hyperplasia: 3 (massive synovial cells proliferation and in some cases also severe destruction of synovial membrane), cartilage destruction: 2 (necrosis of cartilages), and bone destruction: 3 (numerous resorption lacunae); (c) dexamethasone: inflammatory infiltration: 0, synovial hyperplasia: 0, cartilage destruction: 0, and bone destruction: 0; (d) 50 nmol/kg/day SkQ1: inflammatory infiltration: 3, synovial hyperplasia: 3, cartilage destruction: 3 (destruction of cartilages with formation of pannus), and bone destruction: 3; (e) 250 nmol/kg/day SkQ1: inflammatory infiltration: 2 (moderate inflammatory infiltration), synovial hyperplasia: 2 (foci with more than two layers of cells in synovial membrane), cartilage destruction: 0, and bone destruction: 0; (f) 1250 nmol/kg/day SkQ1: inflammatory infiltration: 1 (mild inflammatory infiltration), synovial hyperplasia: 1 (foci with more than one layer of cells in synovial membrane), cartilage destruction: 1 (erosive surface of cartilages), and bone destruction: 1 (rare resorption lacunae). Inflammatory infiltration in joint tissues and exudation in articular cavity (black asterisks), foci of cartilage destruction (white arrows), bone resorption lacunae (black arrows), and necrotic or hyperplastic synovia (white asterisks).

reported to suppress activation of the NLRP3 inflammasome in a model of dextran sulfate-induced colitis [29]. This mechanism could lead to a complete prevention of arthritis in the pretreatment experiment but could hardly help in treatment (postexposure administration) protocols. However, in the model of autoimmune arthritis, there are likely additional targets of SkQ1. Administration of the compound at the day of disease onset (day 14) in Series I could partially suppress the development of arthritis. At this stage, the initial response to collagen has been long mounted, and the late-stage inflammation has commenced. One known effect of SkQ1 is the inhibition of various stages of the NF-κB-signaling pathway, including IκBa phosphorylation and p65 translocation into the nucleus [30]. This pathway, induced by TNF-α or other stimuli, is one of the key players in RA pathogenesis [31] and therefore may be a target of SkQ1.

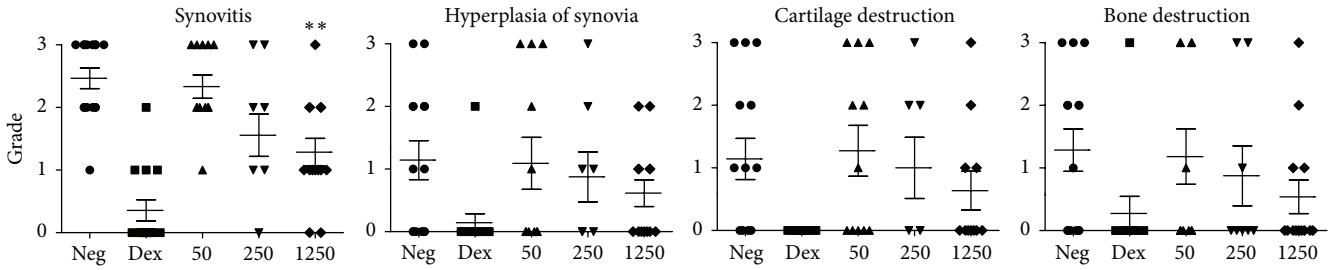

Figure 5: Scoring of pathological findings in SPF Wistar rats in experimental Series II that were treated with various doses of SkQ1 daily from day 1 after arthritis induction and sacrificed 30 days after arthritis induction. Typical lesions and examples of scoring are shown in Figure 4. Several fields of view and slides were examined per each animal. Statistical significance of difference between negative control and pretreated group was tested with Kruskal-Wallis test (not including dexamethasone group) with Dunn's postcorrection; $^{**}p < 0.01$.

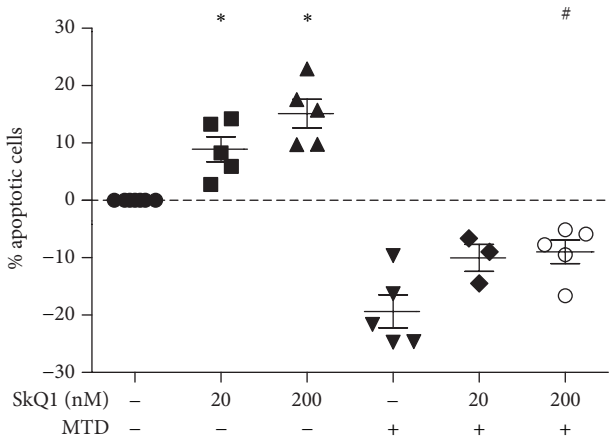

Figure 6: SkQ1 promotes neutrophil apoptosis in absence and in presence of a survival signal provided by mitochondrial debris (MTD). Neutrophils were incubated for 2 h in the absence or presence of 2.5 mg/mL MTD, and then SkQ1 was added at the indicated concentrations. Apoptosis was measured 22 hours after addition of SkQ1. Seven independent experiments were done using blood of different donors. Percent of apoptotic cells in individual donors varied between 49 and 75%. y-axis shows difference between percent of apoptotic cells in control and experimental wells. Not all tests were done for each serum, but each experimental group was reproduced 3–5 times with distinct blood samples; dots represent these distinct experiments. ANOVA with Dunn's postcorrection, $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus MTD-treated cells without SkQ1.

It should be noted that there is a controversy regarding the role of ROS in development of RA, because in certain settings NADPH oxidase (NOX2) generated ROS were shown to have a protective effect in RA [32], and abundance of reduced SH groups was correlated to increased survival of T lymphocytes and their capacity to transfer arthritis [33]. This could explain some controversial results obtained with conventional antioxidants in RA, which could have opposite effects at different stages of disease induction and at different levels of ROS metabolism. High specificity of mitochondria-targeted antioxidants can provide their beneficial role on regulation of inflammation, while avoiding potential controversial effects.

Tissue and cell injury often results in release of intracellular compounds termed damage-associated molecular patterns (DAMPs), which are recognized by the innate immune system and act as endogenous danger signals to promote and exacerbate the inflammatory response. Mitochondrial debris (MTD) have emerged as a potent source of DAMPs [34]. We have shown that the neutrophil apoptosis delay that was caused by the proinflammatory action of MTD was attenuated by SkQ1 (Figure 6). Therefore, SkQ1 may promote resolution of inflammation and prevent progressive tissue destruction. Importantly, SkQ1 prevents apoptotic and/or necrotic cell death in many cellular models at comparable concentrations [12, 21, 35]. So far, induction of apoptosis by SkQ1 at nanomolar concentrations was observed only in neutrophils. Thus, the proapoptotic effect of SkQ1 on neutrophils and the antiapoptotic effect on other cell types could hypothetically cooperate to reduce joint damage in arthritis. It is possible, however, that SkQ1 could also exert its effect on pathogenesis of experimental RA at other levels.

It is also of interest that, in experimental Series I, bone destruction was suppressed much more than the cartilage destruction. Differentiation of osteoclasts is regulated by ROS, probably via an autocrine positive-feedback loop [36], which can make them more responsive to other inflammatory stimuli by costimulating the NF-κB signaling pathway [37]. Correspondingly, antioxidants have been shown to inhibit osteoclast proliferation [38, 39]. The effect of SkQ1 on osteoclast differentiation could add to its protective effect on bone destruction in the RA model.

5. Conclusion

Mitochondrial antioxidant SkQ1 ameliorated inflammatory infiltration and damage to cartilage and bone in a rat model of autoimmune arthritis. These pharmacological effects were observed at a dose that is about 100 times less than the maximum tolerated dose. The magnitude of these effects does not justify use of SkQ1 as a monotherapy of rheumatoid arthritis. It is commonly acknowledged that successful treatment of RA should involve blocking of multiple inflammation signaling pathways, and SkQ1 could be a valuable supplement to the main therapy.

Competing Interests

Maxim V. Skulachev is a board member of Mitotech S. A. company that owns all IP rights for SkQ type compounds. M. V. Skulachev, A. N. Lukashev, R. A. Zinovkin, M. V. Egorov, and M. V. Lovat are inventors but do not have property rights in patents regarding SkQ class compounds.

Authors' Contributions

Alexander A. Andreev-Andrievskiy and Nataliya G. Kolosova contributed equally to the paper.

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Review Article

HCV-Induced Oxidative Stress: Battlefield-Winning Strategy

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About 150 million people worldwide are chronically infected with hepatitis C virus (HCV). The persistence of the infection is controlled by several mechanisms including the induction of oxidative stress. HCV relies on this strategy to redirect lipid metabolism machinery and escape immune response. The 3β-hydroxysterol Δ24-reductase (DHCR24) is one of the newly discovered host markers of oxidative stress. This protein, as HCV-induced oxidative stress responsive protein, may play a critical role in the pathogenesis of HCV chronic infection and associated liver diseases, when aberrantly expressed. The sustained expression of DHCR24 in response to HCV-induced oxidative stress results in suppression of nuclear p53 activity by blocking its acetylation and increasing its interaction with MDM2 in the cytoplasm leading to its degradation, which may induce hepatocarcinogenesis.

1. Introduction

Hepatitis C chronic infection is a major public health issue and continues to make annually around 500.000 deaths due to hepatitis C-related liver diseases [1]. Hepatitis C virus (HCV) is a positive single stranded RNA hepacivirus (family Flavivirus, genus *Hepacivirus*); the genome size is 9.6 kb, flanked by highly conserved untranslated regions (UTRs) at 5′ and 3′ ends and encoding a large polyprotein of 3010 amino acids, that will be co- and posttranslationally processed into 3 structural (core and envelope proteins E1 and E2) and 7 nonstructural (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) mature proteins. The translation of this polyprotein is initiated by an internal ribosomal entry site (IRES) harbored in the 5′-UTR [2]. HCV has a hepatic tropism and a cytoplasmic life cycle; however, it was established that HCV is able to initiate an abortive cycle in dendritic cells (DCs) and B lymphocytes [3, 4]. E1 and E2 envelope proteins play an important role in HCV-hepatocyte attachment and entry and are involved in direct cell surface interactions with cellular receptors. Several cell receptors were identified as being potential HCV cell entry to the cell, mainly transmembrane lectins as dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and its liver and lymphatic endothelium homologue (L-SIGN), scavenger member 1 receptor-class B (SR-BI), tight junction proteins such as claudin-1 (CLDN-1) and occludin (OCLN), cluster of differentiation 81 (CD81) protein, and, recently, the very-low-density lipoprotein receptor (VLDLR) [5, 6]. The entry into the host cell is followed by uncoating and release of the viral genome, translation of viral proteins, replication of the viral genome, and assembly and secretion of virions. All these events take place in the cytoplasm of the host cell. Uncoating allows exposure of the viral genome to cellular mechanisms. The viral RNA serves first as messenger RNA (mRNA) for viral protein translation. This step occurs in the endoplasmic reticulum (ER). After translation of proteins required for viral replication, the viral RNA serves as a template for synthesis of positive single stranded RNA progeny, in association with intracellular membranes. Although data on the late stages of viral replication is limited, it is currently recognized that the assembly and maturation of viral particles occur in the endoplasmic reticulum and in the Golgi apparatus, to be excreted afterward in the extracellular medium and the bloodstream [7, 8]. This scheme lines the direct involvement of HCV proteins in oxidative stress induction. The exact mechanisms triggering the establishment of chronic infection remain little known, although several mechanisms were propounded, mainly the disruption of interferon (IFN) response, inhibition of DCs and natural killers (NK) cell functions,

induction of autophagy, and promoting chronically infected cells throughout modulation of complement system [5, 7, 9]. Several evidences suggest a strong association between HCV chronic infection and metabolic disorders such as steatosis, insulin resistance, and iron load disregulations causing "a specific HCV-associated dysmetabolic syndrome (HCADS)" as introduced by Lonardo in a correspondence to the Editor of Hepatology [10–12], and all these pathologies have been related, in one way or another, to the oxidative stress. Indeed, a complex interconnection between HCV, oxidative stress, insulin resistance, and steatosis exists: HCV-induced oxidative stress affects the insulin signaling in hepatocytes after the dephosphorylation of AMP-activated kinase via activation of protein phosphatase 2A (PP2A), an inhibitor of Akt protein. Insulin resistance in turn may contribute to steatosis by inducing sterol regulatory element binding transcription factor 1 (SREBF1) that will lead to an increase in fatty acid biosynthesis. Likewise, steatosis might exacerbate both insulin resistance and oxidative stress and accelerates the progression of fibrosis [13–16].

Redox signaling is primordial for the proper functioning of the cell, and the generation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) could be a normal process in the life cycle. Nonetheless, the transiently or chronically enhanced production of ROS may disturb the cellular metabolism and its regulation and damage cellular constituents leading to a situation of oxidative stress, defined simply as an imbalance in the ratio of oxidant/antioxidant particles [17]. This imbalance is often due to an increase in oxidant particles production (ROS/RNS) and a deficiency in antioxidant defense, via either deregulation of enzymatic systems (superoxide dismutase (SOD), glutathione peroxidase/reductase (GPX1/GSR), glutathione transferase (GST), thioredoxin reductase (TXNR), catalase (CAT), heme oxygenase (HMOX), peroxiredoxin (PRDX), and paraoxonase 1 (PON1)) or decrease in antioxidant (vitamin C/E, glutathione, carotenoids, flavonoids, transferrin, albumin, bilirubin, and uric acid) [18, 19]. In the liver, ROS/RNS species including superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\bullet}$), nitric oxide (NO), nitrogen dioxide (NO_2), and nitrate (NO_3) are endogenously produced mainly by mitochondria, though other sources could be strongly involved in oxidative stress induction, particularly endoplasmic reticulum, via cytochrome P450 metabolism [19–22]. They could directly interact with biological molecules including proteins, lipids, and DNA or induce hepatocytes and other liver cells damage via fibrosis, apoptosis, or cell necrosis. Indeed, the activation of stress-induced signaling pathways, mainly mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NF-κB) pathways, modulates protein expression and exposes hepatocytes to further oxidative stress [18, 23, 24].

2. Oxidative Stress and Hepatitis C Virus

Based on animal models and culture systems studies, it was shown that HCV expressed proteins seem to impair directly the mitochondrial respiratory chain through an overproduction of ROS, which alter both mitochondria's structure and function of infected hepatocytes [20, 24, 25]. Almost all HCV proteins were involved in the mitochondrial oxidative stress, with a focus on core and NS5A proteins as the main oxidative stress inducers, in opposition to the idea stating that a viral proteins accumulation is needed to induce oxidative stress [19].

Recently, a review from Garofalo et al. discussed the interaction between mitochondrial raft-like microdomains and disialoganglioside GD3 in the regulation of cell apoptosis. GD3 seems to enhance ROS overproduction, leading to an activation of cytochrome c- (Cyt c-) dependent caspase 3 (CASP3), that could be enhanced by the depletion of glutathione [26].

Likewise, it has been admitted that oxidative stress arises from the dysregulation of calcium signaling in the ER/mitochondria junctions [27]. The evident colocalization of core protein with the mitochondria-associated ER membranes (MAMs) might explain the eventual interaction between HCV core protein with subcellular organelles (mitochondria, ER, and MAMs structures) through modification of calcium redistribution. Indeed, it has been shown that the Ca^{2+} influx regulates the mitochondrial metabolism and MAMs structures ensure the transition of Ca^{2+} ions stored in the ER to the mitochondria, which leads to a reprise of homeostasis or a launching of apoptosis process via Fas ligand pathway. MAMs structures have been recently reported to play an essential role in cell response to stress and controlling HCV replication and persistence. The functional depletion of MAMs associated proteins such as sigma-1 receptor (S1R) could modulate both the viral cell cycle and the host response to stress [19, 24, 26, 28]. However, less is known about the role of these structures in the maturation and infectivity of HCV particles.

At another level, the growing fact that mitochondrial dynamics and mitophagy may be affected directly by HCV sheds the light on a new track of HCV immune escape and persistence strategies [29]. In normal cells, the mitochondrial fission, fusion, and mitophagy are tightly monitored in a control quality loop. However, as previously described, HCV promotes Drp1 phosphorylation and its mitochondrial translocation, which leads to mitochondrial fission promotion and the induction of Parkin-dependent mitophagy. These events attenuate apoptosis of HCV-infected cells and support virus secretion, and immune escape and persistence [30, 31].

3. HCV-Induced Oxidative Stress and Liver Diseases

During acute infection, the incubation phase during the first two months of infection is characterized by high rates of HCV viral load in the serum. The increase in enzyme levels transaminases declares the beginning of an acute phase of infection that lasts a few months before clearance of the virus. In 70–75% of cases, immune response fails to eliminate the virus during the acute phase, and the infection persists. The chronic phase is generally characterized by a stable viral load,

relatively normal transaminase levels, and an inflammatory microenvironment [7, 8].

Currently, the growing evidence that HCV-induced oxidative stress significantly contributes to hepatic disease has been supported by several studies on the correlation between markers of oxidative stress and those of liver injury, in particular, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) which are the main seromarkers of liver disease [32, 33]. The vicious circle controlled by HCV and redox-oriented is commonly activated in major forms of chronic liver disease and plays a critical role in hepatic fibrogenesis and carcinogenesis. During the liver disease course, Kupffer cells trigger inflammation process, and it has been shown that HCV modulates inflammatory responses by inducing interleukin (IL)-1β overproduction and secretion by Kupffer cells and that oxidative stress markers have been shown to correlate with severity of inflammation. Besides, it was supported that the induction of immune response by HCV led to the activation of Kupffer cells, which in its turn contribute to the profibrotic cytokines and ROS release, though less is known about the exact mechanisms triggering Kupffer cells-induced oxidative stress and liver damage [24, 34].

Damaged hepatocytes release ROS in the extracellular environment, leading to the activation of hepatic stellate cells (HSCs). In addition to their role in collagen synthesis, these cells were reported as a supplementary source of ROS during fibrosis process. A series of studies have demonstrated that the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in Kupffer cells and HSCs drives the production of ROS, leading to hepatocytes sensitization to fibrogenic and protumorigenic states [18, 35–37].

Unlike hepatitis B virus (HBV), HCV is not considered as a direct oncovirus, does not possess canonical oncogenes, and is unable to integrate into the host genome. However, the chronic inflammatory episode is the main path leading to HCC development [35–40]. Cytokines and ROS released by either nonparenchymal liver cells (Kupffer cells and HSCs) or immune effector cells (macrophages, mast cells, DCs, and NK cells) recruited during inflammatory response are major mediators of protumorigenic state of hepatocytes. Activated Kupffer cells produce tumor necrosis factor-α (TNF-α) which plays a central role in mediation of proinflammatory immune response and recruitment of blood cells to the site of liver injury. Although a heap of data was published on the role of TNF-α in liver disease, when and how this mediator induces intracellular apoptotic or antiapoptotic pathways are still nonanswered questions. The establishment of the chronology in the loop involving ROS release, oxidative stress occurrence, suppression of JAK/STAT pathway, TNF-α production, and induction of NF-κB pathway might enlighten many dark spots on the oxidative stress-induced HCC mechanism [18, 19, 41, 42].

Although the link between oxidative stress and TNF-α production is well established in alcoholic liver disease, it is still not clearly determined in case of HCV-associated liver disease [42].

4. DHCR24 in Response to Oxidative Stress: An Ally or a Belligerent?

The 3β-hydroxysterol Δ24-reductase (DHCR24) is one of the newly discovered host markers of oxidative stress. The gene coding DHCR24 is located on chromosome 1 (1p33-1p31.1), spans 46.4 kb, and comprises 9 exons and eight introns [43]. Rare mutations (E191K, N294T, K306N, and Y471S) in the DHCR24 gene result in an autosomal recessive disease called desmosterolosis that is characterized by elevated levels of desmosterol in the plasma, liver, and kidneys, developmental malformations, and neuropsychological alterations. It is acknowledged that DHCR24 plays a crucial role in maintaining cellular physiology via the regulation of cholesterol synthesis [44, 45] by catalyzing the conversion of desmosterol to cholesterol in the post-squalene cholesterol biosynthesis pathway. Description of its function as a flavin adenine dinucleotide- (FAD-) dependent oxidoreductase was behind the discovery of its pivotal role in stress response. Furthermore, the ER localization of DHCR24 corroborates the assumption of its direct involvement in oxidative stress response, and this localization changes under oxidative and oncogenic conditions [44]. Throughout its multifunctional backbone, DHCR24 exerts a modulating function in the prevention of stress-induced apoptosis when it is reexpressed at high levels and may exert an antioxidant role via scavenging of ROS [37, 44, 46].

Several studies by our group showed that HCV possess a responsive region in the promoter region of DHCR24, in addition to being the binding site of the transcription factor Sp1 recruited in response to oxidative stress (Figure 1). Further studies showed that this specific region is highly controlled by epigenetic mechanisms since it locates on a CpG island on the promoter region of DHCR24 gene [47–49].

HCV-induced oxidative stress engenders an aberrant cholesterol trafficking and lipid metabolism dysregulation, leading to hepatic fibrosis and progression toward end-stage liver disease, ultimately hepatocellular carcinoma (HCC) which is the most frequent primary liver tumor [50]. The molecular epidemiology of HCC has been characterized by a singular variability between geographic regions depending on several factors, especially risk factors prevailing in each region. In the far eastern region, the region with the highest prevalence of HCC, the prevailing risk factors are HBV/HCV chronic infections [51]. By determining the region-specific molecular context of HCC, many questions could be resolved and many advances in the therapeutic approaches could be achieved. Though remarkable progress has been achieved in cancer molecular studies, mechanisms triggering hepatocarcinogenesis remain poorly known. Initiation of tumoral process needs, typically, a promoting agent which could be viral proteins themselves or viral-induced alterations that could lead to the liver tumorigenesis. Altered hepatocytes with the highest proliferative potential are the origin of the malignancy [52]. Several pathways were propounded to be altered in liver carcinogenesis. However, since 1989, the most studied gene in the class of tumor suppressor genes has been TP53, the gene encoding the phosphoprotein p53 triggering cell cycle checkpoints, apoptosis, senescence, and

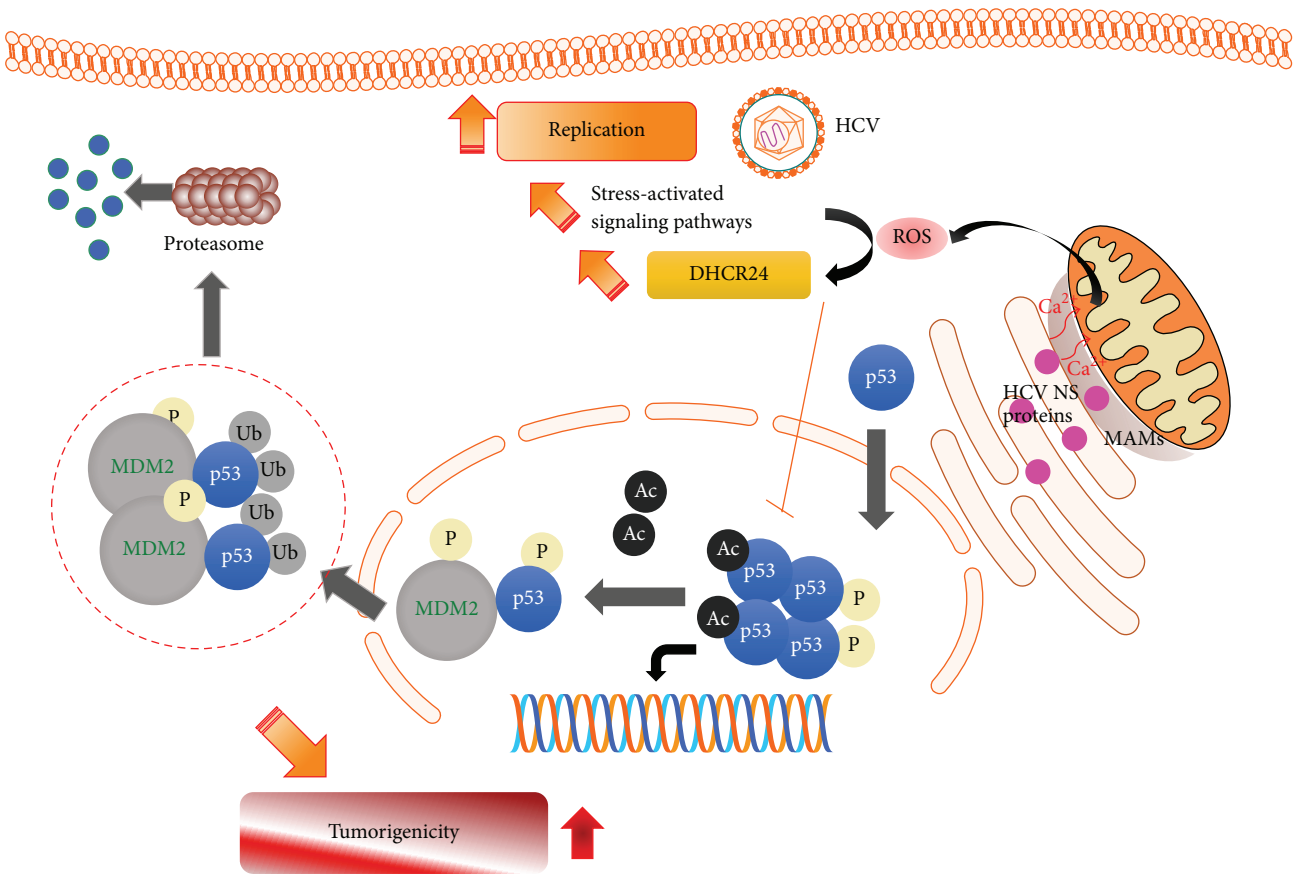

Figure 1: HCV-induced oxidative stress and HCC inception. HCV nonstructural (NS) proteins induced oxidative stress throughout disturbance of mitochondrial metabolism. The generation of ROS activates stress-induced signaling pathways that enhance the overexpression of DHCR24, a pivotal protein for HCV replication and HCC induction. HCV: hepatitis C virus, HCC: hepatocellular carcinoma, ROS: reactive oxygen species, DHCR24: 3β-hydroxysterol Δ24-reductase.

DNA repair, by regulating expression of several target genes [53]. P53 pathway consists of a network of proteins that are induced in response to a signal of intrinsic or extrinsic stress. The second most studied mechanism is related to the regulation of P53 by E3 ubiquitin protein ligase (MDM2) protein. This oncoprotein targets, with high specificity, the P53 protein by binding to its N-terminal transactivator domain and induces its proteasomal degradation. MDM2 gene expression is induced by the wild form of the protein P53 itself through its binding to the first intronic region of MDM2 gene: the P53/MDM2 interaction is a feedback loop regulating the concentration of active p53 [54, 55]. Our data showed that DHCR24 is specifically expressed on the surface of HCV-HCC cell lines and that high levels of anti-DHCR24 antibodies were detected in the sera of patients with HCV-related HCC. These data demonstrate that overexpression of DHCR24 in HCC is specifically induced by HCV. Furthermore, overexpression of DHCR24 impaired p53 activity by suppression of acetylation and increased interaction with MDM2 protooncogene. This impairment of p53 suppressed the hydrogen peroxide-induced apoptotic response in hepatocytes and resulted in upregulation of tumorigenicity in hepatocytes [46, 56].

