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## NITRIC OXIDE IN LIVER DISEASES

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### Abstract

Nitric oxide (NO) and its derivatives play important roles in the physiology and pathophysiology of the liver. Despite its diverse and complicated roles, certain patterns of the effect of NO on the pathogenesis and progression of liver diseases are observed. In general, NO derived from endothelial NO synthase (eNOS) in liver sinusoidal endothelial cells (LSECs) is protective against disease development, while inducible NOS (iNOS)-derived NO contributes to pathological processes. This review addresses the roles of NO in the development of various liver diseases with a focus on recently published articles. We present here two recent advances in understanding NO-mediated signaling, nitrated fatty acids and S-guanylation, and conclude with suggestions on future directions of NO-related studies on the liver.

### Keywords

eNOS; iNOS; endothelial cells; portal hypertension; inflammation

### Nitric oxide in liver physiology and pathophysiology

Nitric oxide (NO) is an important mediator of liver physiology and pathophysiology. NO is generated by three isoforms of nitric oxide synthases (NOSs): neuronal NOS (nNOS; NOS1), inducible NOS (iNOS; NOS2), and endothelial NOS (eNOS; NOS3) [1]. NOS catalyzes the oxidation of L-arginine to NO and citrulline [2]. In the cell, nNOS and iNOS are predominantly found in the cytosol, while eNOS binds to the membrane via palmitoylation and myristoylation [3].

In liver biology, eNOS and iNOS are major players, whereas the role of nNOS is little known. eNOS is mainly expressed in liver sinusoidal endothelial cells (LSECs) and endothelial cells of the hepatic artery, portal vein, central vein, and lymphatic vessels. eNOS is constitutively expressed and produces small amounts of NO in response to stimuli such as

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flow shear stress and vascular endothelial growth factor (VEGF) (Figure 1A). eNOS-derived NO maintains liver homeostasis and inhibits pathological conditions in the liver. In contrast, iNOS is induced in various liver cells, including LSECs, hepatocytes, Kupffer cells (liver resident macrophages), hepatic stellate cells (HSCs), smooth muscle cells, cholangiocytes, and other immune cells [4-6]. Under many pathological conditions, iNOS produces large amounts of NO, which is a major source of reactive nitrogen species (RNS) (Figure 1B). Particularly, peroxynitrite (ONOO<sup>-</sup>) can damage a wide range of cellular molecules including DNA, lipids, and proteins, and can also facilitate protein nitration, affecting structure and function of many target proteins [7] (Figure 1C).

This review summarizes the current understanding of NO biology including two new NO-mediated protein modifications, nitrated fatty acids (NO<sub>2</sub>-FA) and guanine nucleotides (8-nitroguanosine), and the roles of NO in liver diseases, concluding with suggestions on interesting areas of study to be explored in the context of NO and liver biology.

### Mode of action of nitric oxide

The most recognized action of NO is through cyclic guanosine monophosphate (cGMP) as the second messenger. The cGMP-dependent action is initiated by binding of NO to a metal center of guanylyl cyclase (GC), which activates this enzyme, leading to increased production of cGMP. However, cGMP-independent actions of NO, such as protein tyrosine nitration and S-nitrosylation, have also been implicated in various disease conditions. For example, proteomic analysis of the liver has indicated the importance of S-nitrosylated proteins in the regulation of liver function [8, 9]. In pathological conditions, these protein modifications are generally enhanced by increased iNOS-derived NO in conjunction with increased free radicals. In addition to these conventional modes, this section also addresses novel mediators of NO-related action, nitrated fatty acids and nucleotides.

### Metal ions

NO acts as a signaling molecule by binding to metal ions, proteins, lipids, and guanine nucleotides (Figure 2). Among metal ions, a primary target is ferrous heme iron [10], which forms highly stable NO complexes. A classic example is NO binding to ferrous heme iron of soluble guanylyl cyclase (sGC) to produce cGMP as the second messenger [11, 12]. Elevated cGMP directly modulates the activity of phosphodiesterases (PDEs), ion-gated channels, or cGMP-dependent protein kinases to regulate a wide range of physiological functions, including vasodilation, platelet aggregation, and neurotransmission [13].

