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Nitric Oxide and Redox Regulation in the Liver: Part I General Considerations and Redox biology in Hepatitis

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Abstract

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are created in normal hepatocytes and are critical for normal physiological processes including oxidative respiration, growth, regeneration, apoptosis, and microsomal defense. When the levels of oxidation products exceed the capacity of normal antioxidant systems, oxidative stress occurs. This type of stress, in the form of ROS and RNS, can be damaging to all liver cells, including hepatocytes, Kupffer cells, stellate cells, and endothelial cells, through induction of inflammation, ischemia, fibrosis, necrosis, apoptosis, or through malignant transformation by damaging lipids, proteins, and/or DNA. In part I of this review, we will discuss basic redox biology in the liver, including a review of ROS, RNS, and antioxidants, with a focus on nitric oxide as a common source of RNS. We will then review the evidence for oxidative stress as a mechanism of liver injury in hepatitis (alcoholic, viral, non-alcoholic). In part II of this review, we will review oxidative stress in common pathophysiological conditions including ischemia/reperfusion injury, fibrosis, hepatocellular carcinoma, iron overload, Wilson's disease, sepsis and acetaminophen overdose. Finally, biomarkers, proteomic, and antioxidant therapies will be discussed as areas for future therapeutic interventions.

Keywords

nitric oxide; hepatocytes; oxidative stress; reactive oxygen species; hepatitis; ethanol induced hepatitis; fibrosis; ischemia/reperfusion; antioxidants; proteomics

Outline for Part I and II

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2. Redox in Pathologic Hepatocytes

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- **a.** Hepatitis
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- **b.** Ischemia/ Reperfusion Injury
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- **h.** Acetaminophen-Induced liver injury

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   - **a.** Biomarkers/ Redox proteomics
   - **b.** Antioxidants

Redox Regulation in Healthy Hepatocytes

Reactive Oxygen and Reactive Nitrogen Species

Reactive oxygen species (ROS), reactive nitrogen species (RNS), and other free radicals are critical intermediates in the normal physiology and pathophysiology of hepatocytes. ROS, including, H_2O_2, $OH^{\cdot}$, and $O_2^{\cdot-}$, are important in the creation of oxidative stimuli required for normal physiological homeostasis of hepatocytes. When the equilibrium between ROS generation and the antioxidant defense of the cell is disrupted, a net oxidative stress results [143]. In the liver, free radicals triggered by ROS and RNS are created by neutrophils, Kupffer cells, mitochondria, and cytochromes P450 [70]. The damage created by oxidative stress affects hepatocytes, endothelial, Kupffer, and stellate cells by inducing inflammation, ischemia, apoptosis, necrosis, and regeneration. ROS are know to affect lipids, protein, and DNA and have been implicated in the pathophysiology of atherosclerosis, adult respiratory distress syndrome, cystic fibrosis, cataracts, macular degeneration, cancer, liver disease, diabetes, neurological conditions, and ischemia/reperfusion injuries [100] (Figure 1). Currently, it is believed that ROS also affect signal transduction pathways that, when unbalanced, may lead to hepatic inflammation, necrosis, fibrosis and/or apoptosis [64].

ROS include superoxide ($O_2^{\cdot}$-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$), while RNS includes nitric oxide ($NO^{\cdot}$) and peroxynitrite (ONOO-). (Table 1) Of these, those with an unpaired electron are known as “radicals” [59]. Superoxide ($O_2^{\cdot}$-) is a common radical that can serve as a precursor for several other ROS and RNS, although $O_2^{\cdot}$- itself is also a potent oxidant. Superoxide is constitutively produced by mitochondria as a byproduct of oxidative phosphorylation, as the mitochondria will reduce approximately 1–3% of respiratory oxygen molecules to superoxide anions [124]. Inherent in these cells are enzymes and compounds that consume ROS, including superoxide dismutase that converts superoxide anion (O_2-) to hydrogen peroxide (H_2O_2), catalases (H_2O_2 dismutases), and peroxidases that reduce H_2O_2 to water, frequently by employing the reducing power of NAD(P)H (Table 2;Figure 2). Metals including iron and copper can further react with hydrogen peroxide to produce hydroxyl radicals via the Fenton reaction [156]. (Table 2, Figure 2) The Fenton reaction recycles iron from Fe (II) to Fe (III) by oxidiziong superoxide anion to oxygen. Hydroxyl radicals are then

created. Increases in superoxide can increase the levels of bioactive iron. This can occur in Kupffer cells in the liver in pathologic condition such as cirrhosis. The ability of superoxide to activate this reaction makes it an important factor in oxidative stress.

Reactive oxygen species are necessary for many normal physiological functions. Nitric oxide specifically is critically important in microbial defense, neuronal signaling, vascular tone, platelet aggregation, and cardiac contractility. Reactive oxygen species are also implicated in cell signaling and are considered by some to be second messengers which can trigger cytokines, hormones, and growth factors [89]. In this way, ROS can also affect gene expression. ROS are implicated in the normal induction of apoptosis though the exact mechanisms are unclear. Specifically, nitric oxide has been implicated in both the induction and suppression of apoptosis [28,96]. Since ROS and RNS are ubiquitous in the normal physiology of so many processes, it is not surprising that when excess ROS and RNS are produced, many functions of the cell are disrupted.

Antioxidants

To consume excess ROS and RNS, the body utilizes antioxidants. Endogenous antioxidants are usually small molecular weight molecules that are able to prevent or limit oxidative damage by detoxifying ROS and RNS [53]. Common antioxidants in hepatocytes include glutathione (GSH), glutathione peroxidase and reductase enzymes, superoxide dismutase (SOD), catalase, dismutase, thioredoxin, heme oxygenase (HO), peroxidases, and metal binding proteins. (Table 3) Low molecular weight compounds, such as bilirubin, melatonin, lipoic acid, coenzyme Q, and uric acid, also have antioxidant properties.

Glutathione is a ubiquitous tripeptide whose main function is to react with hydrogen peroxide and, via glutathione peroxidase, to create glutathione disulfide (GSSG) (Table 3). GSH also scavenges other ROS and RNS, chelates copper, and prevents oxidation of protein sulfhydryl groups. In addition to glutathione, catalase and peroxidases are able to break down hydrogen peroxide to less reactive metabolites. Catalase converts hydrogen peroxide to water and oxygen, while peroxidase is also able to reduce it to water with reducing equivalents, usually thiol-containing molecules such as thioredoxin. Thioredoxin contains two sulfhydryl groups, which are oxidized to a disulfide.

An antioxidant enzyme is superoxide dismutase (SOD), which catalyzes the reaction of two molecules of superoxide radical anions with each other, leading to the formation of one molecule of molecular oxygen and one molecule of hydrogen peroxide. Peroxide is relatively stable but, in the presence of a transition metal, may form hydroxyl radicals. Because transition metals may create ROS, metal-binding proteins may also serve antioxidant roles. Iron may be bound by transferrin and lactoferrin, while ceruloplasmin and albumin bind copper. Hemoglobin and myoglobin both contain iron and heme. When exposed to excessive oxidative stress, the heme group and the iron may disassociate, thus promoting lipid peroxidation. When this occurs, hemoglobin binding proteins such as haptoglobin and heme binding proteins, like hemopexin, bind these proteins to decrease the level of lipid peroxidation [57].