It has been demonstrated that DHCR24 expression was upregulated in HCC cell lines and tissues from IFN non-responders LC and HCC patients. In a recent work of Ezzikouri et al., a set of patients with HBV/HCV ongoing liver disease has been analyzed for serum DHCR24 antibodies using enzyme-linked immunosorbent assay. The serum anti-DHCR24 antibodies levels were significantly higher in patients with chronic hepatitis C (CHC) than in healthy controls and, interestingly, in early HCV-induced HCC than CHC or liver cirrhosis (LC) patients and in late HCV-induced HCC compared to early HCC-C patients, which demonstrates a stage-related overexpression of DHCR24. The merits gained by DHCR24 as a novel biomarker of HCV-induced HCC rely on the fact that the sensitivity of anti-DHCR24 antibodies detection was shown to be higher than that of other biomarkers (alpha-fetoprotein and protein induced by vitamin K absence or antagonist-II) and that DHCR24 was upregulated in HCV-positive, but not HBV-positive, tissues or HBV/HCV negative HCC specimens [57]. Other data show that DHCR24 enzymatic activity in the cholesterol transport process triggers the DHCR24 surface expression and that DHCR24 possess a binding site of 2-152a MAb that exerts anti-HCV activity and induce the

downregulation of DHCR24 surface expression, shedding the light on the potential function of HCC-surface expressed DHCR24 as carrier of target antitumor agents [46, 58].

The importance of DHCR24 as a novel biomarker of interest rises from its usefulness for early detection of disease progression and specifically HCC. On the other hand, it might represent a new target for HCC therapy leaning on its property of binding to 2-152a MAb, which may be promising tool in the future for the HCC targeting approaches [46]. Nonetheless, there is still too much to learn about DHCR24 biology in the context of HCC, especially posttranslational modifications, how it could affect the HCV life cycle and the disease progression and the human population context, and how it determines DHCR24 expression and functional profile.

5. Conclusion

It is well known that HCV exploit the host lipid machinery to replicate and spread. The available data suggest that HCV-infected cells may become antiapoptotic and replicate efficiently to establish chronic infection through overexpression of DHCR24. Thus, the HCV-induced oxidative stress responsive protein DHCR24 may play a critical role in the pathogenesis of not only HCV persistent infection, but also associated liver diseases as steatosis, steatohepatitis, and cirrhosis. The role of DHCR24 in HCV-associated liver diseases lies in its aberrant expression altering programmed cell death pathways. The sustained expression of DHCR24 in response to HCV-induced oxidative stress results in suppression of nuclear p53 activity by blocking its acetylation and increasing its interaction with MDM2 in the cytoplasm leading to its degradation, which may induce hepatocarcinogenesis.

In conclusion, DHCR24 represents a potential marker of HCV-induced HCC development and might be a prospective target to HCC treatment in regions with high prevalence of HCV- induced HCC.

Abbreviations

ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
CAT: Catalase
CD81: Cluster of differentiation 81
CLDN-1: Claudin-1
Cyt c: Cytochrome c
DC: Dendritic cell
DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
DHCR24: 3β-Hydroxysterol Δ24-reductase
ER: Endoplasmic reticulum
GPX1: Glutathione peroxidase
GSR: Glutathione reductase
GST: Glutathione transferase
H_2O_2: Hydrogen peroxide
HBV: Hepatitis B virus
HCADS: HCV-associated dysmetabolic syndrome
HCC: Hepatocellular carcinoma
HCV: Hepatitis C virus
HMOX: Heme oxygenase
$HO^{\bullet}$: Hydroxyl radicals
HSCs: Hepatic stellate cells
IRES: Internal ribosomal entry site
MAMs: Mitochondria-associated ER membranes
MAPK: Mitogen-activated protein kinases
MDM2: MDM2 protooncogene, E3 ubiquitin protein ligase
NADPH: Nicotinamide adenine dinucleotide phosphate
NF-κB: Nuclear factor kappa B
NK: Natural killer
NO: Nitric oxide
NO_2: Nitrogen dioxide
NO_3: Nitrate
NS: Nonstructural
$O_2^{\bullet-}$: Superoxide anions
OCLN: Occludin
PON1: Paraoxonase 1
PP2A: Protein phosphatase 2A
PRDX: Peroxiredoxin
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
S1R: Sigma-1 receptor
SOD: Superoxide dismutase
SR-BI: Scavenger member 1 receptor-class B
SREBF1: Sterol regulatory element binding transcription factor 1
TNF-α: Tumor necrosis factor-α
TXNR: Thioredoxin reductase
UTR: Untranslated region
VLDLR: Very-low-density lipoprotein receptor.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Basal Glutathionylation of Na,K-ATPase α-Subunit Depends on Redox Status of Cells during the Enzyme Biosynthesis

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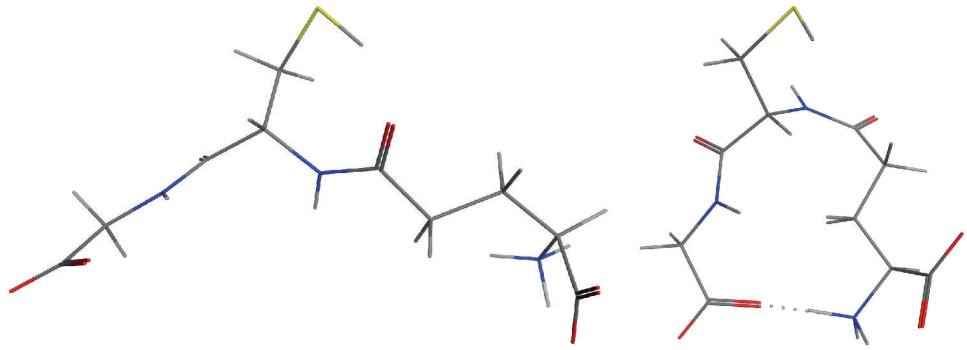
Many viruses induce oxidative stress and cause S-glutathionylation of Cys residues of the host and viral proteins. Changes in cell functioning during viral infection may be associated with glutathionylation of a number of key proteins including Na,K-ATPase which creates a gradient of sodium and potassium ions. It was found that Na,K-ATPase α-subunit has a basal glutathionylation which is not abrogated by reducing agent. We have shown that acute hypoxia leads to increase of total glutathionylation level of Na,K-ATPase α-subunit; however, basal glutathionylation of α-subunit increases under prolonged hypoxia only. The role of basal glutathionylation in Na,K-ATPase function remains unclear. Understanding significance of basal glutathionylation is complicated by the fact that there are no X-ray structures of Na,K-ATPase with the identified glutathione molecules. We have analyzed all X-ray structures of the Na,K-ATPase α-subunit from pig kidney and found that there are a number of isolated cavities with unresolved electron density close to the relevant cysteine residues. Analysis of the structures showed that this unresolved density in the structure can be occupied by glutathione associated with cysteine residues. Here, we discuss the role of basal glutathionylation of Na,K-ATPase α-subunit and provide evidence supporting the view that this modification is cotranslational.

1. Introduction

Viral infections lead to disruption of the redox status of mammalian cells. As a rule, the infections cause oxidative stress [1] and change the thiol redox status, which depends on the ratio of reduced (GSH) and oxidized (GSSG) glutathione [2, 3]. Under normal conditions, the reduced glutathione level in cells (1–5 mM) is 100-fold higher than the oxidized glutathione level. Under oxidative stress, this ratio can be reduced to 1 [4]. The shift of intracellular redox conditions to the oxidized state induces protein glutathionylation that protects the thiol groups of proteins from irreversible oxidation and changes their function [5, 6]. Many viruses that cause oxidative stress induce glutathionylation not only in host proteins but also in their own viral proteins. For example, the HIV-1-protease critical for viral maturation is activated by glutathionylation [7, 8]. Changes in functioning of cells during viral infection may be associated with glutathionylation of a number of key proteins [5, 6]. Notably, the acute (Flu) and chronic (hepatitis) viral infections can lead to the activation of factor Nrf/ARE [9, 10], which causes activation of glutathione transferase participating in glutathionylation of proteins [11, 12].

Na,K-ATPase creates a gradient of sodium and potassium ions necessary for all living mammalian cells. This protein is sensitive to changes in the redox status of cells [13–17]. Earlier we have shown that glutathionylation of Na,K-ATPase catalytic α-subunit is a determinant of the enzyme redox sensitivity [13]. Four cysteines of Na,K-ATPase α-subunit can undergo regulatory glutathionylation in case of GSSG increase, which leads to reversible inactivation of the enzyme, preventing exhaustion of ATP in the cells under oxidative stress. In addition to the regulatory glutathionylation of Na,K-ATPase α-subunit, its basal glutathionylation was found which is not removed by DTT [13]. This leads to the suggestion that the basally glutathionylated cysteine residues are located in a region of protein structure inaccessible to solvent. Basal glutathionylation was identified for



(a) (b)

Figure 1: Three-dimensional models of glutathione used for the Na,K-ATPase, GSH modelling. (a) "Linear" model with elongated shape. (b) "Compact" model, structure forms a loop with hydrogen bond formed between the ends of the molecule.

the Na,K-ATPase α-subunit from various organisms, duck, mole rats, rabbit, and rat, in both cell lysates and purified enzyme preparations [13, 17, 18]. Basal glutathionylation is described for a variety of proteins: inhibitory kappa kinase beta IKKβ, actin, and aldose reductase [5, 6]. Although basal glutathionylation of proteins is a widespread phenomenon, its role remains unclear. Despite the fact that the number of proteins with experimentally observed glutathionylation is growing rapidly [6], there is very small number of crystallographic structures with the identified bounded glutathione; that is, glutathione molecules are not reported in the existing X-ray structures of Na,K-ATPase α-subunit. Identification of glutathione in the structures of glutathionylated proteins is a common problem and a number of approaches are used to achieve this, as in the structure of mitochondrial ABC transporter Atm1 where authors compared the free and glutathione-bound protein structures to prove the presence of bonded glutathione [19]. They showed that there is unresolved electron density in the glutathione-bound protein, and it corresponds to the glutathione molecule. Based on this work, we have assumed that associated glutathiones in the X-ray structures of α-subunit of Na,K-ATPase should be shown as regions with unresolved density. We have analyzed all X-ray structures of the Na,K-ATPase α-subunit from pig kidney and found that there are a number of isolated cavities with unresolved electron density close to the relevant cysteine residues. Analysis of the structures showed that the unresolved density in the structure can be occupied by glutathione associated with cysteine residues. We have shown that acute hypoxia leads to increase of total glutathionylation level of Na,K-ATPase α-subunit; however, basal glutathionylation of α-subunit increases under prolonged hypoxia only. We have found that basal glutathionylation could be removed from fully denatured enzyme only. In this paper, we discuss the role of the basal glutathionylation and provide evidence supporting the view that this modification is cotranslational.

2. Materials and Methods

2.1. Modelling. Models of the glutathionylated Na,K-ATPase were constructed by COOT program using 3B8E, 3KDP, 3WGU, 3WGV, and 4HYT (PDB ids) structures as templates [20–22]. Two model structures of glutathione, the "linear" and "compact," were built and minimized (Figure 1). In order to create models of the glutathionylated Na,K-ATPase, a corresponding model structure of glutathione was manually inserted into the cavity with unresolved electronic density (negative density and blob) using COOT. We assumed that the cavity was isolated when the distance between atoms of the residues forming the cavity was less than the sum of two van der Waals radii. Initially, GSH molecule has been placed in the cavity to verify conformity of the size of GSH molecule and the cavity. Subsequently, the GSH molecule was positioned in the cavity so as to form disulfide bond with a cysteine residue inside the cavity, and there were no structure overlaps. The resulting models of Na,K-ATPase with the S-S bonded glutathione molecule were locally minimized in the MMFF94x force field with the MOE program, version 2009.10. This force field accounts for atomic charges and hydrogen and ionic bonds between neighboring atoms. RMSD values of templates and glutathionylated models were calculated with MOE program (all atom types and parameters of symmetry were taken into account). Analysis of the presence of disulfide bonds in the Na,K-ATPase α-subunit structures was performed using DCCP program [23].

2.2. Cell Culture. Murine fibroblast cells (SC-1 cell line) were grown on DMEM media containing 10% FBS (Fetal Bovine Serum), 2 mM L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humid atmosphere with 5% CO_2. SC-1 cells were grown either for 3.5 h or for 96 h, each at 20% and 0.2% pO_2 (gas mixture contains 5% CO_2 and nitrogen). Cells were lysed in cold RIPA buffer (containing 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail 6 mg/mL, and 25 mM TrisHCl (pH 7.6)) for 60 minutes at 4°C. Cell lysates were cleared by centrifugation at 16 000 g for 10 minutes. Supernatants were collected. Cell lysates were incubated with or without 25 mM reducing agent TCEP (Tris(2-carboxyethyl)-phosphine) (Thermo Scientific) soluble in Tris buffer, pH

7.4, during 30 min at 37°C. Proteins were separated on SDS-PAGE and then α1-subunit of Na,K-ATPase was detected by immunoblotting.

2.3. Immunoblotting. The level of S-glutathionylation of Na,K-ATPase α1-subunit was estimated using immunoblotting. Proteins of cell lysates were separated on SDS-PAGE and transferred to a PVDF membrane. After the blocking procedure, mouse monoclonal anti-glutathione antibody (Chemicon Millipore, MAB5310) was added. Mouse monoclonal anti-Na,K-ATPase α1 antibody clone C464-6 (Upstate Millipore) was applied to detect total amount of α1-subunit, followed by horseradish peroxidase-conjugated secondary antibodies. Membrane was stained using a commercial kit SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific) and chemiluminescence was detected using Bio-Rad ChemiDoc MP instrument. Densitometric analysis was performed by Image Lab (Bio-Rad) program and the results were represented as ratio of glutathionylated α-subunit to total α-subunit band intensity ((GSS-α1)/total α1). The comparison was made between samples applied on the same membrane. The ratio of the bands (GSS-α1)/total α1 in control was taken as 1.

2.4. Immunoprecipitation. Immunoprecipitation was performed on lysates of SC-1 cells. Anti-Na,K-ATPase α1 antibody (3 μg) was added to the cell lysate and the sample was incubated for 15 minutes at 4°C and constant agitation. The resulting immune complex was added to a tube containing protein A agarose and incubated for 2 h at 4°C and constant agitation. The sample was then centrifuged for 1 min at 15000 g and the supernatant was removed. The precipitate was washed with PBS for three times and then heated with 4x Laemmli buffer containing 8 M urea and 8% SDS at 80°C for 5 min to elute the protein. The sample was centrifuged and the supernatant was collected. The supernatant containing Na,K-ATPase α1-subunit was divided into two parts. One part was incubated without and another with 25 mM TCEP dissolved in Tris buffer, pH 7.4, during 30 min at 37°C. Glutathionylation of Na,K-ATPase α1-subunit in samples was detected by immunoblotting.

3. Results and Discussion

3.1. Structures of Na,K-ATPase from Pig Kidney Have Isolated Cavities with Unresolved Density near Several Cysteine Residues. There are eight structures of Na,K-ATPase from pig kidney, but only five of them have resolution better than 4 Å (Table 1), which allow for identifying the isolated cavities containing unresolved electron density near cysteine residues. In these structures, the isolated cavities were identified close to cysteine residues Cys 204, Cys 242, Cys 336, Cys 349, Cys 367, Cys 421, Cys 452, Cys 456, Cys 457, Cys 511, Cys 549, Cys 577, Cys 656, Cys 599, and Cys 698 of Na,K-ATPase α-subunit (Table 2). It has been found that the SH groups of pairs of cysteine residues were oriented into the same cavities: Cys 204, Cys 242; Cys 367, Cys 698; Cys 452, Cys 456 (457); Cys 511, Cys 549. However, using the DSSP program [23], we have shown that there are no disulfide bonds in all structures of Na,K-ATPase α-subunit.

To identify glutathione molecules bonded with cysteine residues, we searched the cavities with unresolved electron density. Unresolved density is a region of relatively high residual electron density that cannot be explained by presence of water [25]. Large areas of unresolved density were found in closed cavities near cysteines by COOT program [25]. The greatest number of areas with unresolved density, similar in shape and size to glutathione molecule, was found near the residues Cys 204, Cys 242; Cys 367, Cys 698; Cys 452, Cys 456, Cys 457; and Cys 599. It should be noted that there is no unresolved density near Cys 421 in all these structures, which coincides with the mass spectrometry data indicating that cysteine residue 421 (Cys 423 of duck α1) has never been found S-glutathionylated [13].

We have analyzed which of the cysteine residues in the pair may be more accessible for glutathionylation. According to [4, 26, 27], cysteine residues located close to positively charged residues (arginine, lysine, and histidine) are more accessible for glutathionylation because they attract the electron density, increasing the probability of glutathione binding. On the contrary, negatively charged residues close to cysteine reduce the probability of glutathionylation. Analysis of the amino acid composition near Cys residues showed that in the pairs of Cys residues pointing into the same cavity one of the cysteines had a positively charged amino acid in its close vicinity, whereas the other Cys had a negatively charged residue (Table 3). Considering these data, cysteine residues 204, 452, 599, and 698 are preferable for glutathionylation. The mass spectrometry data obtained earlier confirmed basal glutathionylation of residues 204 and 698 [13], but it was not possible to assess glutathionylation of residues 452 and 599.

3.2. Glutathione Fits into the Identified Isolated Cavities and Areas with Unresolved Electron Density. Models of the Na,K-ATPase α-subunit with glutathione in isolated cavities were built manually using COOT to verify fitting of glutathione molecules to the cavities. For this purpose, two models of glutathione molecule were used (Figure 1), which were placed in isolated cavities avoiding overlap with the resolved parts of structure. Glutathione molecule was placed in isolated cavities to fit unresolved densities, and thiol group of glutathione has been directed towards the thiol group of a cysteine in the cavity. Areas of unresolved electron density, large enough to insert a glutathione molecule, were found in a number of isolated cavities in Na,K-ATPase near the cysteine residues listed in Table 4. It was found that the electron density capable of accommodating glutathione molecule by shape and size is located in cavities near the cysteine residues Cys 204, Cys 242 (Figure 2) (3WGU); Cys 452, Cys 456, and Cys 457 (Figure 3) (3WGU, 3KDP, and 3B8E); Cys 698, Cys 367 (Figure 4) (3WGU); and Cys 599 (Figure 5) (3WGU, 3KDP, 3B8E, and 4HYT) (Table 4). The role of these Cys residues in the functioning of the enzyme was evaluated previously with point mutagenesis [24]. Replacing one of Cys 367 and Cys 698 residues or all three Cys residues (452, 456, and 457) with alanine or serine resulted in reduced activity by more than

Table 1: Structures of Na,K-ATPase from pig kidney with resolution better than 4 Å.

PDB ID	3B8E	3KDP	3WGU	3WGV	4HYT
Resolution	3.50 Å	3.50 Å	2.80 Å	2.80 Å	3.40 Å
Species, organ	Pig kidney	Pig kidney	Pig kidney	Pig kidney	Pig kidney
Conformation	E2P	—	E1P	E1P with oligomycin	E2P with ouabain
Method	Vapor diffusion and hanging drop	Vapor diffusion	Vapor diffusion and hanging drop	Vapor diffusion and hanging drop	Hanging drop
pH	7.0	7.0	6.2	6.2	6.2
Temperature	292.0 K	292.0 K	283.0 K	283.0 K	292.0 K
Details	14% PEG 2000 mme, 0.2 M choline chloride, 4% glycerol, 4% MPD, 0.04 M DTT, and 0.1–0.4% beta-DDM	14% PEG 2000 mme, 0.2 M choline chloride, 4% glycerol, 4% MPD, 0.04 M DTT, and 0.1–0.4% beta-DDM	Na,K-ATPase was incubated with a buffer, 150 mM NaCl, 1 mM $AlCl_3$, 4 mM NaF, 4 mM ADP, 3 mM $MgCl_2$, 2 mM glutathione, and 20 mM MOPS/n-methyl-D-glucamine (NMDG), pH 7.1, and treated with 1.95% (w/v) octaethylene glycol mono-n-dodecyl ether (C12E8) at a mass ratio (C12E8/protein) of 1.3 and separated from the insoluble fraction by centrifugation at 200.000 g and 10 uC; 17.5% PEG 2000 mme, 10% glycerol, 200 mM NaCl, 50 mM MES-NMDG	Na,K-ATPase was incubated with a buffer, 150 mM NaCl, 1 mM $AlCl_3$, 4 mM NaF, 4 mM ADP, 3 mM $MgCl_2$, 2 mM glutathione, and 20 mM MOPS/n-methyl-D-glucamine (NMDG), pH 7.1, with 0.25 mM oligomycin A and treated with 1.95% (w/v) octaethylene glycol mono-n-dodecyl ether (C12E8) at a mass ratio (C12E8/protein) of 1.3 and separated from the insoluble fraction by centrifugation at 200.000 g and 10 uC; 17.5% PEG 2000 mme, 10% glycerol, 200 mM NaCl, 50 mM MES-NMDG	16-17% PEG 2000 mme, 10% glycerol, 200 mM $MgCl_2$, 100 mM MES-NMDG, pH 6.2, 100 mM urea, 5% *tert*-butanol, 5 mM DTT, and 1.6 mM sucrose monodecanoate
Reference	[20]	[20]	[21]	[21]	[22]

75%, while replacing Cys 204 and Cys 599 residues resulted in reduced activity by more than 50% (Table 4). This indicates that these Cys residues are not critical for the enzyme activity but their glutathionylation could play an important role in regulating the protein function. Substitution of Cys 242 to Ala or Ser residues was lethal to the cells, suggesting the central role of this residue for the functioning of Na,K-ATPase. According to our data, Cys 242 is one of the residues undergoing regulatory glutathionylation in oxidative stress [13].

The mutagenesis data allowed for concluding that the presence of disulfide bridges is not required for folding of the Na,K-ATPase α-subunit and its subsequent activity [24]. Indeed, analysis of the available structures shows that there are no disulfide bonds between the cysteine residues. Moreover, according to our data, closely located cysteine residues

Table 2: Unresolved electron density close to cytosolic cysteine residues in X-ray structures of Na,K-ATPase α-subunit.

PDB ID/Cys position	3WGU	3WGV	3KDP	4HYT	3B8E	S-glutathionylation[a]
204, 242	Density[b]	Density	Small size of density[c]	Small size of density	Small size of density	+
336	Distant density[d]	Distant density	Density	Density	Distant density	+
349	Fragmented density[e]	Fragmented density	nd[f]	nd	nd	+
367, 698	Density	Fragmented density	Distant density	nd	nd	+
421	nd	nd	Small size of density	nd	nd	–
452, 456	Density	Fragmented density	Density	Fragmented density	Density	+
457	Surface	Surface	Surface	Surface	Surface	+
511, 549	Density	Small size of density	nd	nd	nd	+
656	Fragmented density	Fragmented density	nd	nd	nd	+
599	Density	Fragmented density	Density	Density	Density	Not detected
577	Surface	Surface	Surface	Surface	Surface	Not detected

[a] According to MALDI-TOF mass spectrometry data in [13].
[b] "Density": unresolved density that can fit by glutathione.
[c] "Small size of density": unresolved density but too small to fit glutathione.
[d] "Distant density": unresolved electron density at a distance of more than 5 Å from Cys (glutathione fits density, but the density is far from the residue).
[e] "Fragmented": no intact unresolved density.
[f] "nd": no unresolved density close to the residue.

Table 3: Amino acid composition of porcine Na,K-ATPase α-subunit near Cys residues.

Cys position	Neighboring amino acid residues
204	NGCKV
242	TNCVE
367	TICSD
698	EGCQR
452	LKCIE
456, 457	ELCCGS
599	GKCRS

which could form disulfide bond are directed into the cavity containing glutathione. Thus, it can be hypothesized that glutathionylation prevents formation of the intramolecular SS bridges.

3.3. Energy Minimization of the Models. To verify the obtained data, an SS bond has been formed between the glutathione molecule and the nearest cysteine residue and energy minimization of the obtained models carried out using the MOE program. After minimization, in 8 out of 9 models, glutathione not only stayed within the unresolved density but has moved to a more consistent location in the given density. Only in a single case out of nine, the glutathione molecule after minimization was partially located outside the unresolved density (3WGU, Cys 452). Changes in the Na,K-ATPase structure after minimization are given in Table 5. RMSD between the initial and minimized Na,K-ATPase structures is in the range of 0.05–0.09 Å, indicating that that minimization with the bound glutathione does not result in significant changes of the Na,K-ATPase structure.

We have compared the distances between sulfur atoms in the pairs of the cysteines in the initial structure of the protein (3WGU) and in the model structures (obtained by us) with glutathione attached to one of the cysteine residues (Table 6). This comparison revealed that glutathione binding has little effect on the distance between sulfur atoms in the Cys pairs. After local minimization, RMSD value between the initial and glutathionylated Na,K-ATPase structures was smaller then RMSD value between the initial structure of Na,K-ATPase and Na,K-ATPase with Cys-S-S-Cys bridges (Table 5). Thus, shifting cysteine residues towards each other until the distance between them reached SS bridge length (2.04 Å) and subsequent formation of disulfide bridges make greater changes in the structure of the molecule than incorporation of glutathione. Introduction of glutathione in the regions of protein with unresolved density has almost no effect on protein structure and location of cysteine residues determined by crystallography.

Table 4: Cysteine residues with unresolved GSH-like electron density and effect of their substitution by alanine or serine on Na,K-ATPase activity.

PDB ID/Cys position	3WGU	3WGV	3KDP	4HYT	3B8E	Na,K-ATPase activity according to [24], mutant/wild type, %
204	Density	Density	Small density	Small density	Small density	49.2
242						—[a]
367	Density	Fragmented density	Distant density	nd	nd	23.4
698						22.9
452	Density	Fragmented density	Density	Fragmented density	Density	25.3[b]
456						
599	Density	Fragmented density	Density	Fragmented Density	Density	43.5

Abbreviations and all other details are given in the legend of Table 2.
[a]Cells with Cys 242 Ala or Cys 242 Ser mutant of Na,K-ATPase did not survive under ouabain selective pressure.
[b]Activity of Cys 452, Cys 456, and Cys 457 Ser mutant of Na,K-ATPase.

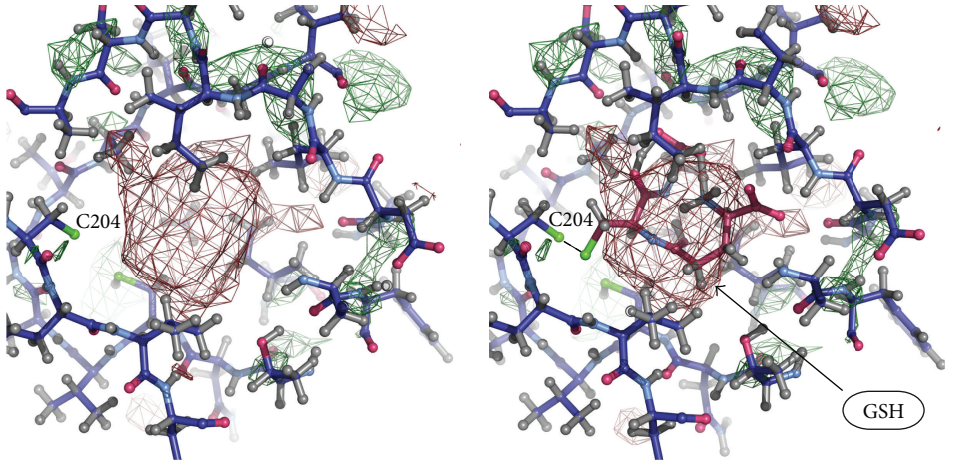


Figure 2: Part of the Na,K-ATPase α1-subunit near Cys204 and Cys242 (PDB code 3WGU). (a) Unresolved density in the isolated cavity. (b) GSH incorporated into the unresolved density.

Table 5: RMSD between initial (PDB ID 3WGU) and model structures of Na,K-ATPase with SS bridges between pair of Cys residues (SS RMSD) and between Cys residue and glutathione (Cys-SSG RMSD) after local and global minimization.

Pairs of Cys residues	SS RMSD, Å local minimization[a]	Glutathionylated Cys	Cys-SSG RMSD, Å local minimization[a]
204–242	0.12	204	0.09
367–698	0.14	698	0.08
452–456	0.12	452	0.05

[a]Local minimization: minimization within a radius of 4.5 Å from the thiol groups of each cysteine residue in MMFF94x force field.