NO also binds to a heme/copper center of cytochrome c oxidase, also known as Complex IV, the terminal enzyme in the mitochondrial respiratory chain [14, 15] and irreversibly inhibits its activity. This inhibition facilitates the reduced state of electron carriers in the respiratory chain, increasing O<sub>2</sub><sup>-</sup> generation as well as reducing adenosine triphosphate (ATP) production [14, 15] and thereby influencing energy metabolism.

Another important example is the binding of NO to the heme of NOS, which inhibits NOS enzyme activity [16]. The binding of NO to Fe(II) or Fe(III) heme attenuates oxygenase activity of NOS. This may serve as a negative feedback system of NOS activity. It is also

possible that increased iNOS-derived NO observed in many types of liver diseases may inhibit eNOS activity through this mechanism, contributing to decreased eNOS-derived NO in LSECs and thus LSEC dysfunction.

### Free radicals

NO reacts with reactive oxygen species (ROS) to form RNS such as  $\bullet\text{NO}_2$ ,  $\text{ONOO}^-$ ,  $\text{HNO}_2$ , and  $\text{NO}_2^+$  (Figure 1C). Perhaps, the most studied RNS-forming reaction is the reaction between  $\bullet\text{NO}$  and superoxide radical  $\text{O}_2\bullet^-$ , which generates peroxynitrite ( $\text{ONOO}^-$ ) [17], a strong biological oxidant generally implicated in toxic effects on cells and tissues.

Mitochondria have been considered the main source of reactive oxygen species (ROS), which mostly originate from the mitochondrial respiratory chain [18]. Excess mitochondrial  $\text{ONOO}^-$  can impair oxidative phosphorylation by inhibiting the respiratory chain complexes (Complex I, Complex IV, and ATP synthase) and MnSOD activity [19]. In addition,  $\text{ONOO}^-$  can oxidize DNA bases, tyrosine residues of proteins, and thiol groups [i.e., cysteine thiol and glutathione (GSH)], causing hepatocyte death [20, 21].

The depletion of the GSH pool and an increase in an oxidized form of GSH (GSSG) are closely related to action of ROS and RNS on cell and liver function [22]. GSH is a triple peptide ( $\gamma$ -L-glutamyl-L-cysteinyl-glycin), synthesized in the cytosol and transported to cellular organelles, including the endoplasmic reticulum (ER), nucleus, and mitochondrion. With cysteine in its backbone, GSH plays an important role in the reduction of electrophiles and oxidants [23]. GSH becomes oxidized to GSSG when it reduces target molecules. Thus, the ratio of GSH to GSSG is a good indicator of oxidative stress and redox balance [24]. The depletion of the GSH pool is associated with the pathogenesis of liver diseases, such as drug-induced liver toxicity (i.e., acetaminophen overdose) [25], chronic alcohol intake [26], non-alcoholic fatty liver disease (NAFLD) [27] and biliary cirrhosis [28]. Chronic alcohol intake, NAFLD or biliary cirrhosis deplete the mitochondrial GSH pool by impairing GSH transport to the mitochondrial matrix, which is largely caused by alterations of lipid compositions (i.e., increased cholesterol contents) in mitochondrial membranes [22].

NOS generates superoxide anion ( $\text{O}_2^-$ ) instead of NO [29, 30] in a condition known as “NOS uncoupling”. NOS uncoupling occurs when tetrahydrobiopterin (BH4), an essential cofactor, is depleted [31]. NOS can convert L-arginine to NO and L-citrulline only as a dimer because BH4 binding site is located at the dimer interface and monomeric NOS cannot bind BH4 or L-arginine [32]. Oxidative stress plays a major role in depleting the cellular BH4 pool, causing NOS uncoupling and further exacerbating oxidative stress [33]. Reduced bioavailability of NO as a result of NOS uncoupling has also been implicated in endothelial cell dysfunction [34].