Exogenous antioxidants can be consumed through the diet; common dietary forms include vitamin C (ascorbate), vitamin E, carotenoids, and plant phenols. Lipophilic α-tocopherol, the active form of vitamin E, scavenges peroxyl radicals and prevents lipid peroxidation [162]. Ascorbate is critical for the biosynthesis of collagen and is able to scavenge superoxide anions, hydroxyl, peroxyl, thiyl, and oxysulfur radicals, and peroxynitrite [12,58]. Carotenoids are a source of vitamin A and also scavenge free radicals [103]. Plant phenols are able to scavenge ROS and RNS and to chelate metals. Many therapeutic interventions, discussed later in this review, have been aimed at increasing dietary antioxidants in order to prevent and/or treat diseases associated with increased ROS, although the results, as of yet, are inconclusive.

Nitric Oxide

Nitric oxide (NO) is a reactive nitrogen species critical in the redox biology of hepatocytes. It is a hydrophobic, freely diffusible, small molecule with a half-life of seconds (or less). It is created by nitric oxide synthase (NOS) which is present in three forms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). nNOS was first discovered in neuronal tissue and is thought to be constitutively active. It has since been identified as having importance in the regulation of skeletal muscle contractions as well. eNOS was first discovered in the endothelium and is constitutively active as well. It is vitally important in the regulation of blood flow and pressure. While initially thought to only be constitutively active, it is now understood that eNOS production may be also be induced by shear stress. iNOS is an inducible NOS found in a variety of cells, although it was first described in macrophages and hepatocytes after treatment with endotoxins and cytokines. Further research has shown that iNOS can also be constitutively expressed as well, particularly in epithelial cells of the respiratory tract and nasal sinuses. Both eNOS and nNOS are calcium dependent, while iNOS binds calmodulin but is calcium independent.

NOS utilizes L-arginine and oxygen, in combination with electrons from NADPH and cofactors FAD, FMN, BH_4, CaM, and heme, to create L-citrulline and NO. (Figure 3) When constitutively expressed, NOS releases small amounts of NO based on calcium. This NO then serves as an intra- and extracellular signaling molecule. In iNOS however, transcriptional activation leads to increased protein levels and increased expression of the enzyme. iNOS is then able to produce NO, independent of calcium and in large amounts (up to micromolar concentrations). This induction of iNOS may be protective or destructive to the cell, depending on the type of stimulus and the amount and duration of iNOS expression.

NO itself interacts with multiple molecular targets including thiols, transitional metals (such as iron), oxygen, and other free radicals [149]. It readily binds to heme, thus affecting the activation or inhibition of various proteins. Specifically, NO activates guanylate cyclase, which results in increased cGMP synthesis. This increase then affects the activation or inhibition of other molecules. NO can also inhibit cytochrome p450, which affects the metabolism of many compounds [72,146,168]. NO can inhibit cytochrome oxidase by binding to the oxygen binding site, thus reducing ATP (adenosine triphosphate) production, [52,141] and can bind and inhibit NOS and catalase [14,56].

The interaction of NO and oxygen results in other reactive nitrogen species (RNS) such as dinitrogen trioxide (N_2O_3), which can nitrosate amines producing N-nitrosamines or may nitrosate cysteines on various proteins (RSH) to form S-nitrosothiols (RSNO) [14,17,149] RSNO can transport and store NO as well as affect protein interaction and activity [41,42, 152]. NO also reacts with a peroxidate at a diffusion limiting rate creating peroxynitrite (ONOO-) which depending on the amount and duration, may have beneficial (antimicrobial) or detrimental (cell damage/death) effects on the cell [80,125]. Peroxynitrite affects many molecules and can result in alterations in DNA, in lipid and protein oxidation and in protein nitration. (Figure 1; Table 2)

Hepatocytes was one of the first human cell types where iNOS was described. It the liver iNOS was found to be critical in the development and propagation of inflammation [25,158]. (Figure 4) In the liver, iNOS is known to be expressed in all cells (hepatocytes, Kupffer cells, vascular endothelial cells, and stellate/Ito cells) and its expression is induced by IL-1β or IL-1β in combination with TNF, IFNγ, and/or LPS [11,34,35,51,140,145]. (Table 4) NF-κB and γ-interferon response elements (γ-IRE) have both been found to affect the promoter of iNOS [138,158,159]. iNOS expression is also known to be down-regulated by steroids, TGF-β, the heat shock response, p53, and NO itself [158]. (Figure 4) Cytokine activation of iNOS leads to peroxynitrite (ONOO-) formation and thus oxidative stress, which can be beneficial in the

setting of microbial infection or deleterious in the setting hepatitis or acetaminophen-induced liver injury. (Table 4)

The role of iNOS in liver injury is complex. The amount and duration of iNOS expression determines the amount of NO and thus, the level of reactive nitrogen species created. The effects of iNOS are also dependent on the other proinflammatory cascades active in the cell at the time of NO production. For example, iNOS activation is protective in both preventing sepsis and by inhibiting apoptosis but it is also associated with deleterious effects in both ischemia-reperfusion injury and hemorrhagic shock due to oxidative damage and activation of inflammatory cascades. [94] When iNOS is activated by TNF-α and N-galactosamine or by CCl_4, it plays a protective role by inhibiting apoptosis and decreasing oxidative stress. In cultured hepatocytes, iNOS is activated by TNF-α and Fas antibody, and NO is protective in these cells as it inhibits caspases and thus inhibits apoptosis. Similarly, hepatocytes stimulated by hydrogen peroxide are protected by NO induced heme oxygenase-1 upregulation. [94] These examples of iNOS activation and subsequent downstream regulation of protein/gene expression is just a sample of the complexity inherent in redox signaling in hepatocytes.

In hepatocytes, eNOS is found primarily in the endothelial cells of the sinusoids (Figure 5). eNOS is critical in the maintenance and regulation of vascular tone by the basal and inducible release of NO. Release of NO by the endothelial cells results in vascular smooth muscle relaxation while NO scavenging, by cell free hemoglobin for example, results in vasoconstriction due to relative NO depletion. eNOS production is critical for healthy hepatocyte blood flow. Loss of eNOS induced NO has been implicated in the pathology of early ischemia/reperfusion injury, as described below.

Redox in Apoptosis and Regeneration

Reactive oxygen species have been implicated in regulation of apoptosis and in the regeneration of hepatocytes. Apoptosis can be induced by direct chemical interactions or indirectly by activation of various ligands, including TGF-β, Fas, TNF-α/D-Gal, or TNF-α/actinomycin D [16,47,74]. Apoptosis may be induced by superoxide or H_2O_2 from exogenous sources, such as occurs in monocytes and neutrophils in the setting of liver injury,[64] or from intracellularly generated ROS/RNS as occurs from bile acids, ischemia, or hepatotoxins including alcohol, acetaminophen, and chemotherapeutic drugs [62]. Many of these drugs are metabolized by the cytochrome P450 isoform CYP2E1, which results in increased oxidative stress and apoptosis.

When appropriately induced, Kupffer cells can express the death ligand TNF-α, TNF related apoptosis inducing ligand (TRAIL) and Fas ligand [19]. TNF-α plays a critical role in physiological apoptosis and in pathophysiological liver inflammation, ischemia, and necrosis. TNF-α induced cell death may involve the binding of type I TNF receptor, activation of caspases, and recruitment of intracellular proteins, although caspase independent pathways have also been described [66]. Normally, hepatocytes are resistant to TNF-α but many become sensitized to it after oxidative stress-induced gene alterations [66]. The Fas/CD95 death receptor pathway, as well as cytoplasmic c-Abl, has also been implicated in ROS induced apoptosis [62,121,155].