3.4. Prolonged Incubation of Cells under Hypoxic Conditions Alters the Basal Level of Na,K-ATPase Glutathionylation. It can be hypothesized that the basal glutathionylation depends on the redox status of cells during protein folding. To test this, we incubated SC-1 cells at 0.2% and 20% pO_2 within 3.5 h and 96 h. Then, cell lysates were treated with the reducing agent TCEP (25 mM) for 30 min. Acute hypoxia (3.5 h) leads to increase of total glutathionylation level of Na,K-ATPase α-subunit (Figure 6(a)) that corresponds to our data, obtained earlier [13, 28]. However, basal glutathionylation of α-subunit does not change at these conditions (Figure 6(b)). In contrast, at prolonged hypoxia (96 h), basal level of Na,K-ATPase α-subunit glutathionylation was significantly higher than that under 20% pO_2 (Figure 6(c)). Since the basal glutathionylation is not removed by reducing agents and the glutathionylated cysteines are located in isolated cavities, we assume that the reaction of glutathionylation occurs during protein folding. If so, then the basal glutathionylation can be removed only from the unfolded protein. We

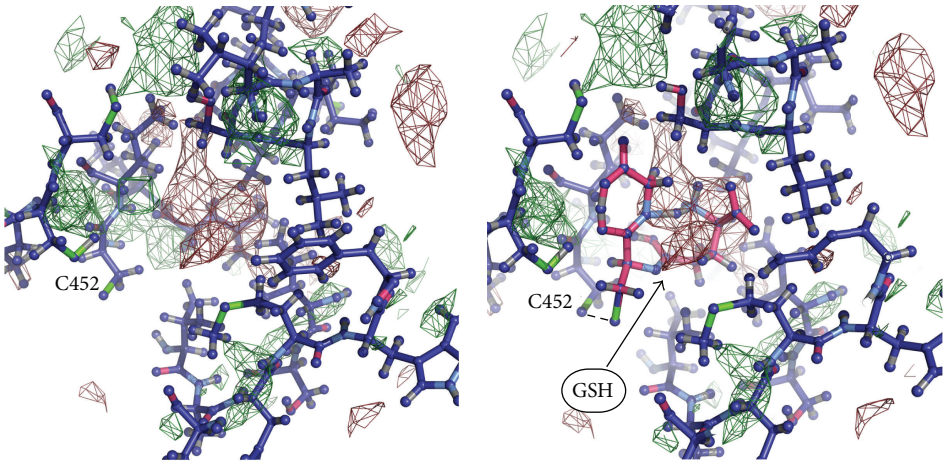


Figure 3: Part of Na,K-ATPase α1-subunit near Cys452 and Cys456 (PDB code 3WGU). (a) Unresolved density in the isolated cavity. (b) GSH incorporated into the unresolved density.

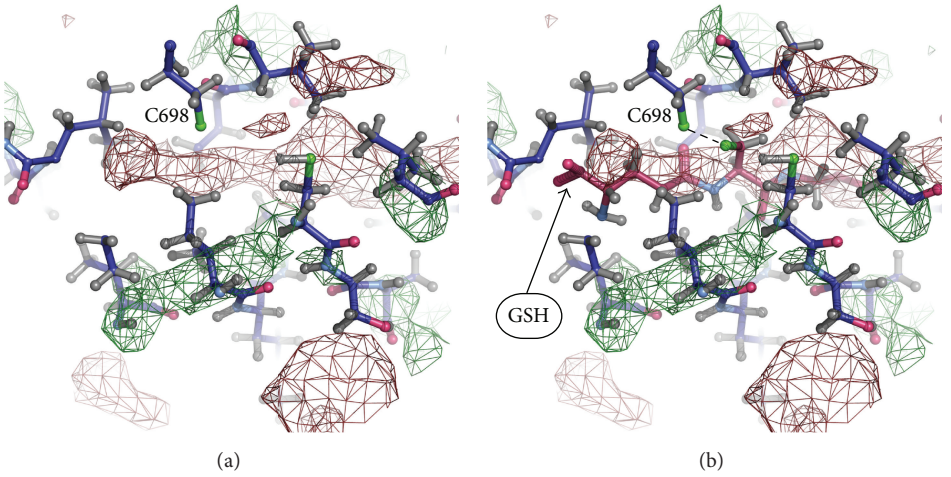


Figure 4: Part of Na,K-ATPase α1-subunit near Cys367 and Cys698 (PDB code 3WGU). (a) Unresolved density in the isolated cavity. (b) GSH incorporated into the unresolved density.

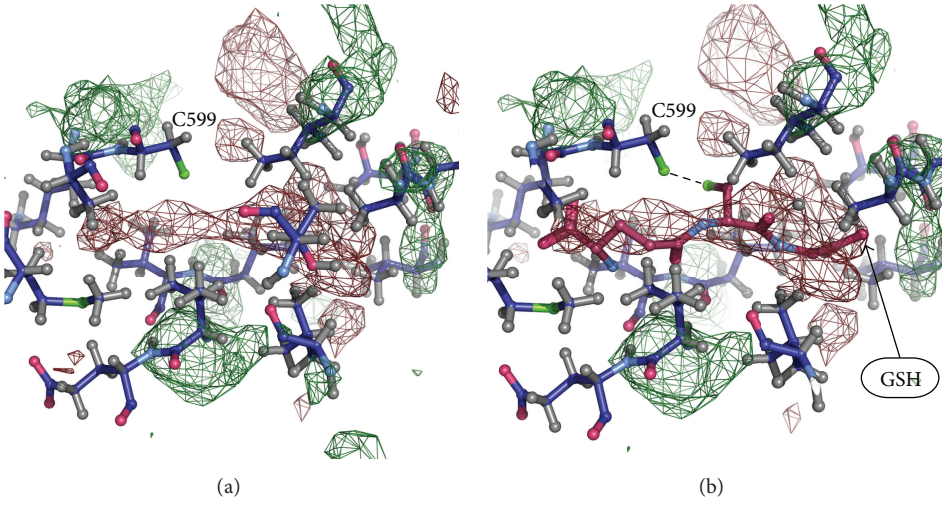


Figure 5: Part of Na,K-ATPase α1-subunit near Cys599 (PDB code 3WGU). (a) Unresolved density in the isolated cavity. (b) GSH incorporated into the unresolved density.

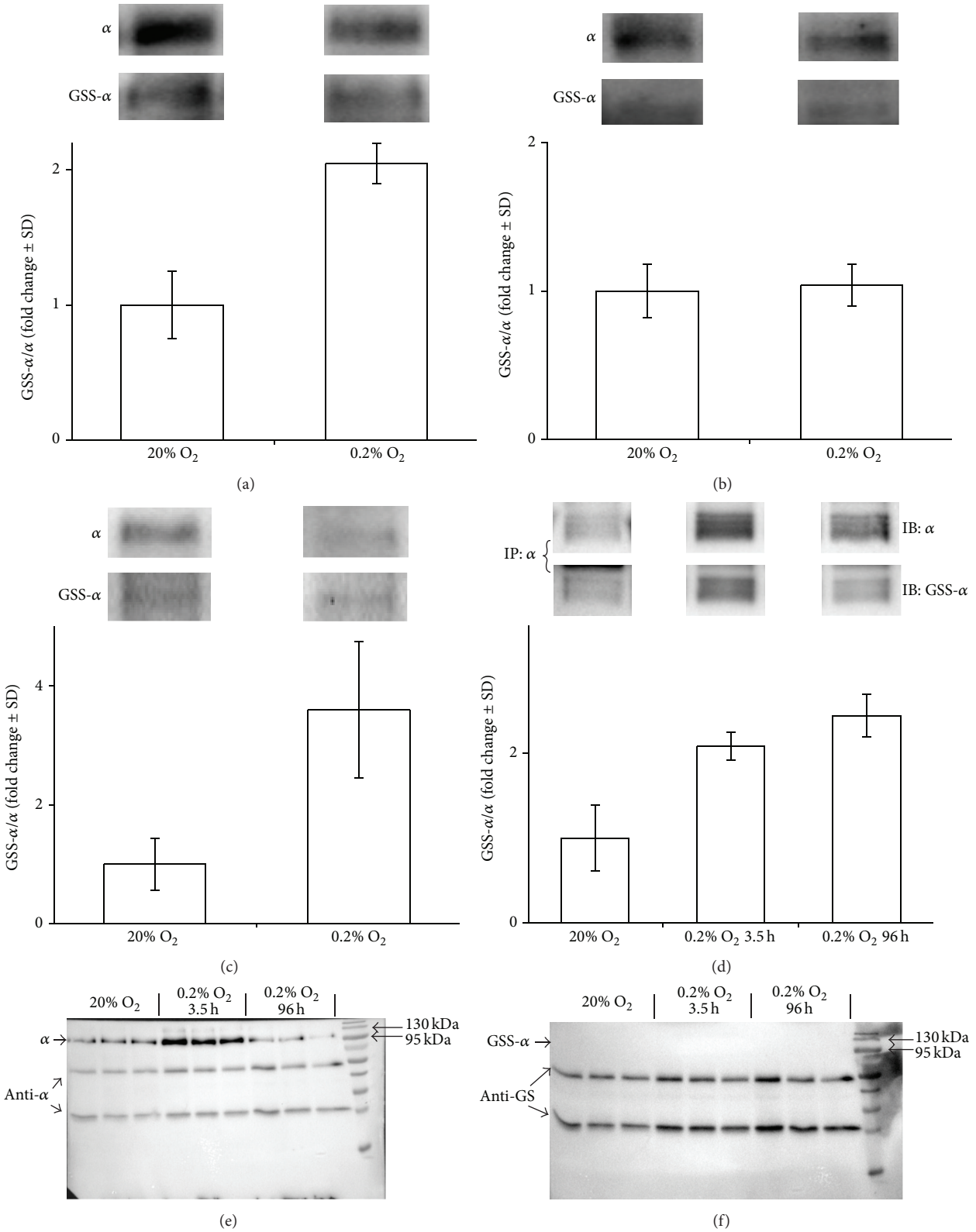


FIGURE 6: S-glutathionylation of α1-subunit of Na,K-ATPase after acute and prolonged hypoxia before and after TCEP (25 mM) treatment. SC-1 cells were grown either for 3.5 h ((a), (b)) or for 96 h (c), each at 20% and 0.2% pO_2. Cell lysates were incubated with ((b), (c)) or without (a) 25 mM TCEP during 30 min at 37°C and α1-subunit of Na,K-ATPase was detected by immunoblotting (IB). (d) α1-Subunit of Na,K-ATPase was immunoprecipitated (IP) from cell lysates by anti-α1 antibodies and glutathionylation was detected with anti-glutathione (anti-GS) antibodies. The original immunoblotting readouts are presented above. Bars represent changes in the S-glutathionylated (GSS-α/α) form of the protein normalized to its total amount. $n = 3$, mean ± SD. ((e), (f)) Immunoblots of Na,K-ATPase α1-subunit after immunoprecipitation with anti-α1 antibodies and TCEP (25 mM) treatment in denaturing conditions (8 M urea, 8% SDS). (e) Detection of total α1-subunit using anti-α1 antibodies. (f) Detection of glutathionylated α1-subunit using anti-GS antibodies.

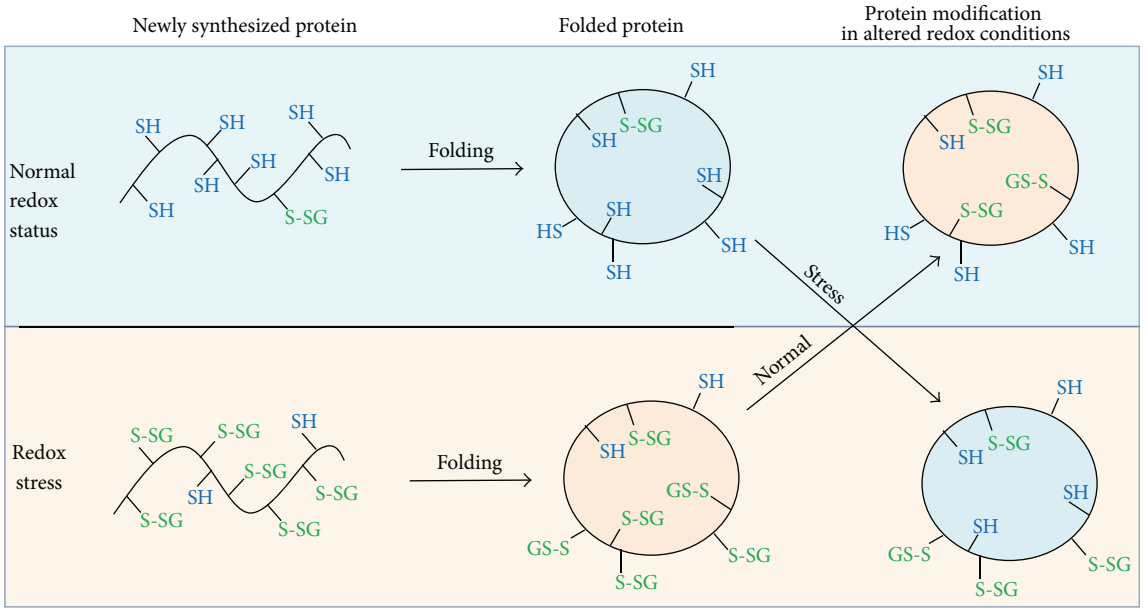


Figure 7: Schematic representation of Na,K-ATPase glutathionylation depending on intracellular redox status. At normal redox status, the level of GSH is about 100 times higher than GSSG. Under these conditions, during biosynthesis, the proteins are slightly glutathionylated. Redox stress leads to the shift in GSH/GSSG ratio that induces protein glutathionylation. At normal redox status and at redox stress, the basal levels of glutathionylation (glutathionylation of solvent-inaccessible cysteine residues) are different. Subsequent change in the redox status does not affect basal glutathionylation, which demonstrates that the protein "memorizes" a cellular redox state during its biosynthesis. In contrast, glutathionylation of the solvent-accessible cysteine residues (regulatory glutathionylation) depends on the current redox status of cell.

Table 6: Distance between Cys thiol groups in initial Na,K-ATPase structure (3WGU) and in model structures with glutathionylated Cys residues.

Pairs of Cys residues	Distance between Cys thiol groups in initial structure, Å	Glutathionylated Cys	Distance between Cys thiol groups in glutathione bound structure, Å
204–242	3.82	204	3.56
		242	3.46
367–698	4.10	367	5.47
		698	5.14
452–456	4.22	452	3.70
		456	5.63

performed immunoprecipitation of Na,K-ATPase α-subunit from lysates of SC-1 cells incubated at 0.2% and 20% pO_2 within 3.5 h and 96 h. Analysis of immunoprecipitated α-subunit revealed increasing of its glutathionylation under hypoxic conditions (Figure 6(d)). Then, we treated Na,K-ATPase α-subunit obtained by immunoprecipitation with TCEP under denaturing conditions (8 M urea and 8% SDS). In this case, the glutathionylation was fully removed (Figures 6(e) and 6(f)). Based on these data, we can conclude that the basal glutathionylation is a cotranslational modification which, for example, is necessary to prevent the formation of disulphide bridges between the neighboring cysteine residues during protein folding. Formation of disulfide bridges can increase rigidity of the structure and prevent conformational lability of the molecule. In particular, residues Cys 204 and Cys 242 are located in the actuator domain of Na,K-ATPase that performs large amplitude transitions during the catalytic cycle, which would be impossible if SS bridges were formed between these residues. In addition, the formation of SS bridge between Cys 204 and Cys 242 residues will prevent the exposition of SH group of Cys 242 to the solvent from the cavity and its regulatory glutathionylation. It is also possible that glutathionylation is necessary for correct protein folding. Only glutathione and oxidized glutathione are required for glutathionylation of α-subunit of Na,K-ATPase [13] without any additional enzymes and cofactors. Thus, the ability of SH group to undergo glutathionylation directly depends on the redox state of cells [5]. Reduced SH groups interact with GSSG, the level of which increases in oxidative stress. In the case of oxidation of thiol groups to SOH, they interact with GSH, the concentration of which in cytosol is 1–5 mM.

Unlike the genetic code and histone code that underlie information storage and utilization, the epigenetic code and redox code modulate operation of the genetic and histone codes in the organizational structure, differentiation, and adaptation of an organism to the environment [29]. Redox signaling and redox control in multicellular organisms evolved and diversified with the increase in atmospheric O_2 about 600 million years ago. We assume that cotranslational glutathionylation allows for "remembering" a cellular redox state during protein biosynthesis, and as a result proteins

synthesized in different redox conditions will have different properties (Figure 7). This phenomenon, which can be termed "redox memory," may be a necessary step of redox regulation and cell adaptation to different oxygen levels and other factors that change the intracellular redox status.

4. Conclusions

Our analysis of the available Na,K-ATPase structures has revealed isolated cavities with unresolved density near several cysteine residues in the catalytic α-subunit of the enzyme; these cavities correspond to the glutathione molecule. Identification of such unresolved density near cysteine residues can be prognosticated for other proteins that can undergo glutathionylation. Basal glutathionylation will depend on the redox status of cell at the point of protein synthesis (Figure 7). Consequently, depending on the redox status of cell, proteins with diverse levels of glutathionylation will be synthesized, and such proteins accordingly will have different properties. Since basally glutathionylated residues are not accessible to the deglutathionylating agents, the pattern of basal glutathionylation reflects the redox status of the cell at the point of protein folding.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Vladimir A. Mitkevich and Irina Yu. Petrushanko conceived the project, coordinated the study, and drafted the paper with contributions from Yuri M. Poluektov, Anastasia A. Anashkina, and Alexander A. Makarov. Irina Yu. Petrushanko, Ksenia M. Burnysheva, and Valentina A. Lakunina performed experiments. Yuri M. Poluektov and Anastasia A. Anashkina analyzed the structural data and modelled glutathionylated Na,K-ATPase. Alexander A. Makarov coordinated the study and contributed to materials and reagents. Vladimir A. Mitkevich, Irina Yu. Petrushanko, and Yuri M. Poluektov contributed equally to this work.

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Research Article

Hepatitis C Virus NS5A Protein Triggers Oxidative Stress by Inducing NADPH Oxidases 1 and 4 and Cytochrome P450 2E1

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Replication of hepatitis C virus (HCV) is associated with the induction of oxidative stress, which is thought to play a major role in various liver pathologies associated with chronic hepatitis C. NS5A protein of the virus is one of the two key viral proteins that are known to trigger production of reactive oxygen species (ROS). To date it has been considered that NS5A induces oxidative stress by altering calcium homeostasis. Herein we show that NS5A-induced oxidative stress was only moderately inhibited by the intracellular calcium chelator BAPTA-AM and not at all inhibited by the drug that blocks the Ca^{2+} flux from ER to mitochondria. Furthermore, ROS production was not accompanied by induction of ER oxidoreductins (Ero1), H_2O_2-producing enzymes that are implicated in the regulation of calcium fluxes. Instead, we found that NS5A contributes to ROS production by activating expression of NADPH oxidases 1 and 4 as well as cytochrome P450 2E1. These effects were mediated by domain I of NS5A protein. NOX1 and NOX4 induction was mediated by enhanced production of transforming growth factor β1 (TGFβ1). Thus, our data show that NS5A protein induces oxidative stress by several multistep mechanisms.

1. Introduction

Hepatitis C virus (HCV) is a blood-borne pathogen with ca. 120–170 million chronic carriers worldwide [1]. Chronic hepatitis C (CHC) infection is often accompanied by various liver and extrahepatic diseases that include fibrosis, steatosis, and insulin resistance and frequently progresses to cirrhosis and liver cancer [1]. Investigation of molecular mechanisms which underlie the pathogenicity of the virus led to the discovery of multiple events in CHC carriers that can trigger metabolic dysfunctions and on the long term carcinogenesis. In particular, studies from several groups pointed out oxidative stress as a phenomenon which is strongly associated with most of the HCV-associated diseases ([2–4] and references herein).

Oxidative stress is a marked increase of highly reactive oxygen intermediates (reactive oxygen species, ROS) including superoxide anion, hydroxyl radical, and hydrogen peroxide [5]. ROS readily modify various biological compounds including nucleic acids, proteins, and lipids, thus presenting a threat to cell fate. In cells, ROS are normally present at low levels and formed by various enzymes. These include the oxidative phosphorylation system in mitochondria, ion channels such as NADPH oxidases (NOX), metabolic enzymes such as xanthine oxidase, enzymes involved in degradation of lipids, biogenic polyamines and cytochromes, and finally members of the protein folding machinery at the endoplasmic reticulum such as the ER oxidoreductins (Ero1) [5, 6]. It has been shown by several groups that five of the HCV proteins, namely, core, NS5A, and to a lesser extent E1, E2, and NS4B [7–9], induce oxidative stress by two major mechanisms. These include alteration of calcium homeostasis leading to mitochondrial dysfunction [10, 11] and induction of NOX1 and NOX4 [12, 13].

To date it has been shown that core protein can induce efflux of Ca^{2+} from ER to mitochondria by several different mechanisms, alter normal functioning of mitochondria

Table 1: Oligonucleotides used for plasmid construction.

Oligonucleotide	Restriction site	Sequence
P1	*Pst*I	5′-ATTCTGCAGTCCGGCTCGTGGCTAAGA-3′
P2	*Hind*III	5′-AATAAGCTTTTAGCAGCAGACGACGTCCTC-3′
P3	*Hind*III	5′-ATTAAGCTTTTAGGAGTCATGACGGGTAGTG-3′
P4	*Pst*I	5′-ATTCTGCAGCCGGACGCTGACCTCATC-3′
P5	*Hind*III	5′-ATTAAGCTTTTATGGAGGTGGTATCGGAGG-3′
P6	*Pst*I	5′-AATCTGCAGCGGAGGAAGAGGACGGTT-3′

Table 2: Oligonucleotides used to assemble short interfering RNA (siRNA).

Target transcript	Abbreviation	Sequence
No	siMock	5′-GUAAGACACGACUUAUCGCdTdT-3′
		5′-GCGAUAAGUCGUGUCUUACdTdT-3′
NOX1	siNOX1	5′-UCAUAUCAUUGCACAUCUAdTdT-3′
		5′-UAGAUGUGCAAUGAUAUGAdTdT-3′
NOX4	siNOX4	5′-GCCUCUACAUAUGCAAUAAdTdT-3′
		5′-UUAUUGCAUAUGUAGAGGCdTdT-3′
COX-2	siCOX2	5′-UGAAAGGACUUAUGGGUAAdTdT-3′
		5′-UUACCCAUAAGUCCUUUCAdTdT-3′

(summarized in [2]), and induce NOX4 enzyme in a transforming growth factor β1- (TGFβ1-) dependent manner [13]. In addition, we have recently demonstrated that HCV core also induces NOX1, cytochrome P450 2E1 (CYP2E1), and ER oxidoreductin 1α (Ero1α), with the latter being a mediator of calcium perturbations and generation of superoxide in mitochondria [14]. In contrast to core protein, the mechanisms by which NS5A induces oxidative stress remain more obscure. The only data available show that similar to core protein, NS5A triggers passive leakage of calcium ions from the ER [9], an event that has been shown to be associated with elevation of ROS levels [15].

The goal of our study was to investigate the mechanisms by which NS5A induces ROS using as model Huh7 cells expressing NS5A protein. A particular focus was to be given to NADPH oxidases 1 and 4 and other ROS-generating cellular enzymes such as ER-residing cytochrome P450 2E1 and ER oxidoreductin 1α. In addition, we aimed to identify the domains of NS5A containing prooxidant activity.

2. Materials and Methods

2.1. Materials. Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA), Dulbecco's modified Eagle medium (DMEM) and antibiotics for cell cultures were purchased from PanEco. Fetal bovine serum (cat #SV30160.03) was obtained from HyClone (Logan, UT, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA), dihydroethidium (DHE), and other reagents were from Sigma (St. Louis, MO, USA), unless otherwise noted. HRP-conjugated antibodies to c-myc tag (18824P) were from QED Bioscience (San Diego, CA, USA), primary antibodies to cytochrome P4502E1 (ab28146), β-actin (ab3280), HCV core (ab58713), and HRP-conjugated anti-rabbit and anti-mouse secondary antibodies, as well as CYP2E1 inhibitor 4-methylpyrazole were purchased from Abcam (Cambridge, UK). Antibodies to NOX1 (Mox1, H75, and sc-25545), NOX4 (H-300, sc-30141), COX-2 (29, sc-19994), and TGFβ1/2 (12Y-1, sc-80346L) were obtained from Santa-Cruz Biotechnology (Dallas, TX, USA). Hybond-ECL membrane was supplied by GE Healthcare; enzymes were from Thermo Scientific (Rockford, IL, USA). qPCRmix-HS and qPCRmix-HS SYBR + ROX master mixes were purchased from Evrogen (Moscow, Russia). The unmodified DNA oligonucleotides were supplied by Litech (Moscow, Russia), the Taqman probes were synthesized by Syntol (Moscow, Russia), and the RNA oligonucleotides were obtained from DNA Synthesis Ltd. (Moscow, Russia). Huh7 cells were a kind gift of Professor R. Bartenschlager (Heidelberg University, Germany).

2.2. Plasmid Construction. C-myc tagged expression plasmids for full-length NS5A protein and N- and C-terminal fragments were constructed based on the pCMV-Tag3 vector (Agilent, Santa-Clara, CA, USA). A full-length NS5A (1–447 aa) fragment encoding a 1b genotype NS5A sequence (AJ238799) was amplified using oligonucleotides P1 and P2 (Table 1). The product was digested with *Pst*I and *Hind*III endonucleases and cloned into the respective sites of pCMV-Tag3. NS5A fragments corresponding to domains I (DI, 1–249 aa), II (D2, 250–355 aa), and III (D3, 356–447 aa) were constructed in a similar fashion using pairs of oligos P1 and P3, P4 and P5, and P2 and P6 (Table 1), respectively. All plasmids were confirmed by sequencing.

2.3. Cell Culture and Transfection. The human hepatoma Huh7 cell line was maintained and transfected as described in [8]. For RNA interference, siRNAs (see Table 2) were annealed in a buffer (5 μM each siRNA in 5 mM Tris-HCl, pH 7.5, 1 mM EDTA) by heating at 65°C for 5 min and then slow cooling to room temperature. Transfection of the cells with

Table 3: Primers used for quantification of gene transcription levels by real-time RT-PCR.

Transcript		Sequence
NOX1	Sense	5′-TTAACAGCACGCTGATCCTG-3′
	Antisense	5′-CTGGAGAGAATGGAGGCAAG-3
NOX4	Sense	5′-GCTGACGTTGCATGTTTCAG-3′
	Antisense	5′-CGGGAGGGTGGGTATCTAA-3′
COX-2	Sense	5′-CCATGTCAAAACCGAGGTGTAT-3′
	Antisense	5′-TCCGGTGTTGAGCAGTTTTCT-3′
TGFβ1	Sense	5′-GCAGCACGTGGAGCTGTA-3′
	Antisense	5′-CAGCCGGTTGCTGAGGTA-3′
CYP 2E1	Sense	5′-GACTGTGGCCGACCTGTT-3′
	Antisense	5′-ACTACGACTGTGCCCTTGG-3′
Ero1α	Sense	5′-GCATTGAAGAAGGTGAGCAA-3′
	Antisense	5′-ATCATGCTTGGTCCACTGAA-3′
Ero1β	Sense	5′-GGGCCAAGTCATTAAAGGAA-3′
	Antisense	5′-TTTATCGCACCCAACACAGT-3′

the obtained siRNA duplexes was performed using Lipofectamine 2000 according the manufacturer's specification using 100 pmol of each duplex per well of 24-well plate or 400 pmol per well of 6-well plate. To prevent expression of TGFβ1-dependent genes, the NS5A-expressing cells were treated with anti-TGFβ1/2 neutralizing antibodies 18 h after transfection and subjected to analysis after additional 30 h. Alternatively, anti-HCV core antibodies were added as a negative control. In addition, in some experiments, the cells were treated with 100 μM 4-methylpyrazole (4-MP), 100 μM BAPTA-AM, or 100 μM Ru360.