### Protein modification (tyrosine nitration and S-nitrosylation)

Nitration of protein tyrosine residues creates irreversible and stable adducts and has been considered an indicator of nitrosative stress (Figure 2). Tyrosine nitration of proteins often occurs in inflammatory conditions and mediates peroxynitrite-induced cell death signaling [35].

Unlike tyrosine nitration, S-nitrosylation is a reversible reaction (Figure 2), in which oxidized NO binds to a specific cysteine thiol anion in proteins and peptides [36]. Protein S-nitrosylation is enhanced by NOS activity, but decreased by S-nitrosoglutathione reductase (GSNOR), a ubiquitous and highly conserved denitrosylase, thus serving as a key regulator of protein S-nitrosylation [37]. eNOS facilitates S-nitrosylation in endothelial cells by specifically localizing at the Golgi apparatus and generating a high concentration of NO pool locally [38, 39].

A number of potential targets, such as CD147 and various mitochondrial proteins, for protein S-nitrosylation have been identified in the liver [8, 9]. It has been suggested that the dysregulation of protein S-nitrosylation, mostly mediated by iNOS, is associated with the pathogenesis of various liver diseases, including hepatocellular carcinoma (HCC) [40, 41], hepatic steatosis [9], and cholestasis [42, 43].

For example, lack of GSNOR resulted in S-nitrosylation, ubiquitination, and proteosomal degradation of O<sup>6</sup>-alkylguanine-DNA-alkyltransferase (AGT). AGT is a key DNA repair enzyme and was reported for protection against dialkylnitrosamine-induced HCC [44]. Both pharmacological inactivation and genetic deletion of iNOS prevented S-nitrosylation and inactivation of AGT in GSNOR knockout mice, suggesting a role of iNOS in S-nitrosylation and liver carcinogenesis in the absence of GSNOR gene [40]. Similarly, decreased expression and activity of GSNOR as well as increased S-nitrosylated proteins were observed in cholestatic livers isolated from mice with bile duct ligation surgery (an experimental model of cholestasis). Treatment with an iNOS inhibitor, S-methylisothiourea, ameliorated hepatocellular injury with restoration of GSNOR activity and decreased S-nitrosylated proteins, suggesting a contribution of increased S-nitrosylated proteins generated by increased iNOS-derived NO to cholestatic liver disease [43].

Another S-nitrosylated protein was also reported for its possible contribution to cholestasis. Taurocholic acid, a form of bile acids, is involved in the emulsification of fat. Its uptake by hepatocytes is mediated by a transporter NTCP/Ntcp, a Na<sup>+</sup>-dependent taurocholate cotransporting polypeptide. NO derived from iNOS S-nitrosylated Ntcp at Cys96 and inhibited taurocholic acid uptake in hepatocytes *in vitro*. This result implies that S-nitrosylation of Ntcp causes sepsis-associated cholestasis by inhibiting taurocholic acid uptake and thus decreasing bile flow [42].

NO signaling also appears to be an important regulator of the bile salt pool in hepatocytes [45]. Excess bile salts stimulated NO production in hepatocytes, leading to S-nitrosylation and nuclear translocation of GAPDH. Interestingly, S-nitrosylated GAPDH (SNO-GAPDH) transnitrosylated HDAC2 and SIRT1 in the nuclei of hepatocytes. S-nitrosylated HDAC2 formed a complex with SHP, which repressed gene expression of CYP7A1, an enzyme that converts cholesterol to bile acids. Thus, this mechanism could work as a negative feedback by decreasing bile acid production.

### Nitrated fatty acids

Mitochondria keep polyunsaturated fatty acids (PUFAs) in their inner membranes [18]. NO-derived species (reactive nitrogen species, RNS), such as •NO<sub>2</sub>, ONOO<sup>-</sup>, HNO<sub>2</sub>, and NO<sub>2</sub><sup>+</sup>,

can oxidize or nitrate PUFAs and yield nitrated fatty acids (NO<sub>2</sub>-FA) (Figure 2). NO<sub>2</sub>-FA is an electrophile, which readily reacts with nucleophilic amino acids such as histidine and cysteine and changes protein function and catalytic activity [46, 47]. Importantly, NO<sub>2</sub>-FA appears to target specific proteins [48]. Although which RNS predominate the reaction is not known, NO<sub>2</sub>-FA production occurs in pathological conditions, such as cardiac ischemia [49], pulmonary hypertension [50], and diabetes [50, 51].