NO appears to play a role in apoptosis but the exact mechanisms are unclear. Hepatic cellular proliferation may be suppressed by NO [90,91] and this effect is prevented by the addition of NOS inhibitors [90,91]. NO induced apoptosis has been suggested by the demonstration of apoptosis in various cells cocultured with NO producing cells [44,87,166]. On the other hand, other studies have demonstrated that NO can protect the liver from TNF-α induced apoptosis [74,165], so the amount of NO and the previous redox conditions of the cell seem to be important in determining the role of NO as an inducer or inhibitor of apoptosis.

Regeneration is associated with increased iNOS expression, which in turn is associated with increased cell proliferation [114]. These protective effects are both cGMP dependent and independent [76]. cGMP analogs decrease caspase activity, while ODQ decreases the inhibition of caspase-3-like activity. NO also results in S-nitrosation of cysteines, causing in inhibition of caspase activity [73,75,76,95,112,113]. These inhibitory effects can be partially reversed by dithiothreitol [76,95]. NO also decreases recombinant caspase, inhibits Bid and Bcl-2 cleavage, and reduces cytochrome c release; all of these are apoptotic stimuli [75,95]. NO induced heat shock protein 70 (HSP70) also protects the liver from apoptosis [74].

Following partial hepatectomy, iNOS expression increases within 4–6 hours [61]. iNOS appears to be critical in the regeneration of hepatocytes after resection, particularly in iNOS knockout mice that show impaired liver regeneration and increased cell death [136]. The NOS inhibitors, aminoguanidine and L-NMMA, demonstrate the need for NO in DNA synthesis [21], while other factors, including cytokine IL-6, TNF-α, NF-κB, and STAT3, also appear to be important in liver regeneration [2,29,33,36,97,169].

In summary, the production of ROS is critical for hepatic homeostasis, but when production overwhelms consumption, oxidative stress develops. Oxidative stress, in turn, can damage all of the cells present in the liver by induction of disruptive processes ranging from inflammation to apoptosis to malignant transformation [105]. The primary sources of this oxidative stress tend to be neutrophils, Kupffer cells, the P450 system of hepatocytes ER, and the hepatocyte mitochondria with nitric oxide playing a critical role. Markers for this oxidative stress include not only increased levels of ROS/RNS, lipid peroxidation, transcription or translational errors, and upregulation of known associated genes/protein expression, but can also include decreased levels of antioxidants including glutathione, vitamin E, ascorbate, and selenium [20,105].

Hepatitis

Alcohol-induced Liver Injury—Oxidative stress is a well-documented cause of alcoholic liver damage. Ethanol is metabolized via alcohol dehydrogenase (ADH), the microsomal ethanol-oxidizing system (MEOS), and catalase in the peroxisomes [98,99,102], although the alcohol dehydrogenase pathway is responsible for a majority of ethanol metabolism. In this pathway, nicotinamide adenine dinucleotide (NAD) is reduced by a transfer of hydrogen to NADH with concomitant production of acetaldehyde. NADP can also be reduced to NADPH and this increase in reducing equivalents in the cytosol (NADH and NADPH) changes the redox potential of the cell. Hydrogen equivalents from ethanol, but not NADH, are transferred from the cytosol to the mitochondria via a shuttle mechanism such as the malate cycle, the fatty acid elongation cycle, and/or the α-glycerophosphate cycle. This replaces the citric acid cycle as the source of hydrogen and the mitochondria become more reduced.

Metabolism of ethanol occurs in the CYP2E1 of the endoplasmic reticulum within hepatocytes. This oxidative metabolism uses NADPH oxidase to generate superoxide anion, hydrogen peroxide, and hydroxyethyl radicals, which can lead to lipid peroxidation [22,37,118,148, 163]. (Figure 6) Acetaldehyde is also created as a product of the ADH reaction and is then oxidized to acetate via mitochondrial aldehyde dehydrogenase. Acetate has been associated with up-regulation of transcription factors such as AP-1 (activator protein-1) and NF-κB, which can upregulate chemokines, inflammatory cytokines (IL-1β, IL-6, IL-18, and others), adhesion molecules, and Fas ligands [137]. This can further lead to apoptosis via caspase cascades as well as necrosis from ATP depletion [133].

One of the mechanisms of alcohol-related liver damage is through oxidative stress and cytokine production leading to hepatic necrosis and fibrosis [131]. Animal models of alcoholic liver disease have demonstrated that increasing oxidative stress increases the severity of liver injury, [71,106,122] while reducing oxidative stress may prevent liver injury [101,167]. Chronic

alcohol exposure can lead to mitochondrial abnormalities including megamitochondria and can result in increased CYP2E1 activity. Ethanol induces an isoform of the cytochrome P450 family, CYP2E1, resulting in oxidative stress in hepatocytes and Kupffer cells [13,82–84], while polymorphisms of CYP2E1 may correlate with susceptibility of alcoholic liver disease [164]. This isoform generates more ROS than do other P450 isoforms via reduction of molecular oxygen through superoxide anion to hydrogen peroxide [6,39]. Animal models of mice that overexpress CYP2E1 showed enhanced alcohol-induced liver injury, although CYP2E1 knockout mice can still develop alcohol-induced liver injury [117]. This increased CYP2E1 activity is noted in animals and humans exposed to chronic intoxication as measured by increased amounts of 1-hydroxyethyl free radicals [6,79]. These react with macromolecules, thiols, and epitopes that affect both structure and function. In models of intoxicated mice, inhibition of CYP2E1 is associated with decreased lipid peroxidation and hydroxyethyl radical production [4,119].

Since mitochondria lack catalase, glutathione (GSH) becomes very important in protection against oxidative stress. GSH is imported into the mitochondria from the cytosol and chronic alcohol exposure results in an impairment of this transport, possibly a result of the accumulation of acetylaldehyde. Mitochondria then become glutathione depleted [104]. Decreased mitochondrial GSH from decreased GSH synthase sensitizes hepatocytes to TNF-α, resulting in increased hepatic damage [31,107]. Glutathione is synthesized from S-adenosylmethionine (SAMe) via methionine synthase (MS) and methionine adenosyltransferase (MAT). In patients with alcoholic liver disease, decreased hepatic SAMe and GSH were observed, and the administration of SAM can reverse the effects of ethanol exposure by increasing liver GSH availability [129]. Patients who drink ethanol heavily are also noted to have decreased levels of a number of antioxidants including selenium,[38] vitamin A,[92] vitamin E,[161] and Coenzyme Q [10] perhaps from decrease intake, decrease absorption, or both.

In the setting of oxidative stress, NO production is also increased, which leads to further damage [171]. Alcohol and lipopolysaccharides (LPS) together create increased hepatic damage, but the addition of aminoguanidine decreases this damage by decreasing NO synthesis [24]. GSH depletion and increase in transaminase activity could be prevented by the inhibition of iNOS [3]. The NO induced vasodilation counters the alcohol-induced vasoconstriction so it is not only the ROS generated by NO, but also the vasoactive properties, that play a role in liver biology [127].