2.4. Measurement of Reactive Oxygen Species. ROS levels in cells grown in 24-well plates were measured using two low molecular weight dyes: 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and dihydroethidium (DHE), whose fluorescence is dependent on levels of H_2O_2 and $O_2^{\bullet-}$, respectively. In case of both dyes the cell culture medium was removed 28 h after transfection and replaced with the medium containing 25 μM H_2DCFDA or DHE. After incubation at room temperature for 30 min the media was removed, and the cells were washed 10 times with PBS (500 μL/well). The fluorescence intensities (FLI) were measured in PBS (200 μL/well) using Plate CHAMELEON V reader (Hidex Ltd.) with excitation at 485 nm and emission at 535 nm in case of H_2DCFDA or with excitation at 510 nm and emission at 590 nm in case of DHE.

2.5. Western Blotting. Western blotting was performed as described previously [8]. Primary antibodies were used at the following concentrations: anti-β-actin: 0.2 μg/mL; anti-NOX1: 0.2 μg/mL; anti-NOX4: 0.4 μg/mL; anti-COX-2: 0.4 μg/mL; and anti-CYP2E1: 0.5 μg/mL. The HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were used at concentrations of 0.2 μg/mL. The HRP-conjugated antibodies to the c-myc tag were used at 0.2 μg/mL concentration.

2.6. Quantitative Real-Time PCR (qRT-PCR). Total RNA was purified and cDNA was synthesized as described previously [8]. Levels of human NOX1, NOX4, COX-2, TGFβ1, CYP2E1, Ero1α, and Ero1β transcripts were quantified according to SYBR Green approach using qPCRmix-HS SYBR + ROX mixture and the primers listed in Table 3. Levels of β-actin transcript as the loading control were determined using previously described Taqman probes [8]. Thermal cycling conditions for both types of real-time PCR included activation at 55°C (5 min) and 95°C (10 min) followed by 40 cycles each of denaturation at 95°C (10 s) and annealing/elongation at 57°C (1 min).

2.7. Quantification of TGFβ1 in Culture Medium by ELISA. Huh7 cells were grown on 24-well plates and transfected as described above. Forty-eight h after transfection the cell culture medium was collected, cell debris was removed by centrifugation (3,000 rpm, 10 min), and concentrations of TGFβ1 were quantified by Human TGF-beta1 Platinum ELISA kit (eBioscience, San Diego, CA, USA) according to manufacturer's specification.

2.8. Statistical Analysis. Statistical analysis was performed using BioStat 2009 software (AnalystSoft, Vancouver, Canada). The results are presented as means ± SD. Shapiro-Wilk W test was used to confirm normal distribution of the data. Homogeneity of the variance was studied by Levene's test using SPC for Excel Software (BPI Consulting, LLC, Cypress, TX, USA). Significant differences were determined using one-way ANOVA followed with a Tukey-Kremer *post hoc* analysis. Statistical differences between treated and untreated cells in experiments with neutralizing antibodies were determined using a paired Student's t-test.

3. Results

3.1. Domain I of NS5A Protein Induces Oxidative Stress. The expression levels of full-length (1–447 aa) NS5A or its individual domains 1 (1–249 aa), 2 (250–355 aa), and 3 (356–447 aa) (Figure 1(a)) were verified by western blotting using an antibody recognizing an N-terminal c-myc tag, displayed by all NS5A proteins, upon transfection of Huh7 cells (Figure 1(b)).

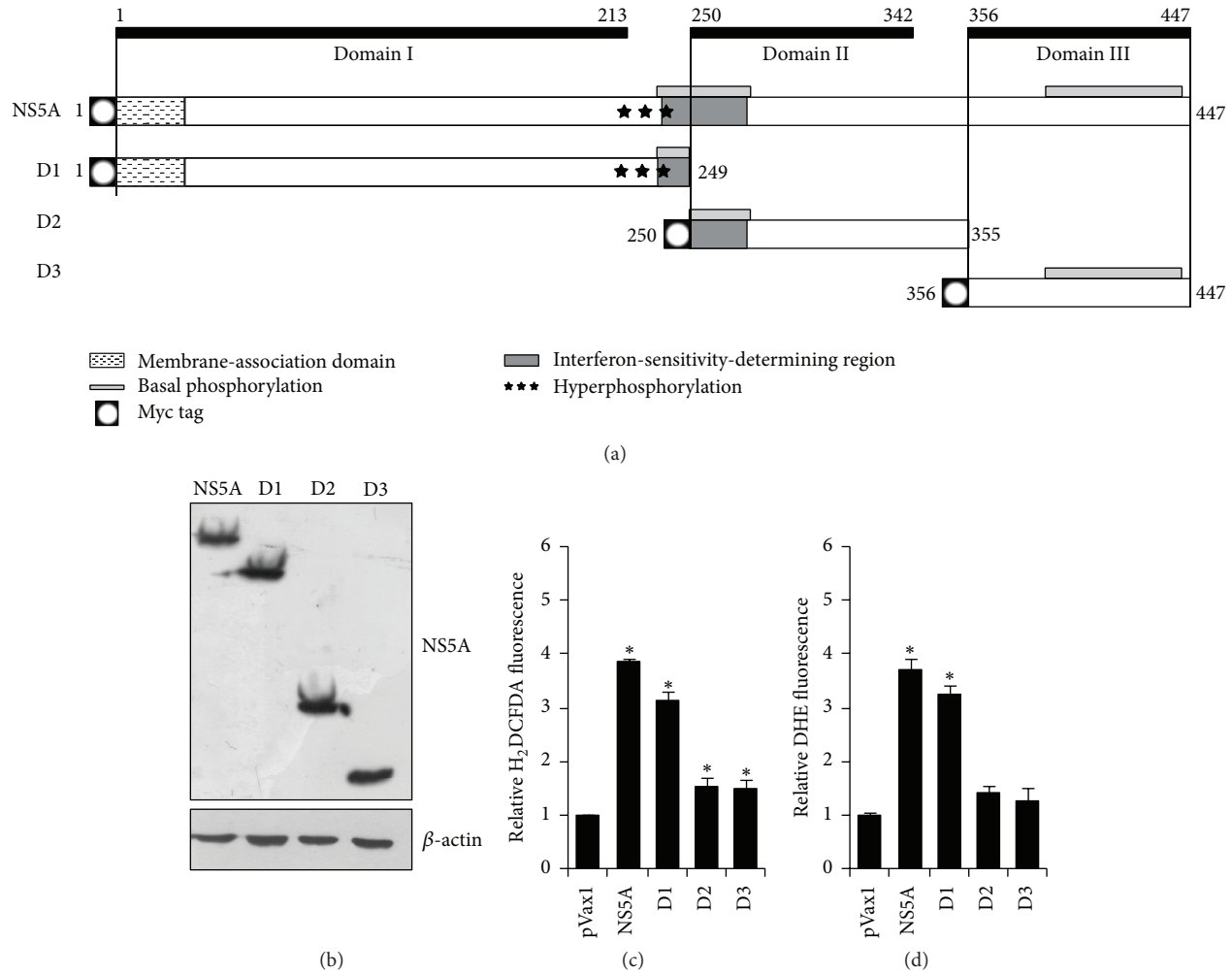

Figure 1: Domain I of HCV NS5A protein is responsible for induction of oxidative stress in Huh7 cells. (a) Schematic representation of truncated forms of NS5A used in the study. Note that all NS5A expression constructs contained an N-terminal c-*myc* tag. The obtained plasmids were transfected into Huh7 cells, which were subjected to detection of NS5A by Western-blot 48 h after transfection using an anti-*myc* antibody (b) or to quantification of ROS levels in H_2DCFDA (c) or DHE (d) assays as described in Section 2. (c and d) Results are presented as mean ± SD from seven replicates. $^*P < 0.001$ *versus* pVax1 (ANOVA with Tukey-Kramer *post hoc* analysis).

Full-length as well as fragments of NS5A were expressed in Huh7 cells to comparable levels.

The ability of individual domains of NS5A protein to induce oxidative stress was investigated with two low molecular weight dyes, namely, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and dihydroethidium (DHE). H_2DCFDA is absorbed by cells and then converted into its deesterified form H_2DCF, which is oxidized into a fluorescent product in an H_2O_2-dependent fashion [16]. Treatment of Huh7 cells transfected with NS5A-expressing plasmids revealed that the full-length protein as well as domain I (D1) induced ROS production (Figure 1(c)). In contrast, domains II and III only slightly affected the level of fluorescence compared to cells transfected with the empty control vector pVax1 (Figure 1(c)). The same results were obtained with DHE, a superoxide anion-specific dye [16]. Again, a pronounced increase in production levels of superoxide anion was observed only for the full-length NS5A and its domain I (Figure 1(d)). Thus NS5A as well as D1 induce ROS.

3.2. Induction of Oxidative Stress by NS5A Protein Is Partially Mediated by NOX1, NOX4, and Cyclooxygenase 2. It has previously been reported that HCV infection provokes oxidative stress by inducing expression of NOX1 and NOX4, and in case of NOX4 the effect has been shown to be mediated by both structural and nonstructural proteins of the virus [12, 13]. Here, the role of NS5A on expression of NOX1 and NOX4 was studied by real-time RT-PCR and Western-blot analysis. As shown in Figures 2(a) and 2(b), the full-length NS5A protein and domain 1 induce expression of NOX1 and, to a lesser extent, of NOX4 as well as of TGFβ1 and cyclooxygenase 2 (COX-2) (Figures 2(a) and 2(b)). Induction of TGFβ1 production was verified by ELISA (Table 4). These two latter proteins have previously been

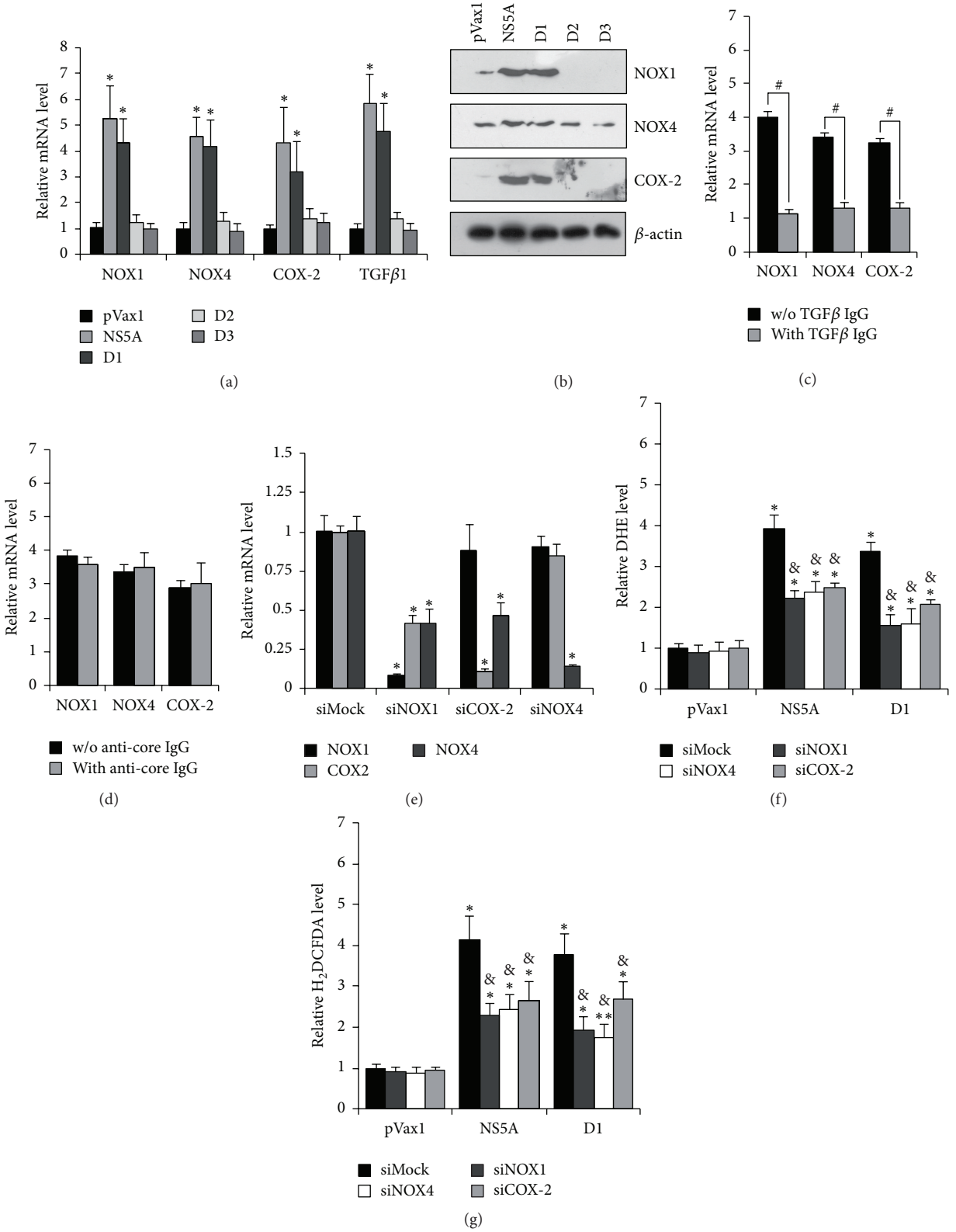

Figure 2: Domain I of NS5A protein triggers ROS production *via* activation of the TGFβ1 → NOX1 → COX-2 → NOX4 cascade. (a, b) Huh7 cells were transfected with NS5A-expression constructs and subjected to gene expression analysis by real-time RT-PCR (a) or Western blotting (b). (c, d) NS5A-expressing cells were treated with anti-TGFβ1/2 antibodies (c) or anti-HCV core antibodies as negative control (d), and after an additional 30 hrs gene expression was quantified using RT-qPCR. (d–f) Huh7 cells expressing full-length NS5A were transfected with siRNA and 48 hrs later subjected to gene expression analysis by RT-qPCR (e) or measurement of ROS levels using the DHE (f) or H_2DCFDA (g) assays. Results are mean ± SD from six replicates; $^{*}P < 0.01$ or $^{**}P < 0.05$ versus DMSO-treated cells transfected with pVax1 (ANOVA with Tukey-Kramer *post hoc* analysis); $^{\&}P < 0.01$ versus DMSO-treated cells transfected with the respective NS5A form (ANOVA with Tukey-Kramer *post hoc* analysis); $^{\#}P < 0.01$ (*t*-test).

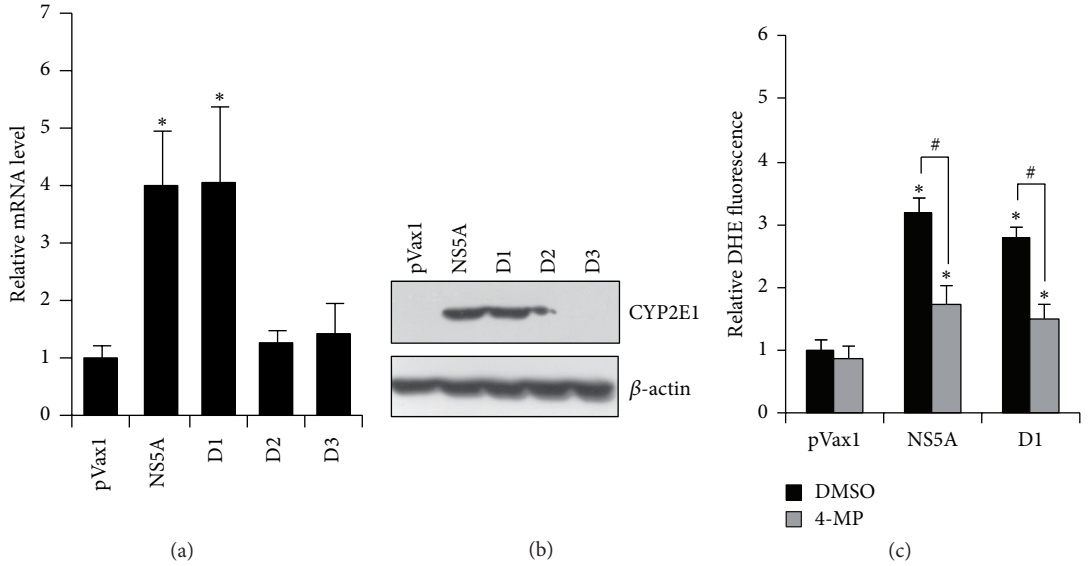

Figure 3: HCV NS5A protein induces expression of cytochrome P450 2E1 which contributes to ROS production. Huh7 cells were transfected with NS5A-expressing constructs and subjected to quantification of CYP 2E1 levels by real-time RT-PCR (a) or Western blotting (b) 40 hrs after transfection. (c) 18 hrs after transfection NS5A expressing Huh7 cells were treated with 100 μM 4-methylpyrazole (4MP) for additional 12 hrs prior to quantification of ROS levels in DHE assay. Results are mean ± SD from six replicates; $^{*}P < 0.01$ versus DMSO-treated cells transfected with pVax1; $^{\#}P < 0.01$ (ANOVA with Tukey-Kramer *post hoc* analysis).

Table 4: Secretion of TGFβ1 from Huh7 cells transfected with pVax1 or plasmids expressing full-length NS5A or its domain 1.

	pVax1	NS5A	D1
TGFβ1 (pg/mL)	<375*	690 ± 137	565 ± 80

*The value was below sensitivity of kit for TGFβ1.

implicated in the regulation of NOX1 and NOX4 in response to inflammatory stimuli [17]. The role of TGFβ1 and COX-2 in NS5A induced induction of NOX1 and NOX4 was verified using neutralizing antibodies and RNA interference approaches. Treatment of NS5A-expressing cells with anti-TGFβ1/2 antibodies prevented activation of both NOX1 and NOX4, as was revealed by RT-qPCR (Figure 2(c)). At the same time, anti-COX-2 siRNAs suppressed expression not only of cyclooxygenase 2 but also of NOX4 (Figure 2(d)). In addition, anti-NOX1 siRNA caused a decrease of NOX1, NOX4, and COX-2 transcripts (Figure 2(d)) supporting the existence of a TGFβ1 → COX1 → COX-2 → NOX4 cascade.

Next, the same approaches were employed to reveal the role of these proteins in NS5A-induced oxidative stress. Indeed, anti-NOX1, NOX4, and COX-2 siRNAs decreased ROS production by 1.6–1.8-fold, as was revealed in DHE and H_2DCFDA assays (Figures 2(e) and 2(f)) but did not eliminate it down to background levels. Thus, NADPH oxidases 1 and 4 represent an important, but not the only, source of ROS, in HCV NS5A expressing cells.

3.3. CYP2E1 Is Another Source of ROS in NS5A-Expressing Cells. Cytochrome P450 2E1 is considered as a significant source of ROS [5]. Using real-time RT-PCR and Western-blot analysis it was revealed that NS5A and its domain 1 cause a pronounced increase in CYP2E1 expression (Figures 3(a) and 3(b)). In contrast, domains 2 and 3 again had no detectable effect on cytochrome expression. Treatment of NS5A-expressing cells with 4-methylpyrazole, a low molecular weight inhibitor of CYP2E1 enzymatic activity, suppressed production of superoxide anion by app. 40% (Figure 3(c)). Thus, CYP2E1 represents a novel source of ROS which is induced by HCV NS5A protein.

3.4. HCV NS5A Protein Does Not Affect Expression of ER Oxidoreductins 1α and 1β. Another probable group of enzymes involved in induction of oxidative stress are the ER oxidoreductins 1α and 1β (Ero1α and Ero1β) which produce H_2O_2 and regulate redistribution of calcium ions between ER and mitochondria [18]. Previously we found that HCV core induces Ero1α at transcriptional level [14]. To unveil a role for both enzymes in oxidative stress, their expression in naive and NS5A-expressing Huh7 cells was measured by real-time RT-PCR. However, neither full-length NS5A nor its individual domains affected the transcript levels of these enzymes (Figure 4(a)).

3.5. Efflux of Calcium Ions from ER Does Not Contribute Significantly to NS5A-Induced Oxidative Stress. Next, the role of calcium ions in NS5A-mediated induction of oxidative stress was estimated. It was performed using two compounds: BAPTA-AM, a general cell-permeable Ca^{2+} chelator, and Ru360, an inhibitor of calcium uniporter which mediates influx of the ions from the ER directly into mitochondria. Cytoplasmic calcium chelator BAPTA-AM caused only 30% reduction in ROS production in DHE assays (Figure 4(b)).

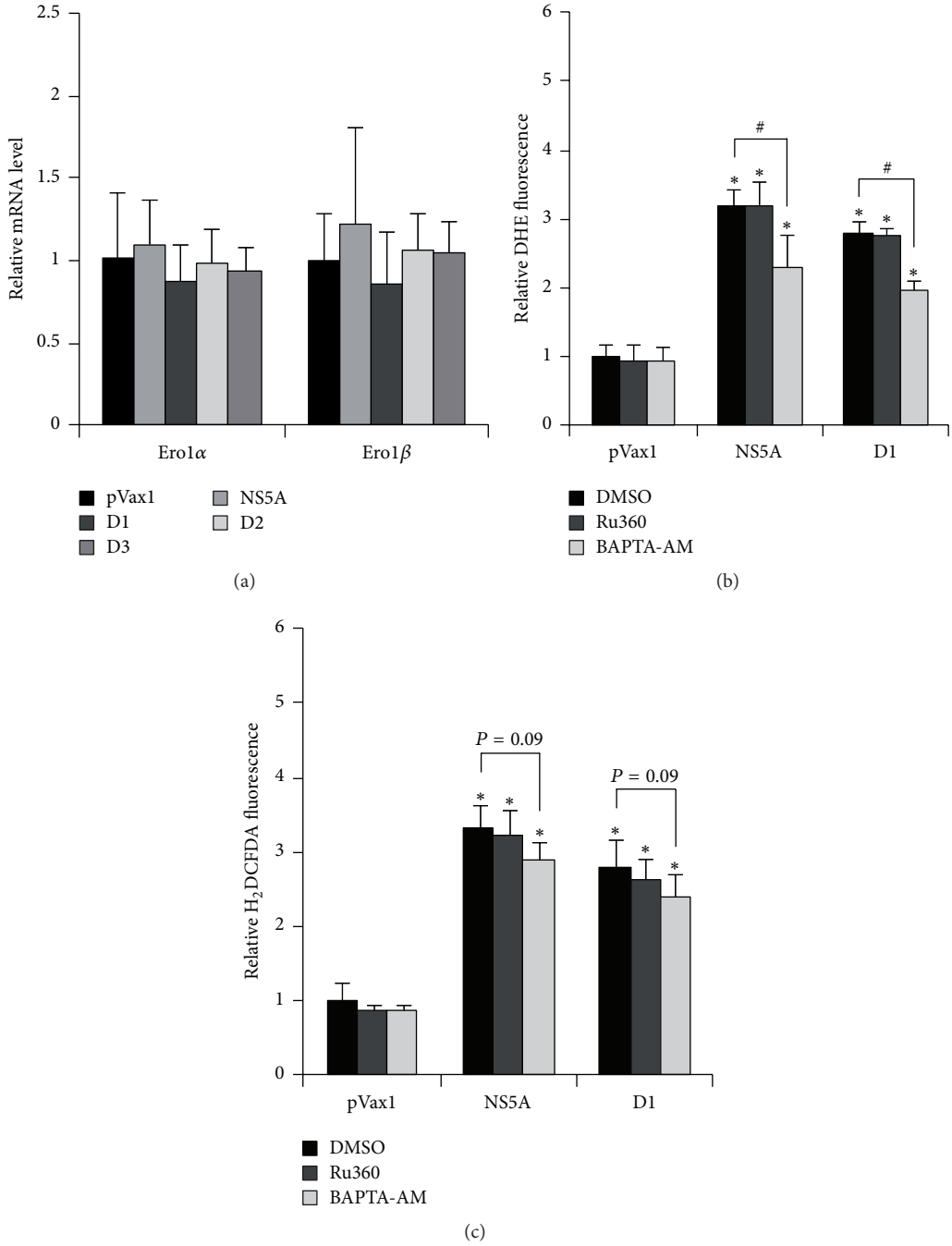

Figure 4: ER oxidoreductins 1α and 1β and calcium signaling have no significant role in induction of oxidative stress by NS5A protein. (a) Huh7 cells were transfected with NS5A-expressing plasmids, and levels of Ero1α and 1β transcripts were analyzed 40 hrs after transfection by real-time RT-PCR. (b, c) 18 hrs after transfection NS5A expressing Huh7 cells were treated with calcium chelator BAPTA-AM or inhibitor or mitochondria Ca^{2+} uniporter Ru360, and ROS levels were analyzed 28 hrs after transfection in DHE (b) or H_2DCFDA (c) assays as described in Section 2. Results are mean ± SD from six replicates; $^{*}P < 0.01$ versus DMSO-treated cells transfected with pVax1; $^{\#}P < 0.01$ between the indicated groups (ANOVA with Tukey-Kramer *post hoc* analysis).

In H_2DCFDA assays a similar reduction was observed (Figure 4(c)). Ru360 did not affect ROS production at all. Thus, calcium signaling has only minor input in NS5A-triggered oxidative stress.

4. Discussion

Previously it has been reported that one of the key mechanisms by which HCV core protein induces oxidative stress is

the efflux of calcium ions from the ER and their accumulation in mitochondria ([2, 5] and references herein). In the case of NS5A, release of Ca^{2+} from ER stores has also been reported, although this release has been considered to be a consequence of oxidative stress [19]. Our data support these observations, as calcium chelator BAPTA-AM had only a minor effect, whereas Ru360, an inhibitor of direct flux of Ca^{2+} from ER to mitochondria, had no effect at all on NS5A-mediated ROS production. Therefore, HCV core and NS5A proteins are likely to induce oxidative stress by different mechanisms.

The other reported mechanism of HCV-induced ROS production involves induction of NADPH oxidases 1 and 4 [12, 13]. In these studies, the ability to trigger NOX1 expression was assigned to the core protein [12], whereas enhanced expression of NOX4 was observed in case of both structural and nonstructural viral proteins [13]. Our data suggest that expression of both NOX genes can be mediated by NS5A protein, and their induction is achieved through activation of TGFβ1 expression. In addition, we observed that both NOX1 and NOX4 can contribute to superoxide anion production using an siRNA approach, in contrast to data in the literature, which suggest that *in vitro* production of ROS by recombinant NOX4 is limited to H_2O_2 [20]. However, production of H_2O_2 has been described mainly for the recombinant NOX4 [20], whereas various independent groups have also reported decreased superoxide production in cells with downregulated NOX4 [12, 21].

In 2011 Sancho et al. revealed that induction of NADPH oxidases in response to proinflammatory stimuli was regulated by the cascade TGFβ → NOX1 → COX-2 → NOX4 in Chang liver CCL-13 cells [17]. We confirmed the existence of this cascade in NS5A expressing Huh7 cells using anti-TGFβ1/2 antibodies and anti-NOX and COX2 siRNAs; however, we found no evidence for an activation of this cascade in HCV core expressing Huh7 cells [14]. Interestingly, TGFβ induction was earlier reported to depend on calcium signaling, at least in the replicative HCVcc infectious system [22]. However, using the Ca^{2+} chelator BAPTA-AM, we noticed only minor effects on TGFβ induced oxidative stress.

Cytochrome P450 2E1 is a ROS-producing enzyme which is involved in catabolism of an array of endogenous and exogenous compounds including ethanol [23]. It is expressed mainly in the liver and is localized mostly in the ER membrane [23], where HCV replication occurs [1]. Here CYP2E1 was identified as an important source of ROS in NS5A expressing cells. Previously it has been shown that coover-expression of CYP2E1 with HCV core protein activates ROS production in response to xenobiotic treatment much more potently than any of these proteins expressed alone [24]. Elevated CYP2E1 expression was also described in liver of chronic hepatitis C patients with mild fibrosis [25]. Thus our data present the first evidence that HCV NS5A can directly induce CYP2E1 expression which in turn contributes to oxidative stress.