Unlike other electrophiles that exert cytotoxicity or pro-inflammatory effects by oxidizing lipids or lipoproteins in the vasculature [52], NO<sub>2</sub>-FA has been reported to exhibit anti-inflammatory properties through inhibition of leukocyte and platelet activation [53], vascular smooth muscle proliferation [54], and lipopolysaccharide (LPS)-stimulated macrophage cytokine secretion [55]. Among an increasing number of NO<sub>2</sub>-FA activated signaling pathways, reactions with transcription factors, such as Nrf2/Keap 1 (nuclear erythroid 2-related factor 2/Kelch ECH associating protein) and NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), are particularly important. Keap 1 binds to a transcription factor Nrf2 and prevents its nuclear translocation. Covalent binding of NO<sub>2</sub>-FA to cysteine residues of Keap1 allowed nuclear translocation of Nrf2, inducing expression of antioxidant-associated genes, such as glutathione S-transferases, heme oxygenase-1, thioredoxin, and components of the glutathione synthesis pathways [56]. Impaired Nrf2/Keap1 signaling has been observed in various liver abnormalities including hepatomegaly [57, 58], liver cirrhosis [59], and immune mediated hepatitis [56]. Thus, it is interesting to examine the involvement of NO<sub>2</sub>-FA.

NFκB induces major inflammatory cytokines involved in liver diseases. NO<sub>2</sub>-FA was shown to cause nitroalkylation of p65 subunit (an active subunit of NFκB) and inhibit its activity, thereby decreasing expression of inflammatory cytokines [55]. Supplementation of NO<sub>2</sub>-FA could therefore protect the liver from injury like the case of diabetic nephropathy in which administration of NO<sub>2</sub>-FA via osmotic pump showed moderate amelioration [60].

NO<sub>2</sub>-FA could also facilitate glucose homeostasis and lipid metabolism as a ligand of peroxisome proliferator-activated receptor-γ (PPAR-γ) [61]. NO<sub>2</sub>-FA bound to the ligand-binding domain Cys285 of PPAR-γ [62] and induced PPAR-γ downstream gene expression [61]. Since nitrated linoleic acids, a type of NO<sub>2</sub>-FA, increased glucose uptake and adipocyte differentiation through PPAR-γ activation [61], it is tempting to speculate that NO<sub>2</sub>-FA could be involved in the pathological process of non-alcoholic fatty liver disease (NAFLD). NO<sub>2</sub>-FA and nitrated guanine nucleotides, which are discussed below, are relatively new discoveries of NO's modifications. Their roles in liver biology are largely unknown and should be explored.

### **Nitrated guanine nucleotides (8-nitroguanosine)**

Nitration of guanine nucleotides was originally found to occur in microbial infection in an iNOS-derived NO-dependent manner [63]. Among nitrated guanines, 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP) showed the highest biological activity [63, 64] and readily reacted with sulfhydryls of glutathione and proteins to form 8-thioalkoxy-cGMP adducts (Figure 2). Similar to NO<sub>2</sub>-FA, this NO-mediated protein modification, termed "S-guanylation" [65], occurs in two steps. Using cGMP as an example, the first step is the

nitration of GTP (i.e., 8-nitro-GTP), which is then catalyzed by guanylyl cyclase (GC) to generate nitrated cGMP (i.e., 8-nitro-cGMP) [66]. The second is the modification of target proteins by nitrated cGMP (i.e., 8-nitro-cGMP). A number of target molecules for S-guanylation have been identified [67]. One remarkable finding was the role of 8-nitro-cGMP in autophagy in macrophages [68]. 8-nitro-cGMP bound to cysteine residues (i.e., S-guanylation) of invading bacteria such as group A streptococcus. Then, these S-guanylated bacteria were tagged with polyubiquitin, a molecular tag known to allow selective delivery to autophagosomes for degradation, resulting in the clearance of the bacteria.