The role of NADPH oxidase and iNOS in Kupffer cells is a compelling source of oxidative stress in alcohol-induced liver injury. NADPH oxidase (p47phox) knockout mice are resistant to alcohol-induced liver damage [81]. Mice given diphenyleneiodonium sulfate (an NADPH oxidase blocker) and alcohol were protected against alcohol-induced liver damage. Ethanol treated mice were noted to have severe liver injury involving gut-derived endotoxin, CD14 receptor, free radicals (detected by ESR), activation of NF-κB, and release of TNF-α from activated Kupffer cells. In contrast, NADPH oxidase-deficient mice had no notable liver pathology, no increase in free radicals, no activation of NF-κB, and no increase in TNF-α. This suggests that NADPH oxidase is important in the development of alcohol-induced liver injury as it relates to NF-κB and TNFα expression [81].

iNOS appears to be required for the formation of alcohol-induced liver injury by McKim et al. [116] Studies on mice reveal that after weeks of ethanol exposure, serum alanine aminotransferase (ALT) levels were significantly increased in wild-type mice but blunted in iNOS knockout mice. Similarly, if wild-type mice were treated with N-(3-aminomethyl) benzyl-acetamindine (1400W), an iNOS inhibitor, their levels of hepatic dysfunction were also attenuated. The administration of ethanol in turn induced inflammation, fatty accumulation, and necrosis in the wild-type mice but not in iNOS knockout mice. The iNOS knockout mice

also did not have accumulation of lipid peroxidase proteins (4-hydroxynonenal) or reactive nitrogen species (3-nitrotyrosine) [116]. These results suggest that iNOS is critical in the development of alcohol-induced liver injury.

Measures of lipid peroxidation, 4-hydroxy-2,3-nonenal (HNE) and malonaldehyde (MDA) protein adducts, are noted in the plasma of alcoholics who have with no signs of hepatic dysfunction. MDA is noted in both protein and lipid free solution of alcoholic patients [30], while HNE is noted by immunohistochemistry of the liver of alcoholics[128], and the magnitude of lipid peroxidation correlates with the degree of liver injury [144], being most prevalent in the perivenular region where liver injury is usually most significant [128]. Oxidation of n-6-polyunsaturated fatty acids produces hydroxyalkenal HNE; male rats chronically intoxicated have increased HNE levels in mitochondria and microsomes [68]. HNE and other hydroxyalkenals can also induce the up-regulation of procollagen type I gene in human stellate cell cultures, thus resulting in increased transcription and synthesis of collagen type 1 [130,133]. HNE induces expression and synthesis of the fibrogenic cytokine transforming growth factor β1 (TGFβ1) in rat liver and macrophage-derived cell lines [93]. HNE also activates the transcription of the heat shock protein (hsp70) in HepG2 cells, a human hepatoma cell line [18]. All of these processes have been implicated in the development of alcoholic liver fibrosis. Kupffer cells, in particular, are involved in the generation of protein adducts with both acetaldehyde and ethanol-induced lipid peroxidation products in alcoholic liver disease [123].

Animal models have also used electron spin resonance to detect free radicals seen in alcohol-induced liver injury [6,7,81]. When hepatocytes were incubated with NADPH, ethanol and the spin trapping agent 4-pyridyl-1-oxide-t-butyl nitrone (4-POBN) produced an electron spin resonance (ESR) signal. The free radical formation was dependent upon the activity of the microsomal monoxygenase system and increased with ethanol and oxide, while catalase and P-450 inhibitors decreased it [6]. This free radical formation in part appeared to be initiated by the hepatic Kupffer cells and was associated with activation of the transcription factor NF-κB, and release of cytotoxic TNF-α from activated Kupffer cells [81].

Impairment of proteasome function, appearing as the loss of the ability to break down oxidized proteins, is also associated with alcohol-induced liver damage. Proteasome inhibition has been associated with increased TNF-mediated hepatocyte death and inflammation [67] and TNF can cause oxidative stress and impair mitochondrial function. Monocytes of alcoholic hepatitis patients produce TNF and have an increased TNF response to endotoxins or lipopolysaccharides (LPS) [115]. Animal models have demonstrated increased LPS stimulated serum TNF levels, while increased Kupffer cell TNF production has been observed in alcohol-fed rats. Inhibition of TNF secretion by antisense oligonucleotides prevented liver injury in ethanol-fed rats [134].

The development of hepatocellular carcinoma has also been associated with alcohol-related liver injury. As noted previously, oxidative stress appears to be important in the development of alcohol-induced liver injury and alcohol-induced liver injury is associated with an increased incidence of hepatocellular carcinoma, although data correlating these two conditions is lacking. Rodents chronically exposed to alcohol produce an ethanol-derived α-hydroxyethyl radical (CH3C·HOH) which may form neoantigens [150], although the role of these neoantigens in the development of carcinoma is unclear. While many studies have documented an association of oxidative stress with alcohol-induced liver injury, and there is clinical evidence of the association between alcohol-induced liver injury and hepatocellular carcinoma, there is little evidence for a mechanism by which alcohol-induced liver injury could lead to hepatocellular carcinoma.

In addition to injury, chronic alcohol exposure is also related to apoptosis of hepatocytes [9, 54], which can be inhibited by the administration of antioxidants. Specifically, mice with chronic ethanol exposure were noted to have an increase in the number of apoptotic bodies, which was depend on the duration of ethanol exposure. After a period of abstinence, this increase in apoptosis was reversed [54]. The increased apoptotic bodies seen involved the parenchymal cells and were often observed with adjacent mononuclear infiltration. This histology was also significant for structural alterations of hepatocytes, mitochondrial pleomorphisms, increases in smooth endoplasmic reticulum and increased lipid deposition.

Viral Hepatitis—Hepatitis B and Hepatitis C virus (HBV/HCV) are common causes of viral hepatitis that result in hepatic inflammation, steatosis, fibrosis, and malignant degeneration. This damage is caused by the virus itself and the subsequent increase in inflammation and oxidative stress. Patients with HCV may experience increased oxidative stress due to activation of NADPH oxidase, increased production of mitochondrial ROS/RNS, decreased antioxidants, iron overload, increased cytokines, and increased Cox-2 and CYP2E1 [15,27,55,126].

Clinically, the serum of HCV patients may show increased ROS [26]. Levels of lipid peroxidation products are increased in serum, white blood cells, and liver specimens in HCV patients. Increased levels of 8-OH-dG in leukocytes are a reliable marker of oxidative stress in patients with chronic HCV infection and this marker was correlated with clinical diagnosis, ferritin levels, and amount of liver steatosis [43]. Mahmood et al. also noted increased levels of 4-hydroxynonenal (HNE) and 8-hydroxyguanosine levels, both of which are measures of oxidative stress, in HCV patients [110]. Serum of HCV patients also contained elevated serum thioredoxin levels that correlate with the severity of disease. Patients who became HCV-RNA negative after 14 days of interferon therapy had lower pretreatment thioredoxin levels that did those who remained positive [154]. These patients were also noted to have increase 8-hydroxydeoxyguanosine, a measure of DNA damage [77].