The ER represents an organelle which produces significant amount of ROS mostly by the protein folding machinery [5]. ER oxidoreductins 1α and 1β are implicated in disulphide bond formation, with H_2O_2 being a major by-product [26]. In addition, it has recently been discovered that Ero1α is also involved in a control of Ca^{2+} translocation from ER directly to mitochondria through mitochondria-associated membranes (MAM) [18]. To date there is no literature on regulation of Ero1α or β by viral infections, except our recent report for the case of HCV core protein. The only two pieces of evidence that have been reported are the suppression of Ero1 expression in epithelial cells from HIV-infected individuals on highly active antiretroviral therapy [27] and our recent data on induction of Ero1α by HCV core which contributes to the oxidative stress [14]. Here in the case of HCV, NS5A has no effect on Ero1α or β expression. Thus, HCV core and NS5A proteins exhibit different effect of Ero1α expression and on consequent calcium signaling.

NS5A represents a 447 aa polyprotein containing three distinct domains: 1 (aa 1–213), 2 (250–342), and 3 (356–447) (Figure 1(a)) [28]. The present study was based on NS5A fragments containing one of these domains: aa 1–249, 250–355, and 356–447 (Figure 1(a)). Our data suggest that domain 1 (D1) contains a strongly prooxidant activity. Previously it was shown that this zinc-binding domain has a distinct conformation and can form homodimers [29], whereas domains 2 and 3 are unfolded [30, 31]. However, the ability of D1 to enhance ROS production may also be due to its localization to the ER; indeed, in D1, the first 30 N-terminal aa of NS5A form a hydrophobic membrane-associating α-helix [32]. The phosphorylation status of NS5A is unlikely to contribute to the generation of oxidative stress, because all phosphorylation sites are localized within D3. Furthermore, hyperphosphorylation is known to require coexpression of other nonstructural viral proteins, which were absent in our study [1, 28]. Finally, the prooxidant activity of NS5A is unlikely to be associated with ability of the protein to ensure HCV escape from interferon α, as the interferon-sensitivity-determining region (ISDR) is localized between D1 and D2 [1].

Altogether, our data show that HCV-induced oxidative stress can be mediated not only by altered calcium signaling and mitochondrial dysfunctions, but also by overexpression of nonmitochondrial proteins including NADPH oxidases 1 and 4, cyclooxygenase 2, and cytochrome P450 2E1. Therefore, special attention should be paid to the investigation of the role of these proteins in viral pathogenesis as well as in nonvirally induced pathologies that are characterized by oxidative stress. Currently both NOX1 and NOX4 and CYP2E1 are implicated in development of inflammation and liver fibrosis [4, 33–35], whereas pharmacological inhibitors of these enzymes have been shown *in vivo* to prevent hepatocyte death and attenuate fibrosis progression [36, 37]. CYP2E1 can also contribute to aggravated progression of liver disease in CHC patients addicted to heavy alcohol consumption [3]. Apart from virus pathogenicity, certain attention should be given in the future to dissecting role of various sources of ROS in virus life cycle. It has been shown that exogenous H_2O_2 affects virus replication; however no attempts have been reported to estimate the impact of endogenous ROS production and its sources/localization on replication capacity. We have recently demonstrated that ROS can affect infectivity of the newly formed virions, which is prevented by HCV-triggered induction of glutathione peroxidase 4 (GPx4) [38]. But again, no data exist in literature on possible role of cellular

ROS-producing enzymes on early or late stages of virus propagation. Last but not least, detailed investigation of the mechanisms by which viral proteins induce oxidative stress can be used to develop effective DNA vaccines. Indeed, previously, we showed for HIV reverse transcriptase that the prooxidant activity of its various forms correlated with its ability to induce specific interferon γ response thus linking redox alterations and immunogenic properties of virus proteins [39].

In conclusion, we identified mechanisms of oxidative stress induction by HCV NS5A protein.

Abbreviations

HCV: Hepatitis C virus
CHC: Chronic hepatitis C
NOX: NADPH oxidase
CYP2E1: Cytochrome P450 2E1
Ero1α: ER oxidoreductin 1α
TGFβ1: Transforming growth factor β1.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

DNA Tumor Viruses and Cell Metabolism

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Viruses play an important role in cancerogenesis. It is estimated that approximately 20% of all cancers are linked to infectious agents. The viral genes modulate the physiological machinery of infected cells that lead to cell transformation and development of cancer. One of the important adoptive responses by the cancer cells is their metabolic change to cope up with continuous requirement of cell survival and proliferation. In this review we will focus on how DNA viruses alter the glucose metabolism of transformed cells. Tumor DNA viruses enhance "aerobic" glycolysis upon virus-induced cell transformation, supporting rapid cell proliferation and showing the Warburg effect. Moreover, viral proteins enhance glucose uptake and controls tumor microenvironment, promoting metastasizing of the tumor cells.

1. Introduction

Development of cancer is a multistep process. Cancer cells differ from normal cells by genetic, metabolic, and histological features. Cancer cells have to fulfill their needs for continuous prol iferation. Hence, they acquire various hallmarks during the process of tumor progression, such as self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastases [1].

Viruses play an important role in cancerogenesis. Globally, it is estimated that approximately 20% of all cancers are linked to infectious agents [2]. The viral genes transcribed or expressed in infected cells modulate the physiological machinery of cells that leads to cell transformation and development of tumor. One of the important adoptive responses by the cancer cells is their metabolic change to cope up with continuous requirement of cell survival and proliferation. In this review, we will focus on how DNA viruses alter the glucose metabolism of cancer cells during carcinogenesis.

2. DNA Tumor Viruses: An Overview

In 1960, Sweet and Hilleman discovered a new virus in cultures of kidney cells of rhesus monkeys, producing vaccines to poliovirus [3]. This virus was named Simian vacuolating virus (SV40). Two years later, the tumorigenic potential of this monkey virus was revealed [4]. At the same time, it was also shown that human adenoviruses could induce tumors in newborn hamsters [5]. For now, many DNA tumor viruses are known; they are grouped in four families, namely, SV40 and polyomavirus, papilloma viruses (HPV), adenoviruses, and herpesviruses. Because of their relatively small genomes and striking biological effects, it is generally assumed that DNA tumor viruses have evolved to target the minimal number of cellular nodes and pathways required for transformation. Studies of DNA viruses have led to the identification of viral genes responsible for cancer induction and paving the way to our current understanding of cancer at the molecular level [2]. In their life cycle, viruses replicate, inducing the cytopathic effect in the host cells and forming new viral particles. Herpesviruses are able to establish persistent infection transforming the host cells. HPV, adenoviruses, and polyomaviruses induce the host cell transformation while infecting nonpermissive cells and integrating into the host genome (see Table 1).

3. Glucose Metabolism in General

It is well known that tumor cells differ from normal cells by glucose metabolism. At the ordinary physiological

Table 1: Human tumor DNA viruses.

Family	Virus	Virus-cell interaction	Associated disease	Level of association %	Size of genome kb
Herpesviruses	Epstein-Barr virus, EBV	Episomal, rarely integrated in transformed cells	Endemic Burkitt's lymphoma (BL) AIDS-asscoiated lymphoma Nasopharengyal carcinoma (NPC)	98 100 100	172
	Kaposi sarcoma herpes virus, KSHV	Episomal, rarely integrated in transformed cells	Kaposi's sarcoma	97	165
Polyoma viruses	John Cunningham Virus, JCV	Episomal, rarely integrated in transformed nonpermissive cells	Progressive multifocal leukoencephalopathy	50–80	5.2
	Virus of B.K. patient, BKV	Episomal, rarely integrated in transformed nonpermissive cells	Nephropathy Nephritis Hemorrhagic cystitis	10–20	5.2
	SV40	Episomal, rarely integrated in transformed nonpermissive cells	Mesothelioma	10–20 cofactor	5.2
Papilloma viruses	HPV	Episomal, integrated in transformed cells	Cervical cancer	71–88 (types 16 and 18)	8
Adenoviruses		Integrated in transformed nonpermissive cells	Small cell lung cancer Childhood ALL	No data	35 kb (type 11)

conditions, one glucose molecule is converted into two pyruvate molecules. Pyruvate oxidation on mitochondria to CO_2 and O_2 results in synthesis of 38 ATP molecules per molecule of glucose [6]. When concentration of oxygen is diminished, no pyruvate oxidation is carried out. Pyruvate is converted to lactate; that is, anaerobic glycolysis is activated. This conversion produces NAD^+, which is required for glycolysis. Glucose is also used by pentose phosphate pathway to produce nucleic acids and NADPH. NADPH is required for anabolic biosynthetic reactions as well as to neutralize ROS [7].

Cells secrete lactate and produce only 2 ATP molecules during glycolysis as compared to pyruvate oxidation [8]. Noteworthy, cancerous cells under normal conditions (in the presence of abundant oxygen) still convert pyruvate to lactate, in parallel to pyruvate oxidation; that is, the Warburg effect is observed (Figures 1(a) and 1(b)). Despite the fact that only 2 molecules of ATP are produced as a result of so-called "aerobic" glycolysis, the rate of reaction is quite high, compared to ATP synthesis on mitochondria (at least nine reactions should be carried out).

Excess lactate production increases the acidity of tumor cell microenvironment and this favors the tumor cell invasion and metastasis [9]. Anaerobic glycolysis is used by tumor cells at hypoxic conditions, which is generally found in solid tumors due to deregulated vasculature. "Aerobic" glycolysis also provides the biosynthetic advantage for tumor cells. Glycolytic intermediates are utilized by proliferating cells to produce fatty acids and nonessential amino acids [10].

In addition to glycolysis, cancer cells exhibit increased gluconeogenesis, glutaminolytic activity, glycerol turnover, pentose phosphate pathway activity, *de novo* fatty acid synthesis, reduced fatty acid oxidation, and modified amino acid metabolism [11]. We have to emphasize that mitochondrial respiration is not hampered in cancer cells [12] but operates at low capacity [13].

Enhanced glucose uptake has also been exploited in FDG-PET technology used clinically for the tumor detection. There are a lot of studies devoted to target the metabolic pathways as anticancer therapy [14].

4. Regulation of Warburg Effect

Many oncoproteins and tumor suppressor proteins can affect the cancer cell metabolism [15]. Transcription factor HIF1A [16] and MYC oncoprotein [17] are involved in upregulation of glucose transporters and many enzymes involved in glycolysis. MYC can also promote the expression of PKM2, resulting in faster proliferation. Tumor suppressor p53 (TP53) can inhibit glycolysis by inducing TIGAR, a regulator of glycolysis and apoptosis [18]. This can support oxidative phosphorylation by inducing SCO2, which is necessary for the formation of electron transport chain [19]. Downstream signaling molecule of PI3 Kinase, AKT1, can enhance glycolysis by various ways. AKT1 promotes glycolysis by increasing expression and membrane translocation of glucose transporters. It also phosphorylates glycolytic enzymes, such as hexokinase and phosphofructokinase 2. AKT1 stimulates

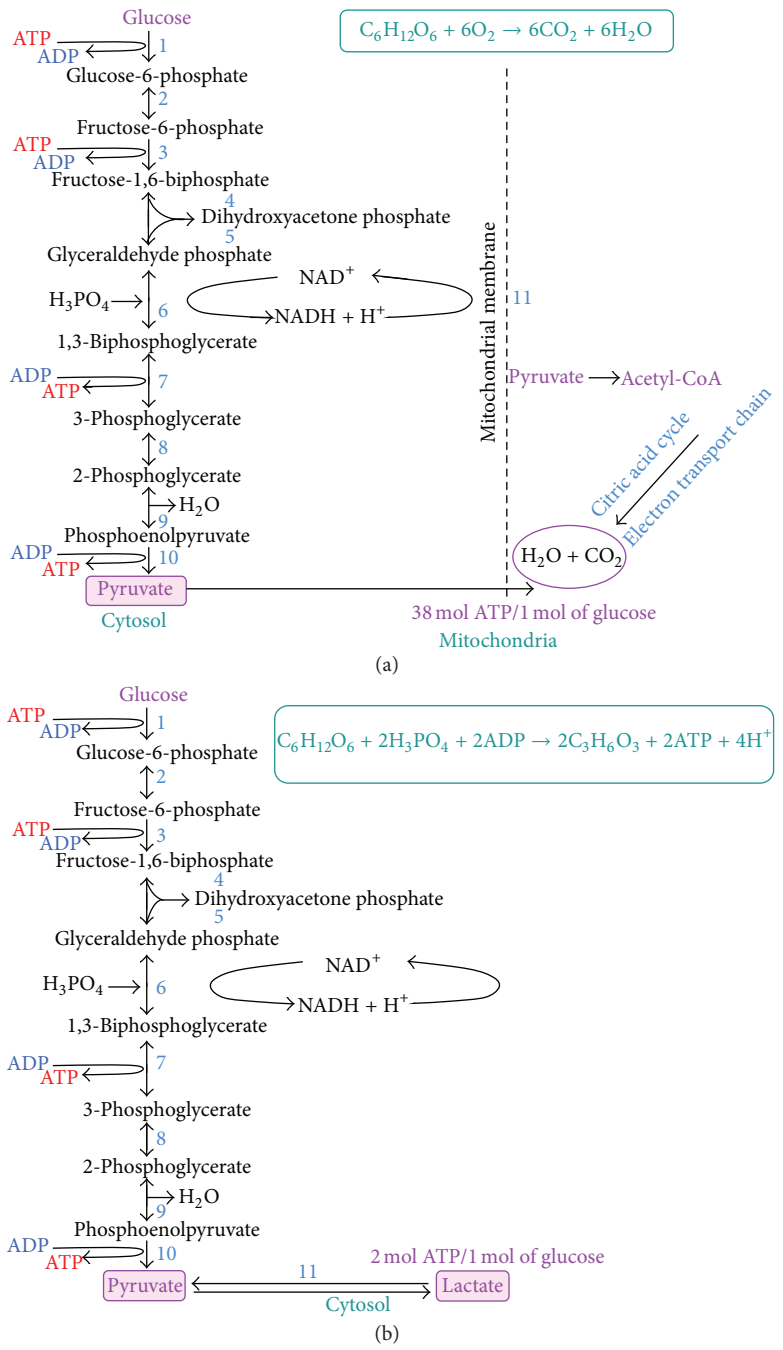

Figure 1: Glucose metabolism at the normal and hypoxic conditions. (a) Glucose is metabolized to pyruvate; the latter undergoes Crebb's cycle in mitochondria and catabolized to CO_2 and oxygen, while 38 molecules of ATP are synthesized. (b) Anaerobic metabolism of glucose, resulting in lactate production and two molecules of ATP. No mitochondria are involved in this process. Cancerous cells use this way of glucose metabolism even at the normal conditions, that is, so-called "aerobic" glycolysis takes place.

mTOR kinase, which activates transcription factor HIF1A even at the normoxic conditions [15]. Loss of AMPK signaling, which is inhibitor of mTOR, also stimulates glycolysis.

5. Virus-Encoded Proteins Play an Important Role in Regulation of "Aerobic" Glycolysis

5.1. Herpesviruses. EBV (HHV4) and KSHV (HHV8) belong to the Herpesviridae family. EBV is associated with BL, a highly aggressive malignancy that is developing from germinal center B-cells [20]. Characteristic of all BL subtypes is enhanced expression of MYC oncoprotein, due to chromosomal rearrangements [21, 22]. Recently we reported that in BL cell lines MYC is the main regulator of "aerobic" glycolysis, while in LCL, with the low expression levels of MYC, HIF1A controls the Warburg effect [23, 24]. HIF1 is a heterodimer consisting of oxygen dependent transcriptional factors HIF1A and ARNT, or HIF1B. The

stability of HIF1A is regulated by oxygen level, while HIF1B is constitutively expressed. At the normoxic condition HIF1A is ubiquitinated by VHL (E3 ubiquitin ligase) at the specific proline residues (402 and 564) [25] that are hydroxylated by prolyl hydroxylases (PHDs) [26, 27]. The ubiquitinated HIF1A undergoes intensive proteasomal degradation. PHD enzymes require iron (Fe^{2+}) and ascorbate as cofactors to perform hydroxylation [28]. At hypoxic condition oxygen is not available for hydroxylation of prolines that results in stabilization of HIF1A [29]. The stabilized HIF1A translocates to nucleus where it forms the HIF1A-ARNT heterodimer and activates transcription of a set of genes and also those involved in regulation of the Warburg effect.

We have reported earlier that even under normal oxygen level EBNA3 binds to PHD2 and EBNA5 to PHD1, thus, inactivating both enzymes. As a consequence, ubiquitination of HIF1A is inhibited, and the Warburg effect is activated [24].

Another herpes virus, KSHV, also promotes the Warburg effect. KSHV induces the expression of miRNA clusters that inhibit the expression of gene, encoding the HIF1A prolyl hydroxylase PHD1 and also the mitochondrial heat shock protein HSPA9 [30]. As a result, the HIF1A is stabilized and transactivates responsive genes. It was shown that one-third of the 194 different biochemicals were altered upon infection of endothelial cells with KSHV, compared with the noninfected host cells, using a metabolomics approach. Noteworthy, the number of altered metabolic pathways was similar to that observed for cancer cells. Pathways include amino acid metabolism and many glycolytic intermediates, such as 3-phosphoglycerate and 2-phosphoglycerate and phosphoenolpyruvate. The pentose phosphate pathway intermediates, such as ribose 5-phosphate, ribulose 5-phosphate, and/or xylulose 5-phosphate, were elevated significantly in KSHV infected samples. Metabolites involved in *de novo* fatty acid synthesis were also increased in KSHV infected cells. Moreover, inhibition of fatty acid synthesis resulted in induction of apoptosis in infected cells [31].

5.2. Polyomaviruses. JCV, SV40, and virus, obtained from a specimen of a renal transplant patient with initials B.K. (BKV), are common polyomaviruses in human populations.

The SV40 transformed rabbit chondrocytes showed alterations in the activities of mitochondria and metabolism. Increases in "aerobic" glycolysis and in activity of glycolytic enzymes were observed in SV40-transformed cells, probably due to chromosomal rearrangements induced by virus [32]. It was also shown, using transfections of primary human fibroblasts with large and small T antigens of SV40 in different combinations along with hTERT and HRAS, that the large T antigen expression leads to decreased dependency of transformed cells on mitochondrial energy production [33]. Noteworthy, the small T antigen of SV40 expression resulted in activation of the AKT signaling, enhancing "aerobic" glycolysis [34, 35]. Interestingly, medulloblastoma cells, expressing the large T antigen of JCV, showed significantly lower mitochondrial respiration and glycolysis. Upon glucose deprivation, T-antigen expression was suppressed due to activation of AMPK, an important sensor of the AMP/ATP ratio in cells. Therefore, the consumption of glutamine increased threefold in cells that expressed the large T of JCV [36]. As was mentioned above, TP53 can inhibit the Warburg effect [37]. It is well known that the large T antigen of polyomaviruses SV40 [38], JCV [39], and BKV [40] binds to TP53 and abolishes functional activity of the latter as transcription factor. Hence, functional inactivation of TP53 not only promotes cell transformation but also induces the metabolic switch.

5.3. Papillomavirus. It was shown that HPV encoded E2 protein is localized predominantly in the nucleus of infected cells. However, in the case of oncogenic (high-risk) strains 18 and 16 the E2 protein can shuttle between cytoplasm and nucleus. It was shown, using mass spectrometry of interactome, that cytoplasmic E2 is associated with the components of respiratory chain in the inner mitochondrial membrane. Electron microscopy showed that E2 alters morphology of cristae and enhances the production of mitochondrial reactive oxygen species (ROS). Such ROS release was found concurrent with stability of HIF1A and increased rate of glycolysis [41]. Another HPV-encoded oncoprotein, E6, also can promote the Warburg effect through inhibiting the binding between HIF1A and VHL. This abolishes VHL-mediated HIF1A ubiquitination, thus stabilizing the latter [42].

5.4. Adenoviruses. The ability of adenoviruses to perform the metabolic shift was demonstrated by infection of primary rat embryonic fibroblasts (REFs) with the oncogenic adenovirus type 12, in comparison with nononcogenic types 3 and 6. REFs, infected with type 12 virus, intensively used glucose at the ordinary conditions; both, "aerobic" glycolysis and pyruvate oxidation, took place. Similar metabolic switch was observed in the hamster sarcoma cells infected with type 12 adenovirus [43].

Recently it was shown that the adenovirus encoded oncogene E4ORF1 can induce *MYC* that plays an important role in glycolysis. Transcriptional activity of the MYC protein is enhanced by E4ORF1. Moreover, the expression levels of enzymes involved in "aerobic" glycolysis, such as hexokinase 2 (HK2), phosphofructokinase 1 (PFKM), GAPDH, and LDHA, are increased [44]. As was discussed earlier, these enzymes are encoded by the MYC-dependent genes.

6. Involvement of DNA Tumor Viruses in Glucose Transport

As was discussed above, glucose is the preferential source of energy for cancer cells; therefore, they need massive supply of glucose compared to normal cells [45]. By hijacking glucose transport system, DNA tumor viruses are able to deliver huge amounts of glucose for proliferating cells, enhancing their tumorigenic capacity [2]. Several viral proteins can facilitate the glucose transport in cancer cells.

Notably, the rise in glucose transport in cancer cells is not due to *de novo* synthesis of a delivery system but by alteration of already existing glucose transport system of cells [46]. Different hypotheses have been proposed to explain this phenomenon, including not sufficient glucose dephosphorylation dependent on glucose-6-phosphatase, increase of HK

expression, and/or the overexpression of glucose transporter (GLUT) proteins [47].

GLUTs are a group of membrane proteins that facilitate the transport of glucose across the plasma membrane. Human genome encodes 14 isoforms of GLUT protein, and GLUT-1,-3,-4 and -12 are involved in cancerogenesis [48]. Expression of GLUT is under the control of activated HIF1A [49]. Infection with DNA tumor viruses leads to elevated expression of GLUT proteins, increasing the glucose uptake. We have shown earlier that expression of *GLUT-1* at mRNA level was induced in EBV positive LCLs and BL cell lines, compared with EBV negative cells [23, 24]. Upon latent infection of human monocytic cell lines with KSHV, GLUT1, and HK expression are increased at the protein level [50].

Not only are the levels of glucose transporter molecules elevated, the trafficking mechanism is also altered to ensure ample supply of glucose. Virus encoded proteins enhance the translocation of GLUT molecules to a cell surface; hence, there is another strategy to increase the glucose uptake. For example, AKT hyperphosphorylation upon KSHV infection correlates with plasma membrane exposure of GLUT1 [51]. Similarly, EBV also induces the translocation of GLUT1 via protein kinase IKKB-AKT pathway [52].

The HPV18-encoded protein E6 participates in stimulation of the SGLT1 activity. By this way, E6 accomplish cellular glucose uptake through Na^+-coupled glucose transport mediated by SGLT1 [53]. It is noteworthy that in SV40 transformed mouse 3T3 cells hexose transporters are relocated from microsomal membranes to plasma membrane, suggesting that oncogenic DNA viruses utilize not only transcriptional regulation of glucose transport but also alterations in transporter trafficking during transformation [54].

7. Effect of DNA Oncoviruses on Secretion of Aerobic Glycolytic Waste

Lactate secreted into an extracellular matrix plays an important role in tumor metastasizing. This process is promoted by lactate-induced secretion of the hyaluronic acid by cancer-associated fibroblasts, thus generating an environment favorable for migration of tumor cells [55]. Moreover, lactate produced by tumor cells helps them to evade immune system by modulating dendritic cell activation and antigen expression that mediate the T cell responses [56, 57].

Activated T cells themselves use glycolysis as a main source of energy [58–60]. Importantly, the immune cells are struggling to get rid of lactate produced by themselves: cellular lactate transport depends on the ratio between the intra- and extracellular concentrations of lactate. Ultimately, leukocytes may be asphyxiated by lactate [61]. Cancerous cells of solid tumors ensure sufficient supply of nutrient and oxygen for rapid proliferation via lactate mediated upregulation of VEGF, thus inducing the angiogenesis [62]. Lactate stimulates the angiogenesis also via PI3K/AKT pathway [63].

The major transporter molecules of lactate in cells are monocarboxylate transporters (MCTs). MCT family consists of 14 members that are encoded by *SLC16A* gene family. The four MCTs (MCT1, MCT2, MCT3, and MCT4) are responsible for proton-linked transport of metabolically important monocarboxylates such as lactate, pyruvate, and ketone bodies [64–67]. MCTs carry 12 transmembrane domains with intracellular N- and C-termini and a large intracellular loop between transmembrane domains 6 and 7. MCT1 and MCT4 require a monotopic ancillary protein, CD147, for plasma membrane expression and function [68]. CD147 is a multifunctional glycoprotein expressed at higher levels by cancer cells and stromal cells in the tumor microenvironment [69]. KSHV-encoded latency associated nuclear antigen LANA either induces CD147 directly, binding to gene promoter, or transactivates *CD147* upon interactions with specificity protein 1 or early growth response protein 2 [70, 71]. Upregulation of MCT4 and CD147 has been also reported in HPV-induced squamous cell carcinoma of the uterine cervix [72]. Importantly, in BL cells MCT4 was also greatly upregulated [23].

In conclusion, tumor DNA viruses modify metabolism of the transformed cells, supporting their rapid proliferation and showing the Warburg effect (summarized on Figure 2). Moreover, viral proteins enhance glucose uptake and controls tumor microenvironment, promoting metastasizing of the tumor cells.