Biological activity of 8-nitro-cGMP could be regulated by cysteine persulfide (CysSSH). CysSSH reacted with 8-nitro-cGMP to generate 8-SH-cGMP. An addition of CysSS-, called electrophile sulfhydration, inhibited the action of 8-nitro-cGMP [69, 70].

8-nitro-cGMP may represent the mechanism of adaptive response to oxidative stress [71]. Similar to NO<sub>2</sub>-FA, 8-nitro-cGMP helped to induce expression of antioxidant-associated genes by binding to Keap1 and thus allowing activation and nuclear translocation of Nrf2. The pathogenesis of liver diseases is largely related to increased oxidative stress. Thus, a role of S-guanylation is anticipated.

## NO and liver diseases

RNS and ROS play a central role in the pathogenesis of a variety of liver diseases. iNOS-derived NO is closely related to this process. This section addresses how NO is involved in the development of various types of liver diseases (Figure 3).

### Non-alcoholic fatty liver disease

NAFLD has emerged as the most common liver disorder in developed countries in recent years. In the US, it is estimated to affect approximately 30% of the general population [72]. NAFLD is a progressive disease that starts with fat accumulation in the liver (steatosis) and can advance to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and eventually cancer over time (Figure 3). It is not fully understood what causes steatosis to progress to NASH and further to cirrhosis and HCC. NAFLD is strongly associated with obesity and type 2 diabetes. NO can be protective or promotive for NAFLD depending on its source as discussed below.

NO produced by eNOS can be protective through the inhibition of inflammatory activation of Kupffer cells [73] (Figure 1A) and enhancement of mitochondrial fatty acid  $\beta$ -oxidation [9]. Simvastatin [3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor], which is known to increase eNOS activity and thus NO production, was also shown to mitigate NASH-related liver fibrosis by inhibiting HSC activation [74]. NO donors also showed preventive roles in hepatic steatosis by facilitating fatty acid  $\beta$ -oxidation [75] and by blocking cytochrome P450 2E1 enzyme (CYP2E1)-mediated oxidative stress [76].

Contrary to the protective effects of eNOS-derived NO or NO donors mentioned above, iNOS-derived NO or its derivatives such as peroxynitrite have been shown to promote NAFLD [77-79]. For example, Arg2<sup>-/-</sup> mice showed increased hepatic steatosis and injury



in response to high-fat diet compared with their wildtype counterparts, accompanied with increased macrophage activation and iNOS expression. Given that arginase 2 competes with iNOS for its substrate (i.e., arginine), lack of arginase 2 favored iNOS induction, contributing to the increased steatosis and injury in *Arg2<sup>-/-</sup>* mice [78].

Leptin, a pro-inflammatory adipocytokine, was shown to contribute to steatohepatic lesions through induction of iNOS and NADPH oxidase, which caused peroxynitrite-mediated oxidative stress and thereby activated Kupffer cells [79]. Insulin resistance, which is commonly found in metabolic syndrome and NAFLD, was also related to iNOS induction [80]. iNOS may cause lipid-induced hepatic insulin resistance by inhibiting key insulin signaling proteins such as Akt, insulin receptor- $\beta$  (IR $\beta$ ), insulin receptor substrate-1 (IRS-1), and 2 (IRS-2), through functional alterations by tyrosine (Tyr) nitration [81].

Overall, eNOS-derived NO or NO donors can be protective, while iNOS-derived NO is generally pathological (Figure 1B). This may be related to the microenvironments at the sites of iNOS induction where generated NO can facilitate the formation of ROS and RNS with other available radicals. While the pathogenesis of NASH remains to be elucidated, a “double-hit theory” has been proposed in which the “first hit” involves hepatic fat accumulation and the “second hit” could be any additional insult to the liver. iNOS-derived NO could be an important second hit.