In HCV, increases in NADPH oxidase generate increased ROS and RNS which may lead to the chronic inflammation seen in these patients [27]. The virus itself, specifically the NS3 protein, can activate NADPH oxidase on membranes and phagosomes leading to increased ROS and thus increased apoptosis and T cell dysfunction [48,160]. The core protein of hepatitis C itself may also inhibit the electron transport chain, increase ROS, increase mitochondrial permeability, increase intracellular calcium levels, and deplete mitochondrial glutathione stores [1,85,86,120,126]. The NS5A protein of HCV can also elicit oxidative stress as demonstrated by increased heme oxygenase-1 (HO-1), increased catalase, increased GSH, activation of AP1, and induction of MnSOD [1,157].

HCV is also strongly associated with the development of hepatocellular carcinoma (HCC). The development and transformation of infection to carcinoma is likely related to oxidative DNA damage from ROS/RNS, specifically from iNOS [111]. Viral hepatitis is associated with an increased iNOS expression [109,111], with a questionable association with iNOS expression and severity of disease [50,69,88]. HBV is able to induce iNOS expression, while iNOS levels are increased in patients infected with HBV compared with levels in patients with other types of hepatic disease [111]. HCV infection can also stimulate the production of NO through activation of the gene for iNOS by the viral core protein and the NS3 protein [109]. HCV patients have increased levels of iNOS, which correlates with high levels of HCV proteins and/or level of disease, as measured by histology [50,69,142].

This increased RNS from increased iNOS expression is associated with DNA damage and, since DNA damage is associated with the development of cancer, NO is implicated in the development of hepatocellular carcinoma. In mice, core protein from HCV can increase ROS, increase lipid peroxidation products, and induce antioxidant gene expression. By blocking

mitochondrial electron transport, the formation of ROS is inhibited. HCV transgenic mice have an increased sensitivity to oxidative stress, in which increased intrahepatic lipid peroxidation products occurs in response to carbon tetrachloride [126]. In patients with HCC, the combined negative expression of iNOS and COX-2 on histology has a significant impact on patient survival [135]. Again, these studies are suggestive but not conclusive as to the relationship between oxidative stress, viral infection, and the development of hepatocellular carcinoma. Further studies are needed to clarify this correlation.

Non-alcoholic fatty liver disease (NAFLD)—Non-alcoholic fatty liver disease (NAFLD), also known as non-alcoholic steatohepatitis (NASH), is syndrome of liver injury similar to that found in alcoholics, but occurs in patients who deny alcohol use. Risk factors include obesity, gender (female), diabetes, and hyperlipidemia. Although it was originally considered a benign disorder, the pathological histology of patients with NAFLD reveals the presence of macrovescicular steatosis, lobular inflammation, fibrosis, and occasionally cirrhosis, suggestive of a pathologic process [65,108].

It is postulated that NAFLS develops because of increased liver fat accumulation, which makes the liver vulnerable to hepatocyte injury. Although the mechanism of NAFLD is not known, oxidative stress appears to play a role via lipid peroxidation, cytokine induction, and Fas ligand induction (Figure 7). Lipid peroxidation causes the release of malondialdehyde (MDA) and 4-hydroxynonenal (HNE), which bind proteins and create neoantigens that can then cause immune reactions, stellate cell activation, and neutrophil chemotaxis [5,170]. Ikura et al. noted a correlation between oxidized phophatidylcholine, a lipid peroxide that serves as a ligand for scavenger receptors, and disease progression [63].

Exposure of free fatty acids to hepatocytes resulted in the translocation of Bax to lysosomes and subsequent lysosomal destabilization, with release of cathepsin B (ctsb), a lysosomal cysteine protease, into the cytosol. The accumulation of free fatty acids was also associated with NF-κB induced cytokine expression, specifically IL-6 and IL-1β [46] (Figure 7). Patients with NAFLD were also noted to have release of ctsb into the cytoplasm, which correlated with disease severity. In a mouse model of NAFLD, either genetic or pharmacological inactivation of ctsb was protective against the development of hepatic steatosis and liver injury [46].

In addition to lipid peroxidation, oxidative stress may be induced in NAFLD by activation or expression of TNF-α or Fas. Crespo et al. noted that obese patients with NAFLD had increased TNF-α expression and increased TNF-α adipose expression, and that this increased expression correlated with degree of fibrosis [32]. TNFα is a potent proinflammatory cytokine that is capable of inducing ROS/RNS and affecting downstream signaling, resulting in more inflammation. Unfortunately, the data suggest only an association, not a causation, so further investigation is necessary. These patients are also noted to have increased Fas-ligand expression as well as active caspases 3 and 7, suggesting increased apoptosis [45].

Animal studies on obese mice indicated an increased production of ROS, increased expression of NADPH oxidase, and decreased expression of antioxidative enzymes. When these mice were treated with a NADPH oxidase inhibitor, ROS production was reduced[49]. Human patients with NAFLD also have higher levels of ROS, including xanthine oxidase, and lower levels of antioxidants [8]. The resulting increase in oxidative stress may lead to increased lipid peroxidation, increased H_2O_2 production, and CYP2E1 induction. Patients with NAFLD and increased cytochrome CYP2E1 were noted to have improved levels after gastric bypass surgery and weight loss [40]. NAFLD patients were also noted to have elevated levels of thioredoxin, a thiol-containing antioxidant, and that thioredoxin levels correlated with severity of disease [153], although in general in NAFLD, ROS could deplete antioxidant enzymes including

glutathione, vitamin E, and ascorbate, thus increasing hepatic susceptibility to oxidative stress [139,151].

Conclusion

Part I of this review discussed the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in normal physiological function of hepatocytes including oxidative respiration, cell signaling, and protein modification required for normal cellular growth, regeneration, apoptosis, and microsomal defense. However, ROS and RNS can damage any cells in the liver causing inflammation, ischemia, fibrosis, necrosis, apoptosis, or malignant transformation. Here we discussed the pathology of hepatitis as it relates to redox biology in the liver. In Part II of this review, we will discuss the pathology of ischemia/reperfusion injury, fibrosis, iron overload, Wilson's disease, sepsis, and acetaminophen overdose as it relates to redox biology. We will also discuss redox proteomics and the potential of antioxidant therapy in the attenuation of disease progression.

Abbreviations

AP	apurinic/apyrimidinic
AP-1	activator protein-1
APE/Ref-1	apurinic/apyrimidinic endonuclease/redox factor 1
ATP	adenosine triphosphate
ALT	aminotransferase
Bcl-2	B-cell lymphoma-2
BER	base excision repair
BH_4	tetrahydrobiopterin
Bid	A BH3 domain-only death agonist protein
CaM	calmodulin
CAT	catalase
cGMP	cyclic guanosine monophosphate
COX-2	cyclooxygenase-2
CSF	colony stimulating factor
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EPO	erythropoietin
eNOS	endothelial nitric oxide synthase
ESR	electron spin resonance
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
γ-IRE	γ-interferon response element
G-CSF	granulocyte colony stimulating factor
GSH	glutathione