Abbreviations

AKT1:	RAC-alpha serine/threonine-protein kinase 1
AMPK:	Adenosine monophosphate kinase
ARNT:	Aryl hydrocarbon receptor nuclear translocator
ATP:	Adenosine triphosphate
BKV:	BK virus
BL:	Burkitt lymphoma
CD147:	Extracellular matrix metalloproteinase inducer
E4ORF1:	Early Region 4 Open Reading Frame 1
EBNA:	EBV-encoded nuclear antigen
EBV:	Epstein-Barr virus
FDG-PET:	Fluorodeoxyglucose (^{18}F) positron emission tomography
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GLUT:	Glucose transporter
HIF1A:	Hypoxia inducible factor 1A
HK:	Hexokinase
HPV:	Human papilloma virus
HRAS:	H-rat sarcoma
HSPA9:	Heat shock protein-A9
hTERT:	Human telomerase reverse transcriptase
IKKB:	Inhibitor of nuclear factor kappa-B kinase subunit beta
JCV:	John Cunningham virus
KSHV:	Kaposi sarcoma herpes virus
LANA:	Latency-associated nuclear antigen
LDHA:	Lactate dehydrogenase A
MCT:	Monocarboxylate transporter
mTOR:	Mechanistic target of rapamycin
NAD:	Nicotinamide adenine dinucleotide

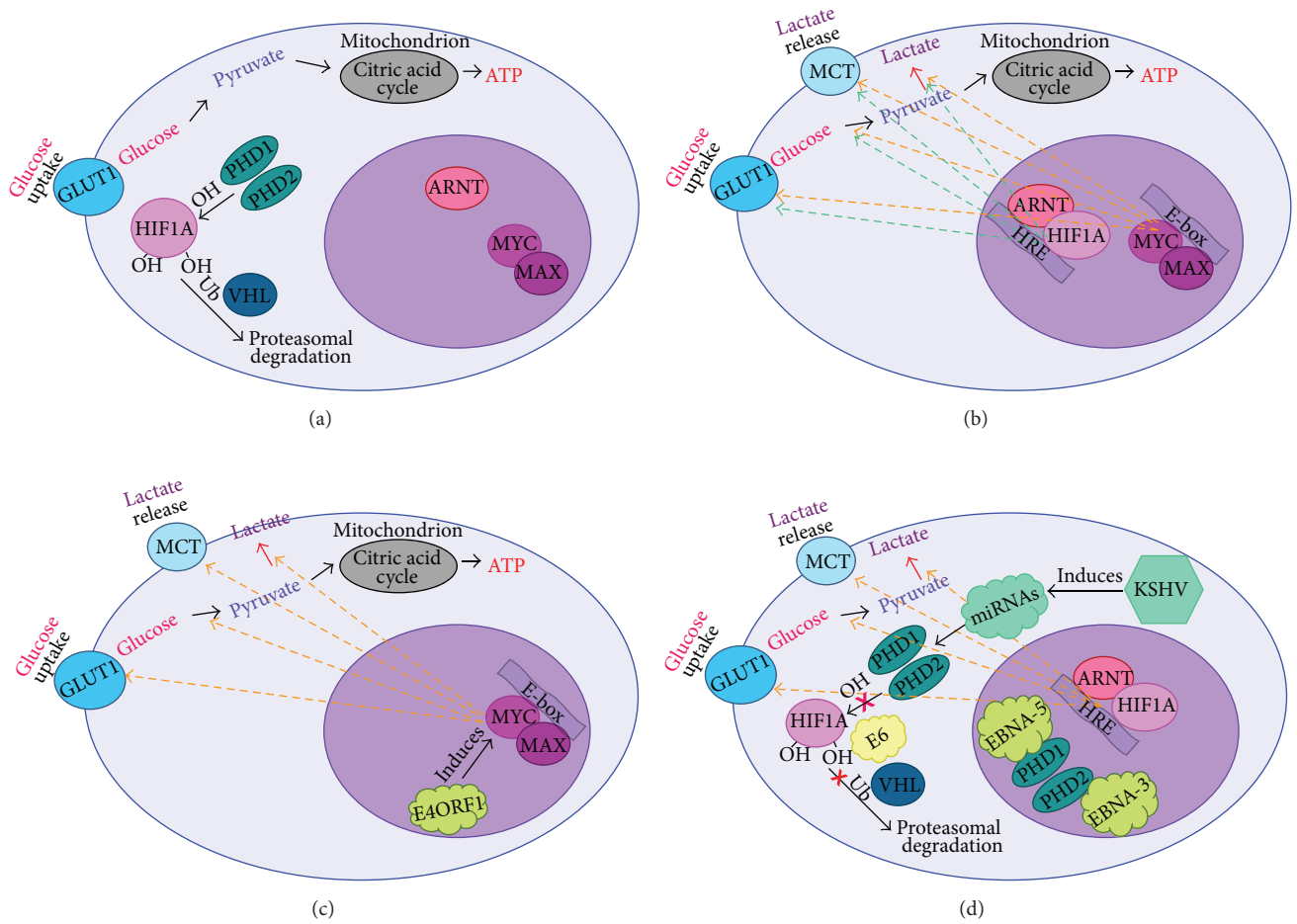

Figure 2: Tumor DNA viruses modify metabolism of the transformed cells. (a) Glucose is metabolized to pyruvate; the latter undergoes Crebb's cycle in mitochondria. (b) "Aerobic" glycolysis takes place, resulting in lactate production. HIF1A-ARNT and MYC-MAX heterodimers induce expression of a set of genes that are involved in glycolysis. (c) E4ORF1 encoded by adenoviruses induces MYC that lead to activation of glycolysis upon infection. (d) HPV-encoded E6 prevents ubiquitination of HIF1A by VHL protein; EBV-encoded EBNA-3 and EBNA-5 bind to PHD2 and PHD2, correspondingly, leading to inhibition of HIF1A hydroxylation; upon infection with KSHV a cluster of miRNAs is activated, resulting in inactivation of PHDs. This leads to stabilization of the HIF1A protein and, hence, to activation of "aerobic" glycolysis.

NADPH:	Nicotinamide adenine dinucleotide phosphate
PFKM:	Phosphofructokinase 1
PHD:	Prolyl hydroxylase domain-containing protein
PI3K:	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKM2:	Pyruvate kinase M2
REF:	Rat embryonic fibroblast
ROS:	Reactive oxygen specie
SCO2:	Cytochrome c oxidase 2
SGLT1:	Sodium-glucose transport protein 1
SLC16A:	Family of proton coupled MCTs
SV40:	Simian vacuolating virus
TIGAR:	TP53-inducible glycolysis and apoptosis regulator
TP53 or p53:	Tumor suppressor p53
VEGF:	Vascular endothelial growth factor
VHL:	Von Hippel–Lindau.

Conflict of Interests

Authors declare no competing interests.

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Review Article

HCV and Oxidative Stress: Implications for HCV Life Cycle and HCV-Associated Pathogenesis

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HCV (hepatitis C virus) is a member of the Flaviviridae family that contains a single-stranded positive-sense RNA genome of approximately 9600 bases. HCV is a major causative agent for chronic liver diseases such as steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma which are caused by multifactorial processes. Elevated levels of reactive oxygen species (ROS) are considered as a major factor contributing to HCV-associated pathogenesis. This review summarizes the mechanisms involved in formation of ROS in HCV replicating cells and describes the interference of HCV with ROS detoxifying systems. The relevance of ROS for HCV-associated pathogenesis is reviewed with a focus on the interference of elevated ROS levels with processes controlling liver regeneration. The overview about the impact of ROS for the viral life cycle is focused on the relevance of autophagy for the HCV life cycle and the crosstalk between HCV, elevated ROS levels, and the induction of autophagy.

1. Introduction

At present, almost 2% of the world population suffers from chronic infection with HCV.HCV (hepatitis C virus) is a major cause of chronic hepatitis, steatosis, fibrosis, liver cirrhosis, and hepatocellular carcinoma. HCV belongs to the Flaviviridae family and contains a single-stranded positive-sense RNA genome of approximately 9600 bases [1, 2]. The viral genome is translated into a polyprotein that encompasses about 3100 amino acids. Cellular and viral proteases process the polyprotein cotranslationally and/or posttranslationally. After proteolytic processing of the amino terminal part of the polyprotein, the three structural proteins, core and the envelope proteins E1 and E2, are formed. The pore forming p7 protein (for a recent review, see [3]) stands between the structural proteins and the nonstructural proteins (NS), NS2, NS3, NS4A, NS4B, NS5A, and NS5B [4–6]. The nonstructural proteins form the replicon complex, localized on the cytoplasmic face of the ER. NS5B is the RNA-dependent RNA polymerase [7] and NS2 and NS3 represent viral proteases. NS3 in addition displays RNA helicase/NTPase activity; NS5A has RNA binding capacity, induces lipid droplet formation, and affects a variety of signal transduction cascades. HCV replication takes place at specialized rearranged intracellular membranes derived from the ER (endoplasmic reticulum), the so-called membranous web [5, 8]. Genome replication does not require the presence of the viral structural proteins enabling the establishment of subgenomic replicons that allow the analysis of genome replication in the absence of virion formation. Before cell culture systems based on the JFH-1 genome [9–11] and Huh7.5 cells were established, the subgenomic replicons were an extremely helpful system to study many aspects of the viral life cycle involved in genome replication [12].

The HCV life cycle is tightly associated with hepatocyte lipid metabolism [13]. The membranous web is enriched in proteins involved in very low density lipoproteins (VLDL) assembly, that is, apolipoprotein (apo)B, apoE, and microsomal triglyceride transfer protein (MTP) [14–17]. Lipid droplets (LDs), organelles for the intracellular storage of neutral lipids, serve as an assembly platform for HCV and thus play an important role for the morphogenesis of HCV [18–20]. LD formation can be induced by core and NS5A. In HCV replicating cells, core is addressed to LDs via diacylglycerol

transferase 1 [21, 22]. HCV assembly and morphogenesis start on the surface of LDs.

There are many reports describing elevated ROS (reactive oxygen species) levels in HCV replicating cells and in liver tissue and lymphocytes derived from HCV-infected patients [23–27]. In this review, the term ROS encompasses various radicals such as the superoxide anion ($O_2^{-\bullet}$), the hydroxyl radical ($HO^\bullet$), or hydrogen peroxide (H_2O_2).

In the liver, high amounts of the antioxidant glutathione (GSH) are found, which plays an important role in phase II metabolism of xenobiotics. In many patients suffering from chronic HCV infection, reduced levels of GSH are found in the serum and in the liver. Moreover, the ratio between the oxidized form (GSSG) and the reduced form (GSH) is elevated, reflecting the partially depleted antioxidant potential [28].

Eukaryotic cells have evolved a variety of strategies to detoxify ROS. Among these are reducing components such as glutathione or small oxidoreductases such as thioredoxin or enzymes that directly detoxify ROS, that is, peroxidases. A central role for the expression of a variety of cytoprotective enzymes plays the transcription factor Nrf2 (NF-E2-related factor 2) [29–31]. In its inactive state, Nrf2 is complexed with its endogenous inhibitor Keap1 (Kelch-like ECH-associated protein 1). This complex is rapidly degraded by the proteasomal system. Elevated levels of radicals or electrophiles affect intracellular SH-groups in Keap1, leading to their modification, which finally leads to a conformational change of Keap1 which results in the release of Nrf2 [32]. Nrf2 enters the nucleus and binds as a heterodimer with sMaf proteins to a conserved sequence in the promoter of cytoprotective genes, the antioxidant response element (ARE), and thereby induces the expression of genes harbouring AREs in their promoter. Nrf2-deficient mice have an impaired liver regeneration [33, 34]. The lack of Nrf2 leads to a diminished expression of cytoprotective genes and therefore to elevated ROS levels triggering an activation of JNK [35]. Activated JNK1/2 leads to a Ser/Thr phosphorylation of IRS1/2 which interrupts insulin receptor-dependent activation of proliferative pathways such as activation of protein kinase B (AKT/PKB). This would require tyrosine phosphorylation of IRS1/2 [36].

On the other hand, constitutive activation of Nrf2 also negatively affects liver regeneration. In transgenic mice, overexpressing caNrf2 liver regeneration is impaired due to impaired proliferation and enhanced apoptosis. An upregulation of the cyclin-dependent kinase inhibitor p15 and of the proapoptotic protein Bim can be observed. There is evidence for a crosstalk between Nrf2 and NF-κB [37, 38]. In neuronal cells, it was observed that activation of Nrf2 is associated with a decreased activation of NF-κB, leading to an elevated sensitivity for apoptotic stimuli [39].

This review aims to provide an overview about the modulation of radical generating pathways in HCV replicating cells and to summarize the interference of HCV with ROS inactivating mechanisms. The relevance of ROS for the HCV life cycle which is focused on the crosstalk of ROS with the induction and the impact of autophagy for the life cycle of HCV is discussed. Finally, the importance of ROS for the onset of HCV-associated pathogenesis with a focus on the interference with pathways affecting liver regeneration is summarized.

2. Sources of Reactive Oxygen Species (ROS) in HCV Replicating Cells

2.1. Increased Mitochondrial ROS Production. Mitochondria play a crucial role for the production of ROS in HCV-infected cells [26]. HCV core protein is considered as a major regulator affecting the release of ROS from mitochondria [40]. The full-length core protein (aa1–191) and the mature form of core (aa1–173) have been described to directly associate with the outer mitochondrial membrane [41–43]. The interaction with the outer mitochondrial membrane is mediated via the C-terminal hydrophobic motif [41, 44]. Moreover, core was found in the mitochondria-associated membrane (MAM) fraction: here a close contact between the membrane of the endoplasmic reticulum (ER) and the mitochondrion exists [41, 45]. Moreover, there is a report that described, based on electron microscopy analysis, an association of core with the inner mitochondrial membrane [46]. On the other hand, a detailed analysis based on confocal immunofluorescence microscopy of HCVcc-infected Huh7.5 cells found no evidence for a direct interaction of core with mitochondria [47, 48], while others described that, regardless of whether core was selectively overexpressed or produced in the context of HCV replication in JFH-1 or J6 positive cells, core is directly associated with the mitochondrion [49]. The detailed analysis of the association of core with the mitochondria is hampered by the fact that much higher amounts of core are localized at the ER and on the surface of LDs. This makes it difficult to discriminate whether a real mitochondrial association is observed or the observation reflects the much stronger signal based on the MAM localization of core.

In addition to core, a fraction of NS3/NS4A, a viral protease complex, is associated with the outer mitochondrial membrane where it cleaves MAVS (mitochondrial antiviral signaling protein). MAVS is tail-anchored at the outer mitochondrial membrane [50, 51]. MAVS [52] is an essential component of the innate immune response pathway: dsRNA is recognized by RIG-I which leads via activation of IRF3 to the expression of IFN-beta.

2.2. Relevance of Elevated Cytosolic Ca^{2+} Levels for Induction of ROS Formation. HCV replication and selective core overexpression induce ER stress [53], leading to Ca^{2+} release from the ER into the cytoplasm [54–57]. In parallel, core inhibits the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) and thereby further contributes to an elevated cytoplasmic Ca^{2+} level. NS5A acts as an additional factor perturbing Ca^{2+} homeostasis by triggering the release of Ca^{2+} from the ER in the cytoplasm [57].

The VDAC (voltage-dependent anion channel) is the major component of the mitochondrial permeability transition (MPT) pore. Interacting with the VDAC core sensitizes the MPT pore and increases the mitochondrial Ca^{2+} uniporter activity [59], leading to increased Ca^{2+} influx in the mitochondria. In vitro experiments support this. Addition of

core to purified mitochondria is sufficient to increase Ca^{2+} uptake and to induce ROS production [44, 59, 60].

This reflects a *vicious circle*: the mitochondrial sensitization to Ca^{2+} along with the opening of the MPT pore triggered by core leads to increased ROS levels which in turn induce MPT pore opening.

2.3. Mechanism of Ca^{2+} Mediated Induction of ROS Formation. The increased uptake of Ca^{2+} into mitochondria leads to perturbation of the electron transport chain [44, 54] by inhibition of the electron transport complex I (NADH:ubiquinone oxidoreductase). Electrons are likely to leak from the electron transport chain (ETC) favouring $O_2^{\bullet-}/H_2O_2$ formation. So [61, 62], the elevated ROS production is based on complex 1 substrates due to decreased complex I activity but not due to a direct interaction of the electron transport complexes with core or other HCV proteins. While the activity/functionality of the complexes II and III are not affected by HCV, the complex IV (cytochrome c oxidase) is the other complex affected by HCV and so it contributes to increased ROS formation by the mitochondria.

Pharmacological interference with ER/mitochondrial Ca^{2+} flux normalizes the electron transport chain complex I activity, restores mitochondrial membrane potential, and normalizes ROS production [54]. Comparable effects can be achieved by chelators [60], indicating the prominent role of Ca^{2+} for mitochondrial dysfunction.

Apart from the direct effects on the respiratory chain, enzymes of the TCA and of the lipid beta oxidation are affected in their functionality [63, 64]. By the resulting decreased amount of NADH/FADH equivalents, the electron transport chain and subsequently the mitochondrial membrane potential are affected, contributing to the increased ROS formation.

Decreased levels of prohibitin are described to lead to impaired assembly and functionality of the complexes of the respiratory chain [65]. The elevated level of prohibitin in HCV replicating cells could reflect the disturbed function of the complexes I and IV of the electron transport chain [66, 67].

3. Impact of NADPH Oxidases for ROS Formation in HCV Replicating Cells

NADPH oxidases (NOX) are multimeric transmembrane enzyme complexes that generate superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) from molecular oxygen, using NADPH as electron donor (recent review: [68]). In mammals, the NOX family encompasses 7 members. In the liver NOX1, NOX2, and NOX4 are found. In case of NOX2, a heterodimeric transmembrane protein encompassing the regulatory subunit p22phox and the catalytic subunit gp91phox, Rac is a further component of the active enzyme complex. Further regulatory components in addition to Rac are p47phox, p40phox, and p67phox. Upon stimulation, these regulatory factors translocate from the cytoplasm to the membrane-bound heterodimeric enzyme complex. In contrast to NOX2, NOX4 requires only p22phox for its enzymatic activity [69, 70].

In the liver, NOX is functionally expressed both in the phagocytic form and in the nonphagocytic form. Various NOX isoforms such as NOX1, NOX2, and NOX4 are distinctively expressed in the specific cell types, including Kupffer cells (KCs), HSCs, endothelial cells (ECs), hepatocytes, and infiltrating leukocytes in the liver [68]. NOX2-derived ROS act in the immune defense in neutrophils and phagocytes.

NOX4 is ubiquitously expressed and inducible. In HCV negative cells NOX4 is found in the cytoplasm and in the nucleus; in HCV replicating cells NOX4 expression is increased and is found preferentially in the nucleus and at the ER [71]. Nuclear formation of ROS by the nuclear NOX4 favours DNA damage.

HCV induces the NOX4 expression: core induces TGF-beta which triggers expression of NOX4. Elevated levels of NOX1 are found in Huh7.5 cells as well as in liver samples from HCV positive patients [72].

4. ER Stress, UPR, and Further Sources of ROS in HCV Replicating Cells

The unfolded protein response (UPR) leads to increased expression of PDI and ER oxidoreductins (Ero1). Ero1 is involved in disulfide bond formation leading to H_2O_2 production as by-product [73]. The induction of ER stress by HCV replication [74, 75] or selective overexpression of HCV structural proteins, E1 and E2 [76], or nonstructural protein, NS4B [77] (overview in [78]), is described. Moreover, Ero1 is involved in the control of Ca^{2+} release from mitochondria [79].

For core-dependent induction of ER stress it was described that both the EIF2AK3 and ATF6 pathways of the unfolded protein response (UPR) were activated by HCV core protein. This contributes to HCV-dependent induction of autophagy [80]. Moreover, HCV replication is increased by upregulation of both WT-PGC-1α and L-PGC-1α through an ER stress-mediated, phosphorylated CREB-dependent pathway [75].

The expression of the ER residing cytochrome P450 2E1 (CYP2E1) is increased in HCV replicating cells and in HCV patients [81]. CYP2E1 is part of the microsomal ethanol oxidizing system (MEOS) which metabolizes ethanol to acetaldehyde in the presence of oxygen and NADPH. CYP2E1 has a significant NADPH oxidase activity, leading to the generation of large quantities of $O_2^{\bullet-}$, H_2O_2, and hydroxyethyl radicals (recent review: [82]). Whether ROS triggers the relocalization of CYP2E1 to the mitochondrion is discussed [83].

5. Interference of HCV with the Nrf2/ARE Pathway

The expression of a variety of cytoprotective genes is controlled by the transcription factor Nrf2. Upon activation Nrf2 is released from the complex with Keap1 and enters the nucleus where it binds as a heterodimer with sMaf proteins to the antioxidant response element, a short cis-acting element which is found in the promoter of Nrf2-dependent genes

and induces the expression of ARE-dependent genes [29, 32]. ARE-dependent genes are involved in the removal of ROI, that is, glutathione-dependent peroxidase (GPx), in the glutathione metabolism, in detoxification of electrophiles by generation of reductive equivalents, or in the removal of misfolded proteins. Examples for ARE-regulated genes are NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione peroxidase (pHGPx), the regulatory and catalytic subunits of glutamate-cysteine ligase (GCLM and GCLC) or glutathione S-transferases (GST), or catalytic units of the constitutive proteasome as PSMB5 [84].

There are conflicting results about the interference of HCV with the Nrf2/ARE system. In a study published by Burdette et al. [85], based on HCV (JFH-1)-infected Huh7 cells, an induction of Nrf2 expression and activation of Nrf2 were described. PDTC as radical scavenger and Ca^{2+} chelating agents abolished HCV-dependent activation of Nrf2. Based on a screen of various kinase inhibitors, p38MAPK and Janus kinase were identified to mediate Nrf2 phosphorylation/activation in HCV replicating Huh7 cells.

In a recent study [86], an increased expression of Nrf2 and nuclear localization of Nrf2 were described for HCV (JFH-1)-infected Huh7.5.1 cells. The HCV-dependent induction of Nrf2 expression is associated with an inhibitory phosphorylation of GSK3-beta which is necessary and sufficient for the induction of Nrf2 by HCV. Moreover, a direct association between GSK3-beta and Nrf2 was described in this study.

Cotransfection of Huh7 cells with expression constructs encoding HCV core, E1, E2, NS4B, or NS5A and an ARE-dependent luciferase reporter construct indicates that selective overproduction of these proteins is able to trigger activation of Nrf2. Further analyses revealed that the activation of Nrf2 by these proteins is due to elevated ROS levels and is transduced by PKC [87].

In contrast to these observations, Carvajal-Yepes et al. [58] found in HCV (JFH-1 and JFH-1/J6) replicating cells and in HCV-infected primary human hepatocytes an inhibitory effect on the activation of Nrf2 and the induction of Nrf2/ARE-dependent genes. In HCV replicating cells, a translocation of the sMafs from the nucleus to the replicon complex occurs, where the sMafs bind to NS3 and thereby are withdrawn from the nucleus. This process depends on the presence of core. In core-deficient mutants this effect is not observed. The replicon-bound sMaf proteins bind Nrf2 and thereby prevent its translocation into the nucleus, leading to an inhibition of Nrf2. In accordance with this, increased sensitivity of HCV replicating cells to ROS-dependent modifications of proteins and DNA (8-OHdG formation) was observed in this study.

These data are in accordance with a transcriptome analysis of HCV replicating cells which revealed a significant reduction of the expression of a variety of Nrf2-dependent genes such as NQO1, epoxide hydrolase 1 (ephx1), catalase (cat), and glutamate-cysteine ligase catalytic subunit (GCLC) and other enzymes of the glutathione metabolism [88]. Moreover, in liver biopsies derived from HCV-infected patients, a decreased expression of heme oxygenase 1 (HO-1), which is Nrf2-dependent regulated, was observed [89, 90]. In contrast to this, in HH4 hepatocytes expressing HCV genome genotype 1a, an increased level of intracellular glutathione and elevated expression of glutathione-S-transferase 3 (GST3) and of metallothionein were described [91]. A recent report described an HCV-dependent induction of glutathione peroxidase 4 (GPx4) in HCV replicating cells and in liver biopsies. The induction is triggered by NS5A via phosphatidylinositol-3-kinase [92].

6. Relevance of ROS for the HCV Life Cycle

The relevance of HCV-dependent induction of oxidative stress with respect to viral genome replication is controversially discussed. On one hand, there are reports describing an inhibitory effect of elevated ROS levels on HCV replication [93, 94] and on the other hand there are reports describing Pycnogenol, a pine extract, which has antioxidant effects and leads to reduced ROS levels and impaired HCV replication [95].

The impact of autophagy on the HCV life cycle is established (for a recent overview, see [96]). This review here focuses on the crosstalk between HCV-dependent induction of ROS, autophagy, and virus morphogenesis.

7. Autophagy

Autophagy, also considered as a "self-eating" process, is a highly conserved and regulated degradation mechanism to maintain cellular homeostasis. Through engulfment of damaged cytoplasmic organelles and protein aggregates, autophagy plays an essential role as a cellular stress response to counteract, for example, ER stress, nutrient deprivation, or a pathogen infection to ensure cell survival [97–99]. Autophagosome formation can be divided into three major steps: the initiation, which begins with the formation of the phagophore assembly site or isolation membranes, the nucleation, and the expansion and enclosure to form double lipid bilayer membrane-bound autophagosomes with an average size of 300–900 nm [100]. Mature autophagosomes then fuse with lysosomes to form autophagolysosomes, where the sequestered cargo is subsequently degraded [101].

The serine/threonine kinase mammalian target of rapamycin (mTOR), a central regulator of the nutrient-sensing pathway, maintains the balance between degradation and synthesis, thereby controlling the growth and starvation response depending on the energy level of the cell [102]. Under nutrient-rich conditions, mTOR suppresses autophagy by phosphorylation of Unc-like kinase 1 und 2 (ULK1/2 complex) [103, 104]. Upon starvation, AMP-activated protein kinase inhibits the mTOR kinase activity, causing an activation of autophagy [105]. This canonical process is regulated by more than 30 autophagy-related genes (Atg) [106]. Four functional units of Atg proteins are involved in the regulation: the Unc-51-like kinase complex (ULK1/2-Atg13-FIP200-ATG101), the class III phosphatidylinositol-3-OH kinase complex (PI3K-Vps-15-Beclin-1-Atg14), and the two ubiquitin-like conjugation systems Atg12 (Atg5-Atg12-Atg16L) and Atg8/LC3 (Atg4-Atg3-LC3) [107]. Upon autophagy induction, the ULK1/2 complex translocates from the cytoplasm to special ER domains, forming

preautophagosomal structure- (PAS-) like structures [108]. Then, in the nucleation step, the activated ULK complex recruits the class III PI3K complex to catalyze the production of autophagosome-specific phosphatidylinositol-3-phosphate (PI3P) [109]. The PI3P effectors DFCP1 (double-FYVE-containing protein 1) and WIPI (WD-repeat protein interacting with phosphoinositides) are recruited, leading to a formation of an ER-associated Ω-like shape, the omegasome, to create the isolation membrane (IM) [110]. Finally, the isolation membrane expands to form the enclosed autophagosome. This process requires the two ubiquitin-like conjugation systems Atg5-Atg12-Atg16L and Atg4-Atg3-LC3. First, Atg12 forms a conjugate with Atg5 by activation through Atg7 (E1-like enzyme) and Atg10 (E2-like enzyme). The Atg12-Atg5 conjugate then interacts with Atg16L to form the Atg5-Atg12-Atg16L complex. This complex serves as an E3-ligase to facilitate the conjugation of cytosolic microtubule-associated protein 1 light chain 3 (LC3) to phosphatidylethanolamine, producing the membrane-bound, lipidated form LC3-II [111]. Finally, the autophagosomes can either fuse with endosomes, forming the hybrid organelle, the amphisome, before fusing with the lysosome or directly fuse with lysosomes, forming the autophagolysosomes to degrade and recycle the sequestered materials [99]. Despite the assumption that the ER is the main membrane source for autophagosome formation, membranes can further originate from ER mitochondria junctions [112], mitochondria [113], Golgi compartment [114], endosomes, and plasma membrane [115].

For the autophagosome-lysosome fusion, the autophagosomal SNARE (soluble N-ethylmaleimide-sensitive factor attachment) protein syntaxin 17 (Stx17) plays a central role [116]. Therefore, Itakura et al. described that Stx17 only translocates on the outer membrane of completed autophagosomes, but not on phagophores, to avoid early fusion with the lysosome. Another regulator of the autophagolysosome formation is the PLEKHM1, (pleckstrin homology domain containing protein family member 1) which controls the fusion through binding to LC3 and HOPS (homotypic fusion and protein sorting) complex, as genetic loss of PLEKHM1 leads to an impaired autophagosome-lysosome fusion [117]. Furthermore, the small GTPase Rab7 is involved in the fusion of the autophagosome with the lysosome [118].

Autophagy is a converging point of different stimuli [119]. As already mentioned, autophagy is a very sensitive process underlying cell responses induced by nearly any stressful condition which influences the cellular homeostasis [106]. Thereby, cells coordinate energy and maintain the nutrient pool for metabolic reactions. If the cell is not able to perpetuate the rate of protein synthesis or to provide the required amount of ATP, autophagy is induced [119]. Furthermore, ROS have been indicated as early inducers of autophagy upon nutrient starvation [120, 121]. It is still ambiguous which reactive species promotes the process, as several publications either indicate H_2O_2 as the molecule generated directly after nutrient deprivation [122, 123] or propose $O_2^{\bullet-}$ as the primary ROS inducing autophagy [124], whereas others just suppose that ROS are indispensable for autophagy, given that treatment with antioxidants partly or completely reverses the mechanism [125].

Although more insights into the complex organization and regulation of autophagy have been gained, further analysis will be needed to better understand the membrane rearrangement processes.

8. Crosstalk Autophagy and the Keap1-Nrf2 Pathway

Autophagy is a major catabolic process that degrades cytoplasmic constituents for the clearance of long-lived or misfolded proteins and damaged organelles. This process functions to keep the cellular homeostasis and to protect the cell against oxidative stress. Starvation as well as oxidative and ER stress can induce autophagy [119, 126]. Another cellular protection system against oxidative and electrophile stress is the Keap1-Nrf2-ARE pathway [127–130].

The crosstalk between autophagy and the Keap1-Nrf2-ARE pathway was uncovered in 2010, when several groups verified the direct interaction between p62 and Keap1 [131–136]. p62 is a stress-inducible cellular protein that has various domains that mediate its interactions with several binding partners, such as LC3-interacting domain (LIR), Keap1-interacting domain (KIR), and ubiquitin-associated (UBA) domain. Furthermore, it operates as a signaling hub and regulates divers' stress responses [137].