GSSG	glutathione disulfide
GST	glutathione S-transferase
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIF	hypoxia-inducible factor
HO-1	heme oxygenase
HNE	4-hydroxynonenal
HSP70	heat shock protein 70
IL	interleukin
INFγ	interferon gamma
im	intramuscular
iNOS	inducible nitric oxide synthase
iv	intravenous
JNK	c-Jun NH2-terminal kinase
KLF6	a zinc finger molecule
L-NIL	N-iminoethyl-L-lysine
L-NMMA	L-N(G)-monomethyl arginine citrate
L-NNA	L-N^{ω}-nitro-L-arginine
LPS	lipopolysaccharides
MAP kinase	mitogen-activated protein kinase
MAT	methionine adenosyltransferase
MDA	malondialdehyde
MDS	myelodysplastic syndrome
MEOS	microsomal ethanol-oxidizing system
MMP	matrix metalloproteinases
MnSOD	manganese-containing superoxide dismutase
MS	methionine synthase
NAC	N-acetylcysteine
NADQI	*N*-acetyl-*p*-benzoquinone imine
NAFLD	non-alcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NOX	nitric oxide scavenger
nNOS	neuronal nitric oxide synthase
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate

NF-κB	nuclear factor-kappa B
NO	nitric oxide; reactive halogen species
NS5A	non-structural 5A protein
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
OLT	orthotopic liver transplant
PEG-poly SNO-BSA	polyethylene glycol-conjugated bovine serum albumin
PMN	polymorphonuclear leukocytes
po	oral
PT	prothrombin time
RHS	reactive hydrogen species
RNA	ribonucleic acid
ROS	reactive oxygen species
RNS	reactive nitrogen species
SAMe	S-adenosylmethionine
sGC	soluble guanylate cyclase
SMAD	mothers against decapentaplegic
SOD	superoxide dismutase
TGFα/β	transforming growth factor α/β
TIMP1	tissue inhibitor metalloproteinase-1
TNFα/β	tumor necrosis factor α/β
TRAIL	TNF related apoptosis inducing ligand
UV	ultraviolet
VEGF	vascular endothelial growth factor
1400W	N-(3-aminomethyl)benzyl-acetamindine
8-OH-dG	8-hydroxydeoxyguanosine

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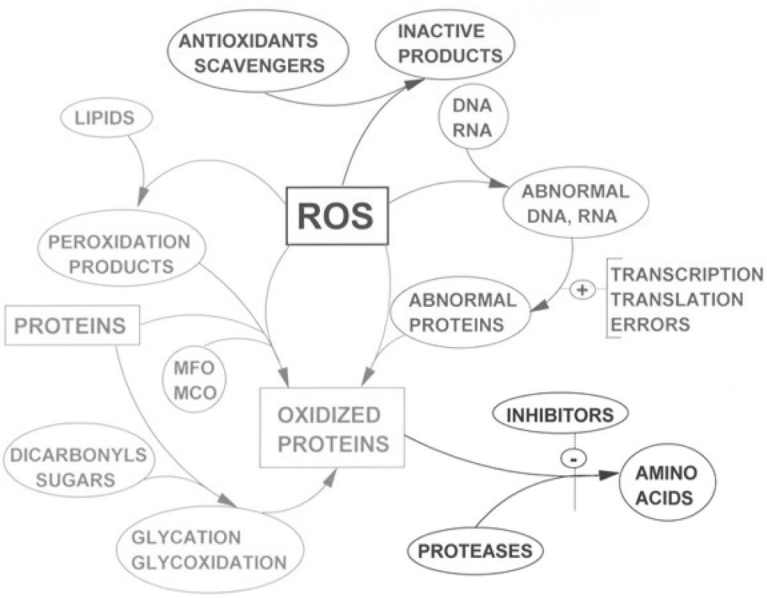

Figure 1.
Reactive oxygen species (ROS) are involved in the oxidation of proteins, lipids, and nucleic acids. Reprinted by permission from dir.nhlbi.nih.gov/labs/lb/es/index.asp. [147]

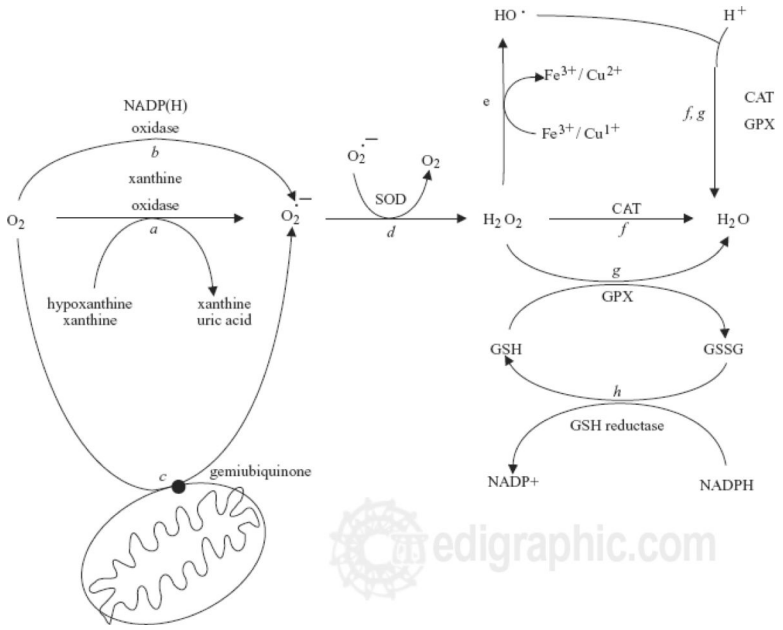

Figure 2.

Pathway for formation of reactive oxygen species. (ROS) Reprinted by permission from Cesaratto L, Vascotto C, Calligaris S, Tell G. The importance of redox state in liver damage. Ann Hepatol. 3:86–92,2004. [23]

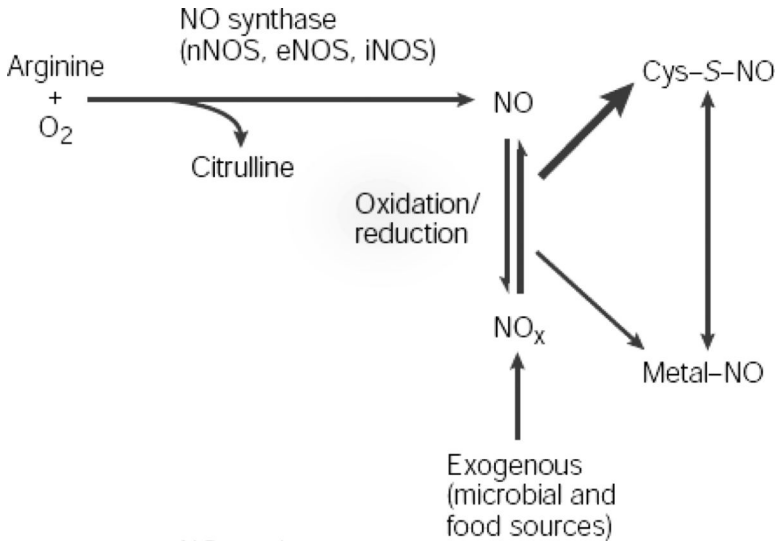

Figure 3.
The formation of nitric oxide. Reprinted by permission from Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. Protein S-nitrosylation: purview and parameters. Nat Rev Mol Cell Biol. 6:150–166,2005. [60]

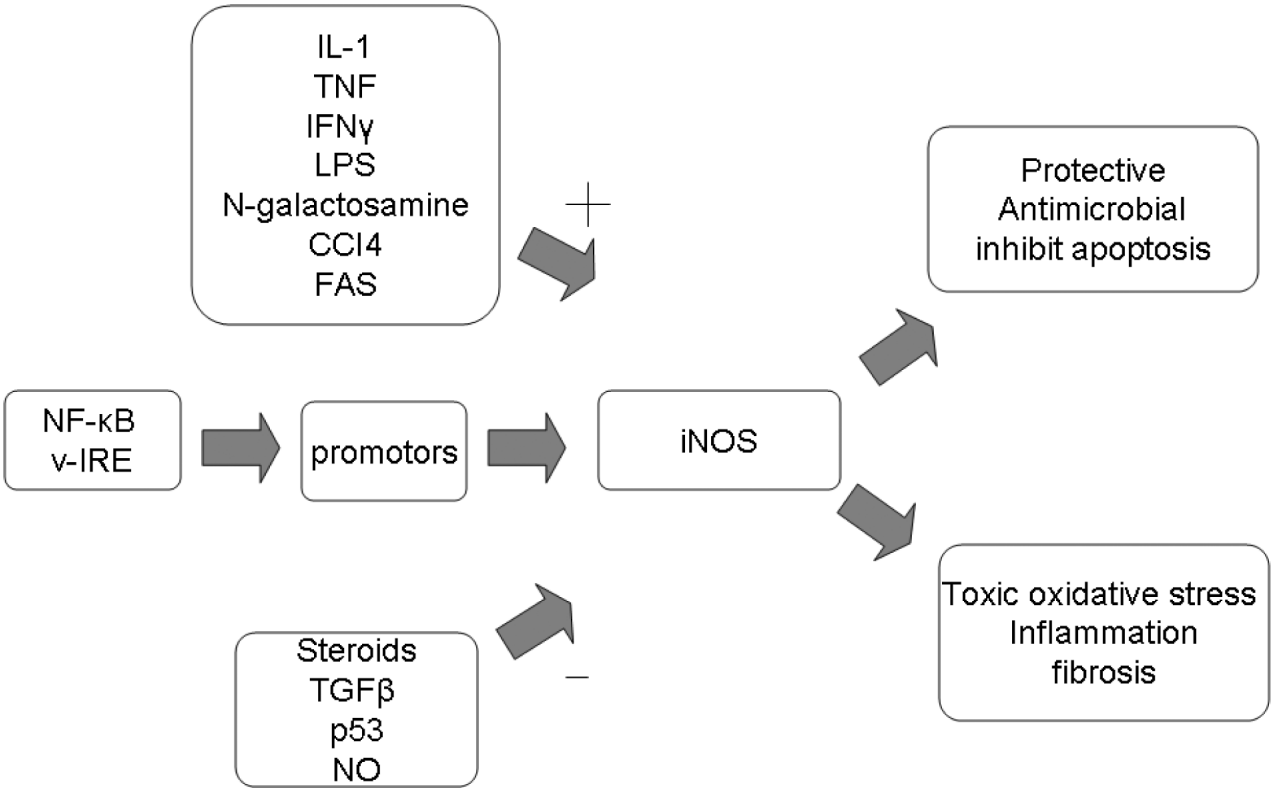

Figure 4.
Important promoter, inhibitor, and effects of iNOS in the liver. iNOS is expressed hepatocytes, Kupffer cells, vascular endothelial cells, and stellate/Ito cells in the liver. The level of iNOS activity is determined by a variety of stimuli. Based on the amount and duration of activity iNOS can be either protective or toxic.

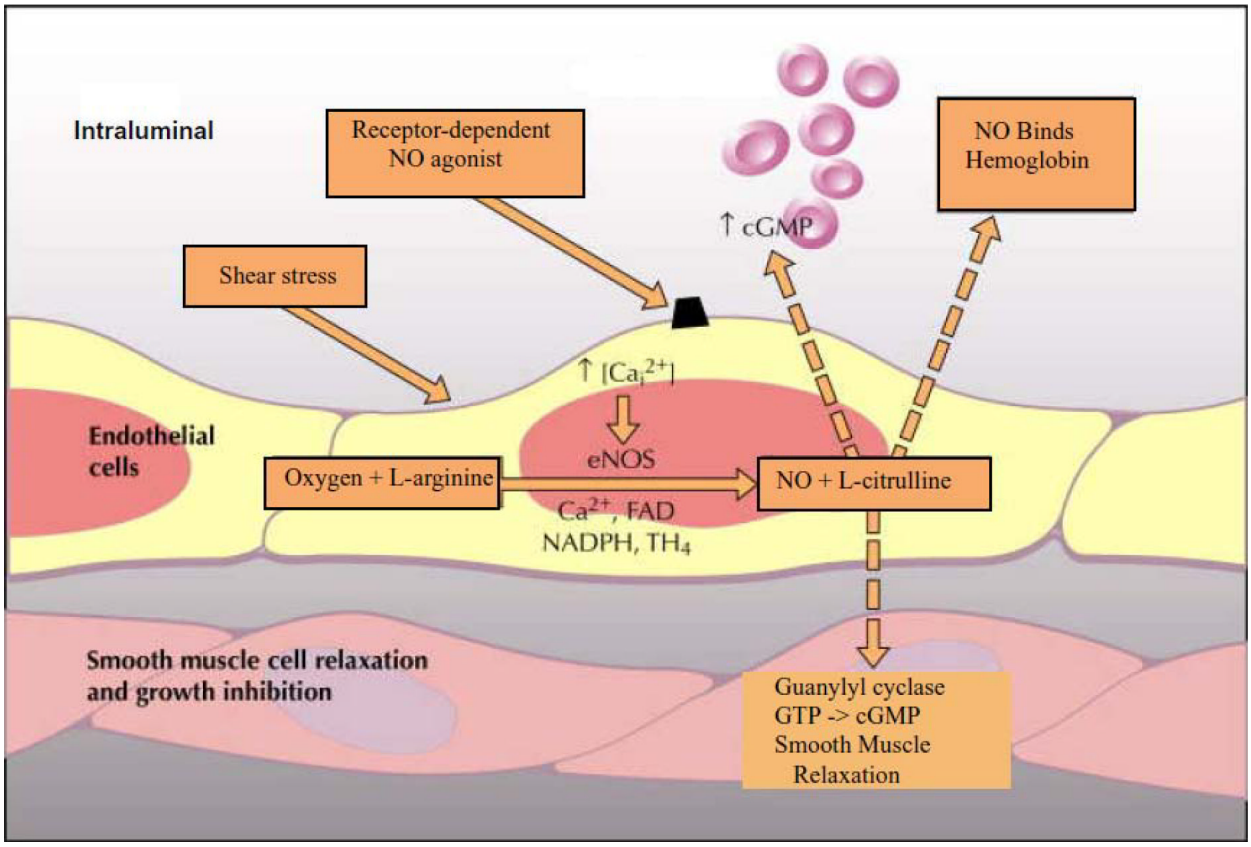

Figure 5.
Critical components in the formation of nitric oxide by eNOS and its role in hepatocytes.