For selective autophagy, p62 plays an important role as autophagy adaptor and binds to ubiquitylated cargo (UBA domain) and guides the cargo towards autophagosomal degradation by interacting with LC3 (LIR domain) [138].

Furthermore, p62 interacts with Keap1 (KIR domain), linking the autophagic- and Keap1-Nrf2-pathway [136]. Thereby, the phosphorylation of p62 of Serine 351 of the KIR increases p62's binding affinity for Keap1 and for this reason competitively inhibits the Keap1-Nrf2 interaction. Hence, Keap1 is sequestered into the autophagosome, leading to an impaired ubiquitylation and subsequent activation of Nrf2 [139]. The kinase phosphorylating p62 on S351 is still unknown. Due to controversial observations, the detailed Keap1-p62 interaction mechanism remains to be solved [133, 134, 139]. Furthermore, it was observed that p62 regulates the degradation of Keap1 by controlling the Keap1 turnover [135].

One can speculate whether the complex mechanism leading to impaired Nrf2/ARE-signaling in HCV positive cells by translocation of sMaf from the nucleus by NS3 to the replicon complexes on the cytoplasmic face of the ER and subsequent sequestration of Nrf2 to the replicon complex-bound sMaf prevents the phospho-p62-dependent released Nrf2 from the entry in the nucleus and thereby inhibits the induction of Nrf2/ARE-dependent genes. This could contribute to an impaired elimination of ROS that contribute to the induction of autophagy which is crucial for the HCV life cycle. This hypothesis is graphically summarized in Figure 1 [58].

It is known that elevated ROS levels can induce the autophagic machinery [120]. In line with this, several studies have shown that in HCV replicating cells the unfolded protein response (UPR) and the autophagic pathway are activated,

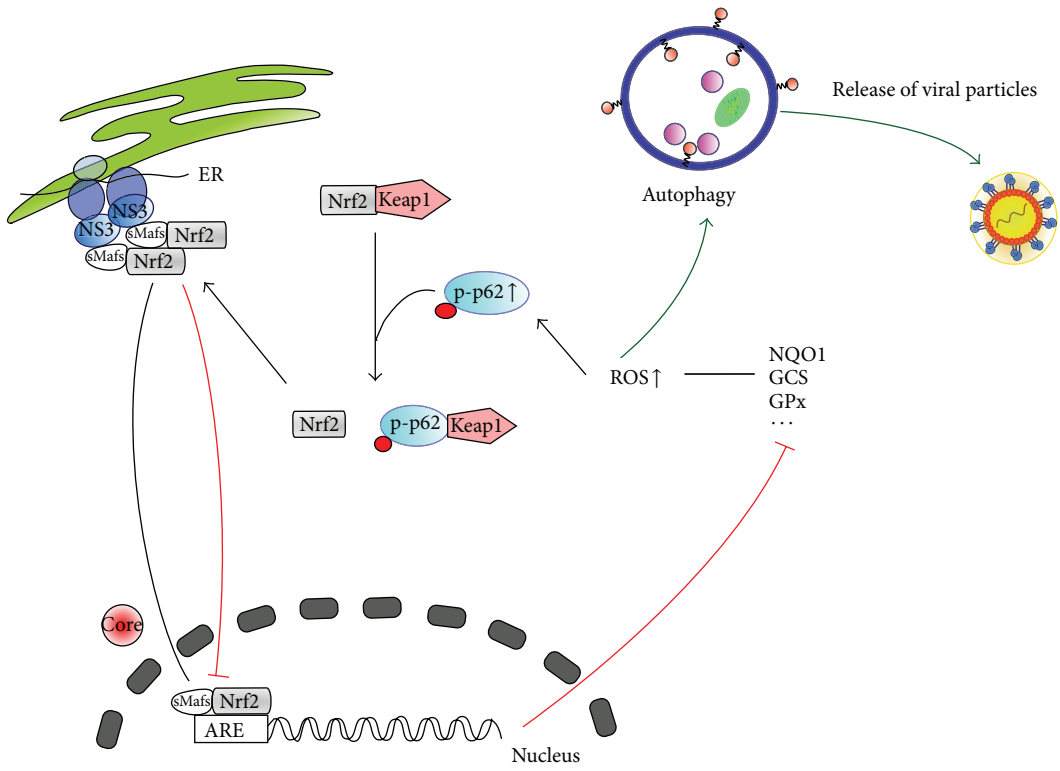

FIGURE 1: Hypothetical model summarizing the interplay between Nrf2, phosphor-p62, ROS, and autophagy. As described by Carvajal-Yepes et al. [58], Nrf2/ARE-signaling in HCV positive cells is impaired by translocation of sMaf from the nucleus to NS3 in the replicon complexes on the cytoplasmic face of the ER. This leads to the sequestration of Nrf2 to the replicon complex-bound sMaf and prevents the phospho-p62-dependent released Nrf2 from the entry in the nucleus and thereby inhibits the induction of Nrf2/ARE-dependent genes. This would contribute to an impaired elimination of ROS that contribute to the induction of autophagy that is crucial for the HCV life cycle.

although the data are conflicting and the detailed mechanism of activation remains to be solved [96].

Several pathways are affected upon HCV infection to result in an induction of autophagy. As already mentioned, HCV infection is associated with oxidative stress and elevated ROS levels. Thereupon, the direct PERK-Nrf2 or the indirect IRE1α-JNK-Nrf2 pathway causes the expression of detoxifying genes [140]. Furthermore, it is supposed that HCV induces ER stress upon activation of UPR in vivo [141] and in vitro [142–145]. Several reports have shown that NS4B plays a central role in UPR and autophagy induction. Li et al. identified that NS4B can induce UPR through activation of the IRE1 or ATF6 pathway, as it interferes with Ca^{2+}-homeostasis, leading to elevated ROS levels [146]. Additionally, Su et al. reported that NS4B interacts with Rab5 and Vps34 and thereby triggers autophagy induction [147]. Furthermore, NS5A was also described to induce autophagy [148]. Moreover, Grégoire et al. demonstrated that different RNA viruses, including HCV, can modulate autophagy by interacting with the human immunity-associated GTPase family M (IRGM) [149]. Taken together, these results showed that a variety of signaling pathways are involved in HCV-induced autophagy. Nevertheless, further investigations are required to understand the complex interplay between HCV and the cellular stress response.

Several studies depicted that autophagy plays a crucial role for the viral life cycle, that is, for the membranous web formation and translation of incoming RNA, replication, and virus release [80, 150, 151]; for recent reviews, see [96, 152, 153].

HCV replication takes place in replication complexes at the so-called membranous web, which consists of ER-derived single-, double-, and multimembrane vesicles and lipid droplets [154–156]. Therefore, an interaction between NS proteins and the autophagic machinery is essential for the reconstruction of the intracellular membranes for the formation of the membranous web [147, 154, 155, 157–165].

The data regarding the role of autophagy for HCV replication are contradictory and yet not fully understood. Several groups demonstrated that HCV induces complete autophagy via the UPR to promote viral replication [142, 143, 145, 155, 166–168], whereas Sir et al. showed contradictory results, reporting that autophagosome maturation seems to be incomplete in HCV replicating cells [165]. A possible explanation for the discrepancy could be the use of different cell culture systems for the studies.

The assembly and release of viral particles are closely linked to the VLDL synthesis and occur via the secretory pathway [169]. Nevertheless, the exact mechanism still remains unclear. Involvement of the autophagic pathway in the release of lipoviral particles was described [16, 17].

Furthermore, Tanida et al. reported that silencing of Atg7 and Beclin-1 inhibits the release of infectious viral particles but does not influence the amount of intracellular viral RNA [167]. Additionally, Grégoire et al. revealed that IRGM interacts with Atg5, Atg10, LC3, and SH3GBL1 and participates in the induction of autophagy and release of viral particles [149, 170].

Summing up, autophagy interferes with various steps of the viral life cycle to promote a permanent viral infection. In light of this, a complex interplay between HCV-induced oxidative stress and the Nrf2/ARE-signaling preserves elevated ROS levels which are necessary for induction of autophagy for HCV life cycle.

9. HCV-Induced Insulin Resistance

HCV infection often comes along with a variety of liver diseases. Oxidative stress is known to play a central role in many of them, as HCV-induced oxidative stress leads to liver damage. A clear correlation between oxidative stress and insulin resistance was reported [171]. Nrf2 is known to be an important regulator of the cellular redox homeostasis, inducing the expression of cytoprotective genes upon elevated ROS levels [34]. Additionally, Beyer et al. identified a further role of Nrf2 in tissue repair [33]. Liver regeneration in Nrf2 knockout mice is delayed after partial hepatectomy or CCl_4-dependent induction of liver damage. This effect was caused by an oxidative stress-mediated resistance to insulin and insulin-like growth factor. Previous investigations have shown an inhibitory effect of ROS on the insulin/insulin growth factor-1 (IGF-1) receptor signaling. This effect was mediated by activation of serine/threonine kinases, that is, JNK, by ROS, which phosphorylate IRS-1 and IRS-2 [172, 173]. Ser/Thr phosphorylation of IRS-1 and IRS-2 leads to dissociation from the insulin receptor, leading to a reduced tyrosine phosphorylation of IRS-1and IRS-2 by the receptor kinase. This results in reduced activation of downstream targets, that is, AKT and p38 [174, 175]. As Nrf2 signaling is impaired upon HCV infection, resulting in elevated ROS levels, this leads to ROS-dependent activation of JNK. Activated JNK phosphorylates IRS1/2 on Ser/Thr residues and thereby impairs insulin signaling.

Shlomai et al. observed an increased expression of peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α), which could also contribute to HCV-induced insulin resistance [176]. PGC-1α is involved in the induction of insulin resistance upon oxidative stress and is a transcriptional cofactor activating the expression of genes involved in the initiation of gluconeogenesis, that is, upregulation of glucose-6-phosphatase (G6Pase), resulting in elevated glucose production [177–179]. Furthermore, an enhanced fatty acid uptake or upregulation of genes involved in lipid and cholesterol synthesis may contribute to oxidative stress-induced insulin resistance [180, 181].

10. Outlook

At present, there exists a lot of information about the fact that HCV infection increases oxidative stress. But the underlying mechanisms and the balance between ROS generating and ROS detoxifying pathways and the regulation of this balance by HCV are not completely understood as reflected by a lot of seemingly contradictory results. To clarify the interplay between these pathways and to reveal the relevance of host factors and of viral factors regarding differences between the HCV genotypes for ROS production/detoxification will be a challenge for the next years. Based on this, whether modulation of this interplay has an impact on the design of antiviral therapy will be analyzed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Liver Damage in Patients with HCV/HIV Coinfection Is Linked to HIV-Related Oxidative Stress

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HIV infection aggravates the progression of liver damage in HCV-coinfected patients, with the underlying pathogenesis being multifactorial. Although high level of oxidative stress has been observed frequently in patients infected with HIV or HCV, the status of oxidative stress in HIV/HCV coinfection and its contribution to HCV liver damage have not been determined. This study involved 363 HBsAg-negative, anti-HCV-positive former blood donors recruited from a village in central China in July 2005; of these, 140 were positive for HIV. Of these 363 subjects, 282 were successfully followed up through July 2009. HIV/HCV-coinfected subjects had higher rates of end-stage liver disease-related death than those monoinfected with HCV. Liver ultrasound manifestations were poor in HIV-positive than in HIV-negative individuals, in both chronic HCV carriers and those with resolved HCV. Serum concentrations of total glutathione (tGSH), malondialdehyde (MDA), glutathione peroxidase (GSH-Px), GSSG, and reduced GSH were higher in HIV-positive than HIV-negative subjects. GSSG concentrations were higher in HIV-infected subjects with abnormal ALT/AST levels than in those with normal ALT/AST levels and were associated with poorer liver ultrasound manifestations. These finding indicated that HIV infection accelerated HCV-associated liver damage in HIV/HCV-coinfected individuals. Increased oxidative stress, induced primarily by HIV coinfection, may contribute to aggravated liver damage.

1. Introduction

Although most individuals exposed to hepatitis C virus (HCV) develop chronic infection, about one quarter to one-third of HCV-infected subjects show spontaneous viral clearance [1, 2]. HCV and human immunodeficiency virus (HIV) share similar routes of transmission, particularly through contaminated blood or blood products. For example, contaminated blood and plasma collection practices in central China in the early 1990s resulted in high rates of HCV and HIV infections among rural farmer donors and more than 90% of HIV-infected subjects coinfected with HCV [3–10]. Hepatotoxicity in HIV-1 monoinfection strongly correlates with CD4+ counts [11] and HIV coinfection can aggravate HCV-related liver damage [7] via mechanisms that include losses of CD4+ T helper cells and neutralizing antibodies [12], dysfunction of dendritic cells (DCs) [13], iron overload [14], and possibly oxidative stress.

Glutathione is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and the amine group of cysteine; this molecule serves as a key antioxidant, preventing damage to important cellular components caused by reactive oxygen species, such as free radicals and peroxides [15]. Glutathione is present in both reduced (GSH) and oxidized (glutathione disulfide (GSSG)) forms. Glutathione reduces disulfide bonds within cytoplasmic proteins to cysteine residues by serving as an electron donor. During this process, reduced GSH is converted to its oxidized form, GSSG [16]. Furthermore, glutathione peroxidase (GSH-Px), a component of the GSH cycle, is one of the most important antioxidant enzymes in humans, catalyzing the breakdown of lipid peroxides and hydroperoxides in both extracellular and intracellular compartments and protecting the integrity of membranes against peroxidative interference and damage [16, 17]. Malondialdehyde (MDA) [18], an important biomarker of oxidative stress, is generated by

the peroxidation of lipids containing polyunsaturated fatty acids and is cytotoxic due to its ability to induce macromolecular crosslinking polymerization.

In vitro studies have shown that oxidative stress is increasing during both HIV [19] and HCV [20] infection. Long-term oxidative stress can lead to an overabundance of free radicals in the liver, contributing to liver fibrosis, cirrhosis, and carcinogenesis. Evaluations of individuals monoinfected with HIV and coinfected with HIV/HCV showed that oxidative stress was greater and levels of serum antioxidants were lower in HIV/HCV-coinfected subjects than in HIV-monoinfected subjects, which is consistent with the observation that fibrosis scores were higher in coinfected subjects compared to HIV monoinfection [19, 21]. These finding hinted that higher oxidative stress status in coinfected subjects was at least partially contributed to HCV infection. However, whether HIV-induced oxidative stress is linked to faster liver disease progression in HIV/HCV-coinfected patients still lacks evidence. We speculated that long-term oxidative stress induced by HIV infection may lead to overabundant free radicals in liver and contribute to liver damage. This study investigated the possible relationship between higher concentrations of circulating free radicals and aggressive liver damage in HIV/HCV-coinfected patients by analyzing the serum levels of oxidants and antioxidants.

2. Materials and Methods

2.1. Study Participants. This study involved 363 HBsAg-negative, anti-HCV-positive former blood donors (FBDs) recruited from a village in central China in July 2005, including 140 coinfected with HIV. In a follow-up survey in July 2009, 282 patients were successfully visited, 36 had died, and 45 were lost to follow-up. Serum concentrations of anti-HCV and anti-HIV antibodies and of HCV and HIV RNA were measured. Subjects positive for anti-HIV antibody were defined as HIV-positive. Subjects positive for anti-HCV antibody and with detectable HCV RNA were defined as chronically infected with HCV, whereas subjects positive for anti-HCV antibody but negative for HCV RNA on two separate occasions at least six months apart were defined as spontaneously clearing HCV. Based on these definitions, the 282 participants were divided into four groups, 102 HIV-negative chronic HCV carriers, 76 HIV-positive chronic HCV carriers, 56 HIV-negative HCV resolvers, and 48 HIV-positive HCV resolvers. A flow diagram of the subjects for this study is shown in Figure S1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/8142431). None of the participants received any form of HCV-specific antiviral therapy, whereas all HIV-positive subjects received regular or intermittent highly active antiretroviral therapy (HAART). HAART regimens included two nucleoside reverse transcriptase inhibitors (NRTIs), either azidothymidine plus didanosine or stavudine plus lamivudine, and one nonnucleoside reverse transcriptase inhibitor (NNRTI), nevirapine. The control group consisted of 18 healthy adults negative for HIV, HBV, and HCV infection.

All participants were interviewed by trained and qualified staff using a standardized questionnaire to collect general information, blood donation history, and use of antiviral or antiretroviral drugs. The demographic and clinical characteristics of all participants are shown in Table S1. All subjects were assessed by routine blood tests and serum biochemical tests.

The study was approved by the institutional review authorities of Peking University Health Science Center (Approval ID: PKUPHLL20090011). All patients provided written informed consent before enrollment in the study.

2.2. Liver Ultrasound Examination. Fasting liver ultrasound examinations were performed using a Convex Ultrasound Scanner (GE LOGIQ 9, GE Medical System, CA, USA). Grey-scale images of the liver were obtained using a 3.5–5 MHz multifrequency transducer. Ultrasonography was performed by two senior physicians, each with more than 10 years of clinical experience. Ultrasound results were classified as normal, altered echostructure, hepatomegaly, diffuse liver parenchyma lesions, and fatty liver. Altered echostructure was defined as intrahepatic hyperechoic and heterogeneous echotexture. Diffuse liver parenchyma was considered an independent factor and was not included in the group with altered echostructure. Fatty liver and its severity were diagnosed according to the criteria from the American Gastroenterology Association, including (1) a diffuse hyperechoic echotexture, (2) increased liver echotexture compared with the kidneys, (3) vascular blurring, and (4) deep attenuation [22].

2.3. HIV and HCV Seropositive Screening and Confirmation. Plasma anti-HCV antibody was detected using Abbott Architect anti-HCV assays (Abbott GmbH & Co. KG, Wiesbaden, Germany) and confirmed by HCV-RIBA assays (Wantai Biological Pharmacy, Beijing, China). HIV infection was screened by ELISA with HIV antibodies (GBI Biotech Co., Ltd., Beijing, China) and confirmed by HIV Blot 2.2 WB assays (Genelabs Diagnostics, Singapore).

2.4. Quantification of HCV-RNA, HIV-RNA, and CD4+/CD8+ T-Cell Counts. Plasma HCV RNA concentrations were measured using the Abbott Real-Time HCV Amplification Kit (Abbott Molecular Inc. Des Plaines, IL, USA), according to the manufacturer's instructions; the limit of detection was 30 IU/mL. Plasma concentrations of HIV-1 RNA were measured with the Standard Amplicor HIV Monitor assay, version 2.0 (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's protocols; the limit of detection was 40 copies/mL.

CD4+ T-cell counts were measured by staining EDTA-treated whole blood for CD3/CD4/CD8/CD45 in TruCount tubes and analyzing with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The absolute numbers of CD4+ T lymphocytes were determined using MultiSET software (BD Bioscience, San Jose, CA, USA).

2.5. Measurements of Oxidative Stress. Three types of glutathione (total and the reduced and oxidized forms) were measured using GSH colorimetric detection kits (Cayman

Table 1: Mortality rates in HCV-monoinfected and HCV/HIV-coinfected patients from 2005 to 2009.

Variable	HCV monoinfection	HCV/HIV coinfection	P value
Total patients in 2005 (n)	223	140	
Total deaths [n (% of total patients)]	7 (3.14%)	29 (20.7%)	<0.001
AIDS-related deaths [n (% of total death)]	0 (0%)	17 (58.6%)	0.005
Non-AIDS-related deaths [n (% of total death)]	7 (100%)	12 (41.4%)	0.005
ESLD-related deaths [n (% of non-AIDS-related death)]	1 (14.3%)	8 (66.7%)	0.027
Other causes of deaths [n (% of non-AIDS-related death)]	6 (85.7%)	4 (33.3%)	0.027

HCV: hepatitis C virus; HIV: human immunodeficiency virus; ESLD: end-stage liver disease.

Table 2: Liver ultrasound manifestations in HIV-positive and HIV-negative patients with/without chronic HCV or resolved HCV.

Liver ultrasound manifestation	HIV^{neg} chronic HCV	HIV^{pos} chronic HCV	P^a value	HIV^{neg} resolved HCV	HIV^{pos} resolved HCV	P^b value
Total patients (n)	102	76		56	48	
Echostructure			<0.001			<0.001
Normal [n, (%)]	73 (71.6%)	24 (31.6%)		40 (71.4%)	16 (33.3%)	
Altered echostructure [n, (%)]	27 (26.5%)	22 (28.9%)		16 (28.6%)	26 (54.2%)	
Diffuse liver parenchyma lesions [n, (%)]	2 (2.0%)	30 (39.5%)		0	6 (12.5%)	
Others						
Hepatomegaly [n, (%)]	1 (1.0%)	2 (2.6%)	0.790	0	1 (2.1%)	0.462
Fatty liver [n, (%)]	7 (6.9%)	6 (7.9%)	0.794	4 (7.1%)	4 (8.3%)	1.00

HCV: hepatitis C virus; HIV: human immunodeficiency virus; P^a comparisons between HIV-negative and HIV-positive groups with chronic HCV infection; P^b comparisons between HIV-negative and HIV-positive groups with resolved HCV.

Chemical, Ann Arbor, MI, USA) after treatment with 2-vinylpyridine. Serum GSH-Px and MDA concentrations were measured with colorimetric detection kits (Jiancheng, Nanjing, China) according to the manufacturer's instructions. Serum concentrations of zinc were measured by the deproteinization method using a Perkin-Elmer 503 atomic absorption spectrophotometer.

2.6. Liver Fibrosis Stages. Stages of liver disease and liver fibrosis were measured using the aspartate aminotransferase to platelet ratio index (APRI) and the FIB-4 fibrosis index [23]. APRI was calculated according to the following formula: AST [IU/L]/(upper limit of normal range) × 100/platelet count (10^9/L). The upper limit of the normal range in this study was 40 IU/L. FIB-4 index was calculated using a formula that included patient age, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations, and platelet counts [24]; that is, FIB-4 index = (age [years] × AST [IU/L])/(platelet count [10^9/L] × ALT [IU/L]$^{1/2}$).

2.7. Statistical Analyses. All statistical analyses were performed using GraphPad Prism for Windows, version 5.0 (GraphPad Software Inc., San Diego, CA). Continuous variables were compared using unpaired t-tests or Mann-Whitney U tests. Categorical variables were compared using Pearson Chi-squared tests, including characteristics of HCV and HIV infection, HCV-related mortality rates, liver echostructure, HIV status, and serum GSSG. Correlations between groups were evaluated by Spearman correlation analysis. All tests were two-tailed, and P values <0.05 were considered statistically significant.

3. Results

3.1. Mortality from 2005 to 2009. Of the 363 HCV-infected participants recruited in 2005, 36 died between 2005 and 2009, including seven HCV-monoinfected and 29 HIV/HCV-coinfected patients. The mortality rate was significantly higher in coinfected than in HCV-monoinfected patients (20.7% versus 3.14%, $P < 0.001$). End-stage liver diseases (ESLDs) were responsible for 66.7% (8/12) of non-AIDS-related deaths in HIV/HCV-coinfected subjects, but only 14.3% (1/7) of deaths in HCV-monoinfected subjects ($P = 0.027$) (Table 1).

3.2. Poorer Liver Ultrasound Manifestations in HIV-Coinfected Subjects. Of the 363 subjects recruited in 2005, 282 (77.7%) were followed up and underwent liver ultrasound examinations in 2009. Ultrasound manifestations of poorer liver histology were observed in HIV-positive compared with HIV-negative individuals, both for chronic HCV carriers and those who experienced spontaneous resolution ($P < 0.001$ each; Table 2). Diffuse liver parenchyma lesions were more common in HIV-positive than in HIV-negative individuals chronically infected with HCV (39.5% versus 2.0%) and in those who showed HCV resolution (12.5% versus

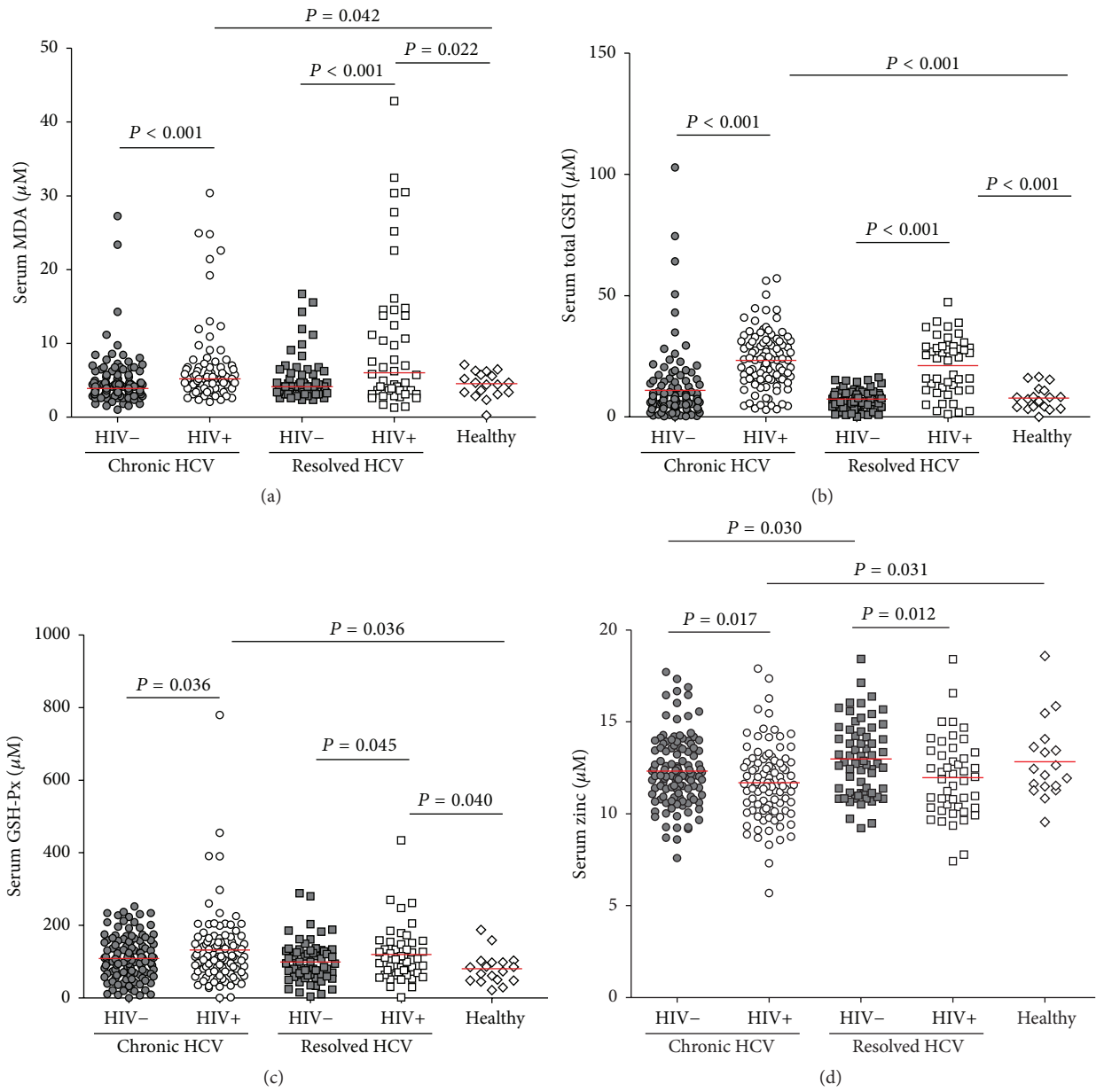


Figure 1: Serum concentrations of the oxidative stress markers MDA (a), total GSH (b), GSH-Px (c), and zinc (d) in HIV-negative chronic HCV carriers (●) ($N = 102$), HIV-positive chronic HCV carriers (○) ($N = 76$), HIV-negative HCV resolvers (■) ($N = 56$), HIV-positive HCV resolvers (□) ($N = 48$), and healthy controls (◇) ($N = 18$). The median value for each group is indicated as a red line. Unpaired t-tests or Mann-Whitney U tests were used for between-group comparisons. All P values were two-tailed, with $P < 0.05$ considered statistically significant.