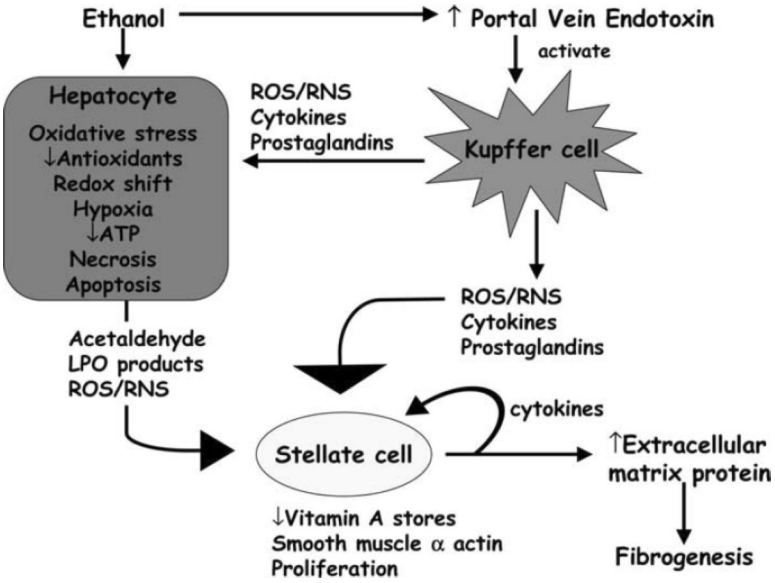

Figure 6.

Mechanism of alcohol-induced liver injury. Alcohol metabolism causes acetaldehyde and reactive oxygen species to be generated, both of which can activate stellate cells. ROS/RNS from Kupffer cells can also active stellate cells causing increasing collagen deposition and eventual fibrosis. Reprinted by permission from Halliwell B, JMC G. Free Radicals in Biology and Medicine. 4th ed. oxford: Oxford University Press; 2007. [59]

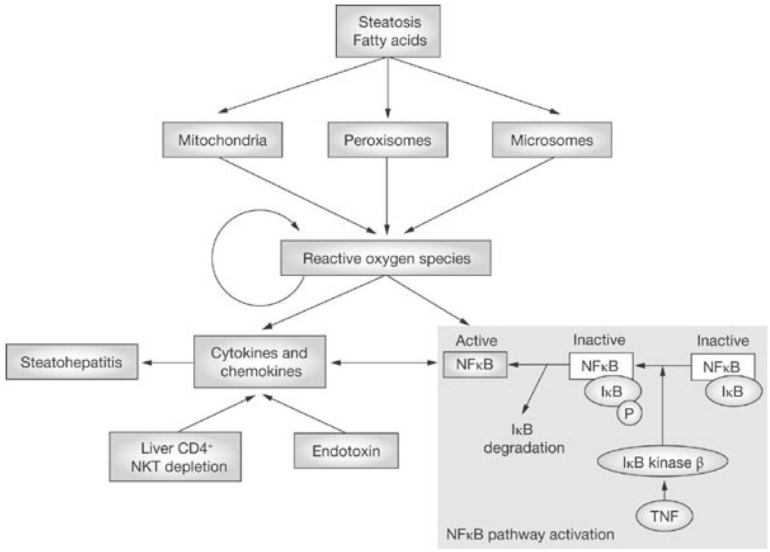

Figure 7.

In non-alcoholic fatty liver disease, the oxidation of free fatty acids increases the production of reactive oxygen species, which, in turn, release cytokines and chemokines, thus causing more inflammation. There is also activation of NF-κB and TNF-α, which again leads to further inflammation. Reprinted by permission from Perlemuter G, Bigorgne A, Cassard-Doulcier AM, Naveau S. Nonalcoholic fatty liver disease: from pathogenesis to patient care. Nat Clin Pract Endocrinol Metab. 3:458–469,2007. [132]

Table 1

List of Common Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

Free Radicals	**Non-radicals**
Reactive oxygen species (ROS)	**Reactive oxygen species (ROS)**
Superoxide ($O_2^{\bullet -}$)	Hydrogen peroxide (H_2O_2)
Hydroxyl radical ($HO^{\bullet}$)	Hypochlorous acid (HOCl)
Peroxyl radical ($RO_2^{\bullet}$)	Hypobromous acid (HOBr)
Alkoxyl radical ($RO^{\bullet}$)	Ozone (O_3)
Hydroperoxyl radical ($HO_2^{\bullet}$)	Organic peroxides (ROOH)
Singlet oxygen ($^1O_2$)	Perioxynitrous acid ($ONOOH^c$)
	Peroxynitrate (O_2NOO^-)
	Peroxynitrite ($ONOO^{-c}$)

Reactive nitrogen species (RNS)	**Reactive nitrogen species (RNS)**
Nitric oxide ($^{\bullet}NO$)	Nitryl chloride (NO_2Cl)
Nitrogen dioxide ($^{\bullet}NO_2$)	Nitrous acid (HNO_2)
Nitrite ($^{\bullet}NO_3$)	Nitrosyl cation (NO^+)
	Nitrosyl anion (NO^-)
	Dinitrogen tetroxide ($N2_2O_4$)
	Dinitrogen trioxide (N_2O_3)
	Peroxynitrite ($ONOO^-$)
	Peroxynitrous acid (ONOOH)
	Alkyl peroxynitrites (ROONO)
	Nitronium cation (NO_2^+)

Table 2

Chemical equations relevant to reactive oxygen and reactive nitrogen species generation.

Reactive oxygen species generation
$O_2 + e^- \rightarrow O_2^{-\bullet}$ (superoxide anion)
$O_2^{-\bullet} + H_2O \rightarrow HO_2^{\bullet}$ (hydroperoxyl radical)
$HO_2^{\bullet} + e^- + H \rightarrow H_2O_2$ (hydrogen peroxide)
$H_2O_2 + e^- \rightarrow OH^- + {}^{\bullet}OH$ (hydroxyl radical)
Reactive nitrogen species generation
L-arginine + $O_2 \rightarrow {}^{\bullet}NO$ (nitric oxide) + L-citrulline
$O_2^{-\bullet} + {}^{\bullet}NO \rightarrow ONOO^-$ (peroxynitrite)
Fenton reaction (catalyzed by transition metals)
$H_2O_2 + Fe^{2+} \rightarrow OH^- + {}^{\bullet}OH + Fe^{3+}$
Haber-Weiss Reaction (catalyzed by transition metals)
$H_2O_2 + O_2^{-\bullet} \rightarrow O_2 + {}^{\bullet}OH + OH^-$

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Table 3

Common antioxidant reactions and the enzymes that catalyze them.

Superoxide Dismutase (SOD)
20_2- + 2H+ → H_2O_2 + O_2
Catalase:
$2H_2O_2 \rightarrow 2H_2O + O_2$
Glutathione peroxidase
2GSH + H_2O_2 → GSSG (glutathione disulfide) + $2H_2O$
Glutathione disulfide reductase
GSSG + NADPH + H^+ → 2GSH + $NADP^+$
Glutathione S-transferase (GST)
RX + GSH → RSG + HX

Table 4

Roles of iNOS production in liver damage.

Condition/Inducers	NO Effect	Mechanism
In vivo		
Endotoxemia	Protective	Inhibits apoptosis
	Toxic	Oxidative stress, circulatory failure
TNFα + N-galactos-amine	Protective	Inhibits apoptosis
CCl_4	Protective	Decreases oxidative stress
Liver regeneration	Protective	Inhibits apoptosis
Ischemia-reperfusion	Toxic	Oxidative damage
Hemorrhagic shock	Toxic	Direct toxicity, activates inflammation
Alcoholic liver injury	Protective	unclear
In vitro (hepatocytes)		
TNFα, Fas antibody	Protective	Inhibits caspase/apoptosis HSP70 upregulation
H_2O_2	Protective	Heme oxygenase-1 upregulation
Acetaminophen	Protective	Modulates GSH levels

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