0%). Altered echostructure was also more frequent in HIV-positive than in HIV-negative individuals with HCV resolution (54.2% versus 28.6%). The frequencies of hepatomegaly and fatty liver were similar in HIV-positive and HIV-negative individuals, both in chronic HCV carriers and in HCV resolvers.

3.3. Higher Serum tGSH, MDA, and GSH-Px Concentrations and Lower Zinc Concentrations, in HIV-Coinfected Subjects. Oxidative stress levels in monoinfected and coinfected subjects were analyzed by measuring serum concentrations of tGSH, MDA, and GSH-Px and zinc concentrations (Figure 1). Overall, HIV-infected patients, both chronic HCV carriers and HCV resolvers, had significantly higher concentrations of MDA ($P = 0.042$ and $P = 0.022$, resp.), total GSH ($P < 0.001$ each), and GSH-Px ($P = 0.036$ and $P = 0.040$, resp.) than healthy controls. Serum concentrations of tGSH ($P < 0.001$), MDA ($P < 0.001$), and GSH-Px ($P = 0.036$) were significantly higher in HIV-positive than in HIV-negative HCV carriers. Similarly, serum concentrations of tGSH ($P < 0.001$), MDA ($P < 0.001$), and GSH-Px ($P = 0.045$) were significantly higher in HIV-positive than

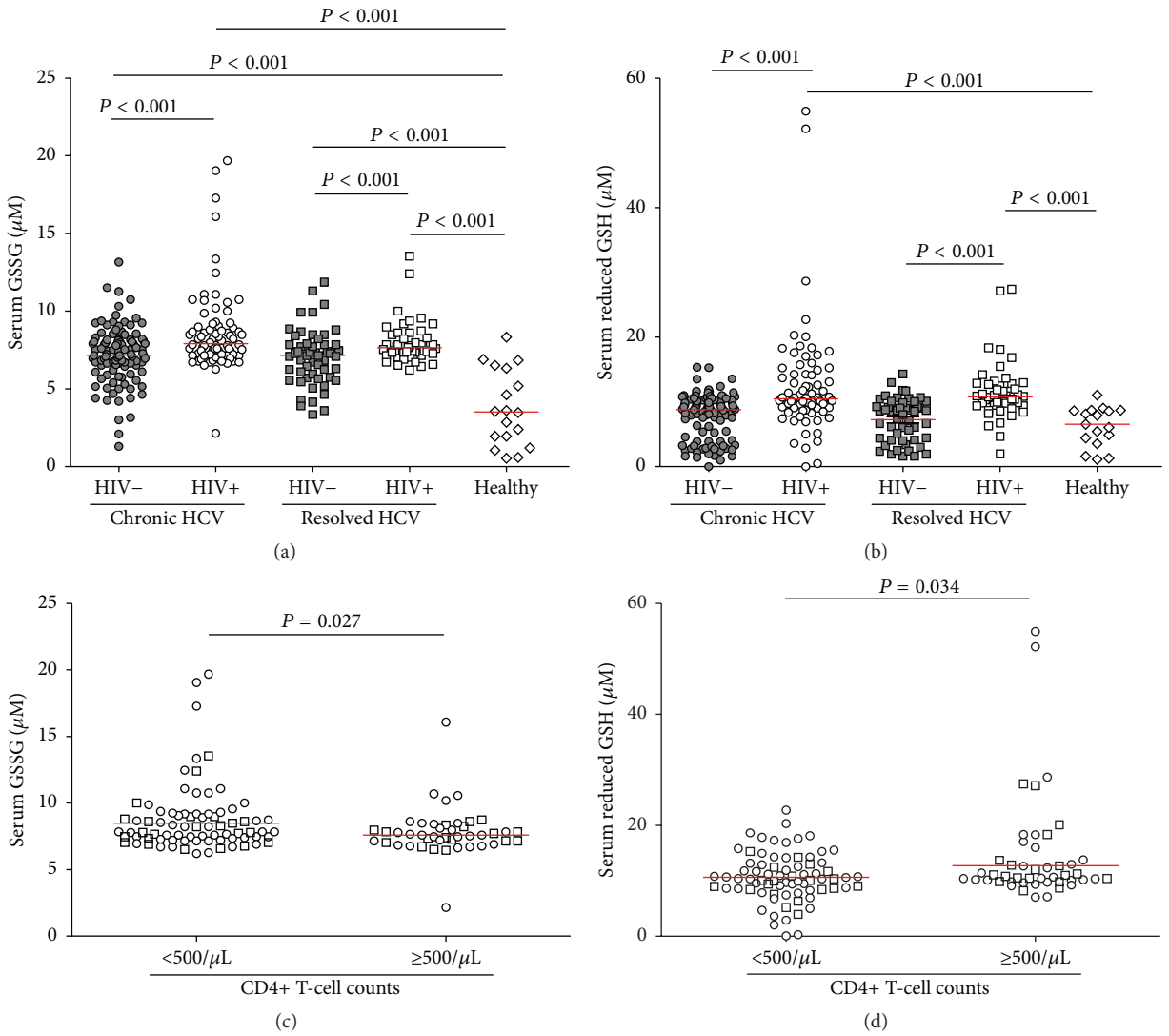


Figure 2: Serum concentration of GSSG (a) and reduced GSH (b) in HIV-negative chronic HCV carriers (●), HIV-positive chronic HCV carriers (○), HIV-negative HCV resolvers (■), HIV-positive HCV resolvers (□), and healthy controls (◇). Serum concentrations of GSSG (c) and reduced GSH (d) in HIV-positive chronic HCV carriers (○) and HIV-positive HCV resolvers (□) were compared in stratified group according to CD4+ T-cell counts (<500/μL versus ≥500/μL). Median value for each group was indicated as red line. Unpaired t-tests or Mann-Whitney U tests were used for between-group comparisons. All P values were two-tailed, with $P < 0.05$ considered statistically significant.

in HIV-negative HCV resolvers (Figures 1(a), 1(b), and 1(c)). By contrast, there were no differences in serum tGSH, MDA, and GSH-Px concentrations between chronic HCV carriers and spontaneous resolvers, either in the presence or absence of HIV coinfection. Lower serum zinc concentrations were observed in HIV-positive than in HIV-negative subjects, both in chronic HCV carriers ($P = 0.017$) and HCV resolvers ($P = 0.012$). Moreover, serum zinc concentrations differed significantly in HIV-negative chronic HCV carriers and resolvers ($P = 0.030$, Figure 1(d)).

3.4. Higher GSSG Was Induced by Either HIV or HCV Infection and Lower CD4+ T-Cell Counts Were Related to Lower Reduced GSH in HIV-Infected Patients. Figure 2(a) shows the concentrations of reduced GSH and GSSG across the five groups of subjects. GSSG concentration was significantly higher in all groups of HCV- and/or HIV-infected subjects than in healthy controls ($P < 0.001$ each), as well as being significantly higher in HIV-positive than in HIV-negative subjects, both for chronic HCV carriers and HCV resolvers ($P < 0.001$ each). No differences were observed between chronic HCV carriers and spontaneous resolvers, either in the presence or absence of HIV coinfection, indicating that oxidative stress remained in the livers of subjects with resolved HCV infection, despite viral clearance.

The concentration of reduced GSH was calculated by subtracting the concentration of GSSG from that of total GSH. Reduced GSH concentration was significantly higher in HIV-positive than in HIV-negative subjects, both among chronic carriers and resolvers, and healthy controls ($P < 0.001$ each,

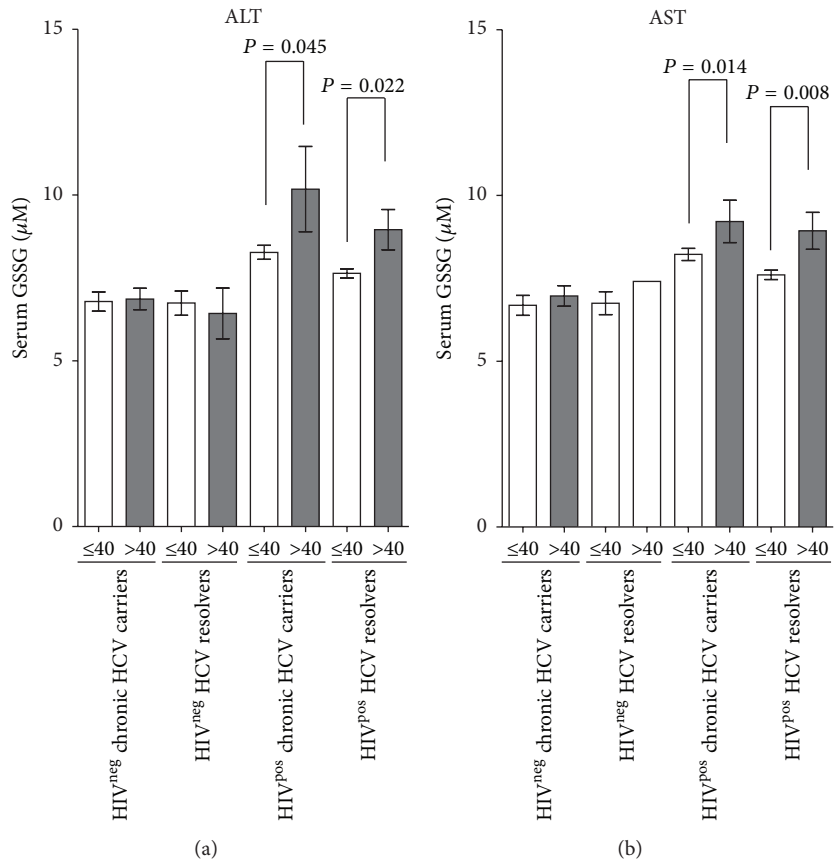


Figure 3: Serum GSSG concentrations are higher in HIV-infected patients with abnormal than in those with normal ALT/AST. Mean ± standard error of the mean (SEM) serum GSSG concentrations in HIV-negative chronic HCV carriers, HIV-positive chronic HCV carriers, HIV-negative HCV resolvers, HIV-positive HCV resolvers, and healthy controls with normal (≤40 IU/L) and abnormal (>40 IU/L) serum ALT (left panel) or AST (right panel). Unpaired nonparametric t-tests were used for between-group comparisons. Average and SEM were indicated for each group. All P values were two-tailed, with $P < 0.05$ considered statistically significant.

Figure 2(b)). These results indicated that the profile of serum reduced GSH was different than that of GSSG, since HIV infection increased GSSG and reduced GSH concentrations, whereas HCV infection increased only GSSG concentration.

In addition, HIV-infected patients (HIV-positive chronic HCV carriers and HIV-positive HCV resolvers) were stratified according to immune status (CD4+ T-cell counts <500/μL and CD4+ T-cell counts ≥500/μL). Analysis showed that HIV-infected patients with lower CD4+ T-cell counts had significantly higher GSSG ($P = 0.027$, Figure 2(c)) while lower reduced GSH ($P = 0.034$, Figure 2(d)) concentrations than patients with higher CD4+ T-cell counts.

3.5. Serum GSSG Was Elevated in HIV-Infected Patients with Abnormal ALT/AST Levels. Serum concentrations of AST and ALT were significantly higher in HCV-monoinfected subjects than in those who experienced spontaneous recovery from HCV ($P < 0.001$ each, Figure S2). Similarly, higher serum AST ($P < 0.001$) and ALT ($P = 0.060$) concentrations were observed in HIV-positive compared to HIV-negative HCV resolvers (Figure S2). Although neither ALT nor AST showed significant correlations with serum GSSG concentrations in both HCV-monoinfected and HIV/HCV-coinfected patients (data not shown), HIV infection of subjects with abnormal (>40 IU/L) AST and ALT levels was associated with significant increases in serum GSSG, both in chronic HCV carriers ($P = 0.045$ for ALT and $P = 0.014$ for AST) and in HCV resolvers ($P = 0.022$ for ALT and $P = 0.008$ for AST) (Figure 3).

3.6. Serum Level of GSSG Was Associated with Poorer Liver Ultrasound Manifestations in HIV-Positive Individuals. Chronic HCV-infected patients may develop liver fibrosis, with some progressing to cirrhosis and even to hepatocellular carcinoma. To evaluate the effects of HIV coinfection on liver fibrosis, APRI and FIB-4 scores were calculated. Compared with HCV resolvers, chronic HCV carriers, whether HIV-negative or HIV-positive, had higher APRI ($P = 0.004$ and $P = 0.007$, resp.) and FIB-4 ($P = 0.028$ and $P = 0.050$, resp.) scores (Figure S3 a and b). In addition, HIV-positive individuals, both chronic HCV carriers and resolvers, had significantly higher APRI ($P = 0.008$ and $P = 0.002$, resp.) and FIB-4 ($P = 0.014$ and $P = 0.027$, resp.) scores

than HIV-negative subjects. Analysis of APRI and FIB-4 scores in different liver ultrasound manifestations (normal, altered echostructure, and diffuse liver parenchyma lesions) in HIV-positive patients showed that APRI score was higher in patients with altered echostructure ($P = 0.031$) and diffuse liver parenchyma ($P = 0.005$) and that FIB-4 score was higher in patients with diffuse liver parenchyma ($P = 0.017$) (Figure S3 c and d). Additionally, negative correlations were found between CD4 T-cell counts and APRI ($P = 0.037$)/FIB-4 ($P = 0.038$) in HIV-positive HCV resolvers (Figure S4 b), but not in HIV-positive chronic HCV carriers (Figure S4 a).

Furthermore, our data showed that serum GSSG was positively correlated with APRI ($r = 0.3567$, $P < 0.001$) and FIB-4 ($r = 0.3277$, $P < 0.001$) indexes in HIV^{pos} groups, but not in HIV^{neg} counterparts ($P > 0.05$) (Figure 4(a)). The relationship of serum GSSG with different liver ultrasound manifestations in the presence or absence of HIV infection was also evaluated. As shown in Figure 4(b), the upper level of normal serum GSSG (9.92 μM) was set as mean plus three times standard deviation based on the values of healthy controls. The results of Chi-square tests showed that serum GSSG was associated with altered echostructure or diffuse liver parenchyma lesions in HIV^{pos} subjects ($\chi^2 = 14.73$, $P < 0.001$) but not in HIV^{neg} individuals ($P > 0.05$). These results indicated that HIV infection contributed at least partially to liver damages in the context of HCV infection, which to some extent ascribed to elevated oxidative stress.

4. Discussion

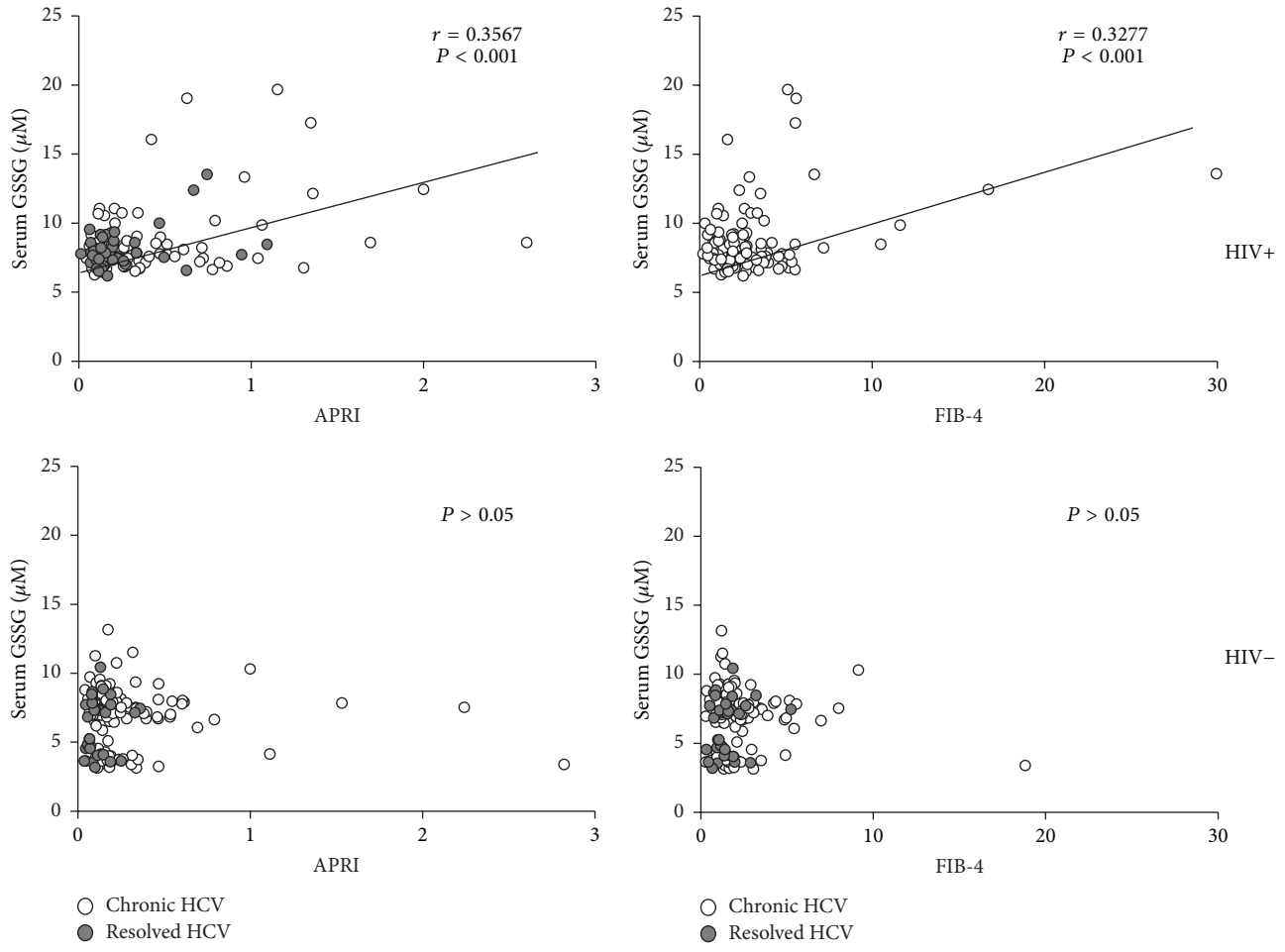
Some studies demonstrated that oxidative stress and liver injury were more pronounced in HIV/HCV coinfection than HIV monoinfection [19, 21]. However, few studies focused on the comparison of oxidative stress between HIV/HCV coinfection and HCV monoinfection. In this study, we demonstrated that HIV/HCV coinfection induced aggravated liver damages compared to HCV monoinfection and a linkage between HIV-induced oxidative stress and the higher incidence rate of advanced liver disease was found in coinfected patients.

In contrast to data from erythrocytes of HCV-infected patients [25] and to our expectations, we found that the antioxidant markers, reduced GSH and GSH-Px, were elevated in patients infected with HIV alone but not in those infected with HCV alone. This discrepancy may ascribe to different sample type used in different studies (serum versus erythrocyte). Serum samples are easier to assay and are likely more accurate in reflecting the status of circulating free radicals. The consistent patterns of GSH-Px and reduced GSH concentrations in subjects infected with HIV and/or HCV support the validity of assaying these markers in serum samples.

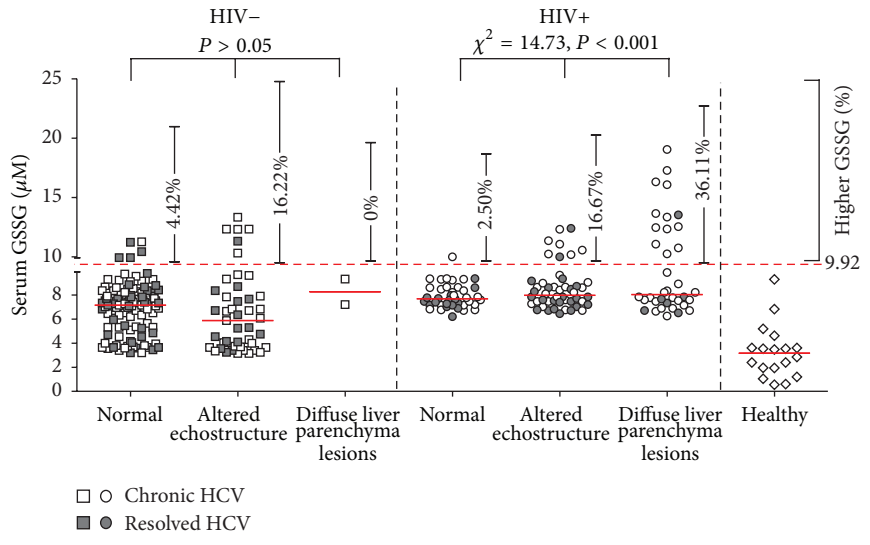
HIV-infected individuals had lower levels of intracellular glutathione in T-cell subsets, with CD4+/CD8+ T-cells having higher levels of intracellular glutathione selectively lost as HIV infection progressed [26, 27]. Our study demonstrated that both serum oxidants (MDA and GSSG) and antioxidants (GSH-Px and reduced GSH) were elevated in chronic HIV/HCV-coinfected patients, but serum zinc was not. The enhanced destruction of lymphocytes and erythrocytes in patients chronically infected with HIV may result in the release of MDA, GSH-Px, GSSG, and reduced GSH. This may partially explain why serum antioxidant indicators were not increased in chronic HCV infection but were markedly increased in HIV/HCV coinfection. However, as an oxidized GSH, serum GSSG was found to be a superior indicator of oxidative stress, during both HCV and HIV infection, as it was increased in chronically HCV-infected, HCV-resolved, and HIV/HCV-coinfected patients.

Hepatotoxicity is a great concern in HIV/HCV-coinfected patients. High HCV-associated morbidity and mortality are common among coinfected patients, even those treated with HAART [28, 29]. This study found that HCV-associated ESLD was a greater cause of death in HIV/HCV-coinfected patients treated with HAART than in HCV-monoinfected patients. It is unclear, however, whether antiretroviral therapy has a negative or positive effect on liver hepatitis in HIV/HCV-coinfected patients [30]. HAART-driven immune reconstitution may retard HIV replication in lymphocytes resident in the liver, thereby theoretically contributing to the ameliorative microenvironment of the liver. Indeed, several retrospective studies have reported a slower progression of liver damage in HAART-treated patients [31, 32]. By contrast, the restoration of immune function may enhance preexisting anti-HCV-specific immune responses in HAART-treated HIV/HCV-coinfected patients. In addition, HAART-associated drug-induced liver injury (DILI) may worsen HCV-related hepatitis in a small number of patients, especially those treated with didanosine, nevirapine, and efavirenz [31–35]. The findings presented in this study clearly showed that HIV coinfection increased serum levels of markers of oxidative stress, both in patients with chronic HCV infection and in HCV resolvers. Although serum antioxidant and oxidant parameters did not correlate with CD4+ T-cell counts in HIV-coinfected patients (data not shown), our data indicated that higher reduced GSH concentrations appeared in HIV-infected patients with higher CD4+ T-cell compared to patients with lower CD4+ T-cell, suggesting that antiretroviral therapy (ART) played a beneficial role in preventing aggravation of oxidative stress status. Similarly, an improvement in the abnormal GSH redox status was found in HIV patients receiving successful ART [36] and HIV infection without ART was associated with lower reduced GSH levels in the lung [37]. We speculated that higher oxidative stress status in HIV/HCV-coinfected patients might ascribe to complicated reasons, including immune system impairment, direct HIV/HCV cytotoxicity, iron load, and/or treatment with HAART.

Ultrasound examination of our patients clearly indicated that aggravated liver parenchymal lesions were more frequent in HIV-positive than in HIV-negative individuals, both in chronic HCV carriers and in HCV resolvers. These patients were not assessed by transient elastography, however, because medical resources at the local medical care were limited at the time the study was initiated. As an alternative, stages of liver fibrosis in these patients were determined using two noninvasive indices of fibrosis, APRI and FIB-4, which have been



(a)



(b)

Figure 4: Association between aggravated liver damage in HCV/HIV-coinfected individuals and higher serum GSSG concentrations. (a) Correlation between APRI (left panel) and FIB-4 (right panel) and serum GSSG concentration in HIV-positive and HIV-negative patients. Correlations of GSSG with APRI and FIB-4 were evaluated by Spearman correlation coefficient. (b) Serum GSSG concentrations in HIV-positive (right panel) and HIV-negative (left panel) patients were compared among patients with different liver ultrasound manifestations (normal, altered echostructure, and diffuse liver parenchymal lesions). The upper level (9.92 μM) of normal serum GSSG was set as the mean plus three times the standard deviation of healthy controls. Trends in GSSG levels among patients categorized by liver ultrasound manifestations were analyzed by Chi-squared tests. All P values were two-tailed, with $P < 0.05$ considered statistically significant.

shown to be valid in evaluating liver fibrosis in numerous studies [23, 38–44]. For example, FIB-4 scores were found to strongly correlate with biopsy-determined stages of HCV-associated liver fibrosis in a large observational cohort of chronic HCV patients [45]. We also found that these two indicators were strongly consistent and correlated with each other in both HCV-monoinfected and HIV/HCV-coinfected patients (data not shown). Both APRI and FIB-4 were higher in HIV-positive than in HIV-negative HCV-infected patients. Interestingly, correlations between APRI/FIB-4 and serum GSSG were observed only in HIV-coinfected patients, not in HIV-negative chronic HCV carriers and HCV resolvers. Similar association was also found between serum GSSG and poorer liver ultrasound manifestation in HIV-infected individual. These findings indicated that, in HIV/HCV-coinfected patients, worsening liver fibrosis status may be associated with HIV infection and may correlate, at least in part, with higher levels of oxidant markers, such as GSSG. Conversely, higher serum GSSG levels were consistent with abnormal levels of serum ALT/AST in HIV/HCV-coinfected but not in HCV-monoinfected patients. Interestingly, a recent study demonstrated that active viral replication induces strong oxidative stress in HIV-1 infected cell lines and a moderate increase of oxidative stress is sufficient to switch HIV-1 from latency to reactivation, which indicated that HIV replication can be a crucial cause of oxidative stress and may contribute to concomitant liver damage, irrespective to HAART [46]. We speculated that higher concentrations of circulating prooxidative components induced by long-term HIV replication *in vivo* may continuously and adversely influence the hepatic microenvironment in HCV-infected patients, resulting in acceleration of the occurrence of fibrosis and cirrhosis, such as poorer liver ultrasound manifestation and elevated APRI/FIB-4 scores in this study. Although HIV induction of a greater degree of oxidative stress was not the sole cause of greater liver damage initially due to HCV infection, it was likely to be at least partially responsible.

Near 100% of the HIV-positive patients in our cohort were also infected with HCV, or were at least positive for anti-HCV antibodies. As only a very small number of HIV-monoinfected patients negative for anti-HCV were found in this village, their number was too small for statistical analysis and they were not included in this study.

5. Conclusions

In conclusion, HCV/HIV-coinfected patients showed accelerated liver damage compared with HCV-monoinfected patients in the present study. This aggravated liver damage was likely due to increased oxidative stress, mainly induced by HIV coinfection.

Abbreviations

tGSH: Total glutathione
GSSG: Oxidized glutathione
MDA: Malondialdehyde
GSH-Px: Glutathione peroxidase
HCV: Hepatitis C virus
HIV-1: Human immunodeficiency virus-1
FBDs: Former blood donors
DCs: Dendritic cells
NRTIs: Nucleoside reverse transcriptase inhibitors
NNRTIs: Nonnucleoside reverse transcriptase inhibitors
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
γ-GT: γ-Glutamyl transpeptidase
APRI: Aspartate aminotransferase to platelet ratio index
FIB-4: Fibrosis index based on four factors
ESLD: End-stage liver disease
HAART: Highly active antiretroviral therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xiangbo Huang and Hua Liang contributed equally to this work.

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