### Alcoholic liver disease

Alcoholic liver disease (ALD) resembles NAFLD but is caused by overconsumption of alcohol. Like NAFLD, ALD also starts with steatosis and can develop into advanced stages (Figure 3). The histological features of ALD are considerably similar to those of NAFLD [82]. Reported roles of NO in ALD are also comparable, showing the beneficial effect of eNOS-derived NO [83] and the harmful effect of iNOS-derived NO [84]. Chronic or acute ethanol overconsumption increases NO levels in the blood and liver [85, 86], with the latter in particular attributed to the increased activity of iNOS.

Caveolin-1, a cell membrane protein, was reported to suppress iNOS induction through inhibition of the epidermal growth factor receptor (EGFR)/signal transducer and activator of transcription 3 (STAT3) signaling cascade in the liver [87], and decrease iNOS-induced nitrosative stress, leading to the protection of the liver from alcohol-induced injury [88].

Argininosuccinate synthase (ASS), the rate-limiting enzyme in the urea cycle responsible for the conversion of L-citrulline to L-arginine (a NOS substrate), was also implicated in iNOS regulation in response to alcohol over-intake. In response to ethanol binge drinking, partial ASS ablation (*ASS<sup>+/-</sup>* mice) resulted in reduced liver injury by decreasing nitrosative stress through lower iNOS induction and less 3-nitrotyrosine (3-NT) protein residues but, in a chronic ethanol-feeding model, enhanced liver injury due to hyperammonemia, increased oxidative stress, and impairment of fatty acid  $\beta$ -oxidation despite no differences in nitrosative stress. The role of ASS may be distinctive depending on the disease process.

Alcohol-related increases of iNOS-derived NO could also cause hepatic injury by changing protein function through S-nitrosylation of important mitochondrial proteins, including

aldehyde dehydrogenase 2 (ALDH2), ATP synthase, and 3-ketoacyl-CoA thiolase. These proteins play important roles in ethanol detoxification, ATP synthesis, and fatty acid  $\beta$ -oxidation, and their activities were inhibited in an experimental model of ALD [89].

### Viral hepatitis

Infections with the hepatitis B and hepatitis C viruses (HBV/HCV) are common causes of hepatic inflammation, fibrosis, and HCC (Figure 3). iNOS-derived NO mediates important pathogenic events, such as DNA damage and gene mutation, in these infectious diseases.

Among the structural and functional components of HBV, hepatitis B virus X protein (HBx), a multi-functional viral regulator, has been implicated in hepatic metabolic disorder [90]. HBx-overexpressing transgenic (Tg) mice exhibited increased hepatic glucose production and hyperglycemia (high blood glucose) due to elevated expression of key gluconeogenic enzymes including *PEPCK*, *G6Pase*, *PGC1 $\alpha$* , and *FOXO1*. However, deletion of the iNOS gene in HBx Tg mice [HBx Tg/iNOS<sup>-/-</sup> mice] corrected these elevated gluconeogenic gene expressions and normalized hyperglycemia. Conversely, treatment with an NO donor, sodium nitroprusside, significantly increased the expression of these gluconeogenic genes in HBx-expressing HepG2 cells (human liver carcinoma cells).

Close connections between HCV infection and iNOS induction have also been described. iNOS induction has been reported in liver tissues from HCV patients [91, 92]. HCV, specifically the HCV core protein or nonstructural protein 3 (NS3), was shown to induce iNOS in human B lymphocytes or Hep G2 cells and cause DNA double-strand breaks that enhance DNA mutation. It was suggested that NO generated by iNOS is responsible for DNA damage and mutations of cellular genes, facilitating HCV pathogenesis and oncogenesis [93].

Current treatment regimens of chronic hepatitis C consist of pegylated interferon alpha (PEG-IFN- $\alpha$ ), ribavirin, and direct-acting antivirals (i.e., viral protease/polymerase inhibitors) [94]. Potential mechanisms of ribavirin's anti-HCV activity are through its anti-angiogenic [95] and anti-inflammatory [96] properties. Ribavirin was shown to inhibit endothelial cell proliferation, endothelial cell tube formation, and vessel formation by decreasing eNOS-derived NO through a sequence of events (decreased intracellular guanosine-5'-triphosphate (GTP) and decreased cofactor tetrahydrobiopterin (BH4)) initiated by the inhibition of intracellular inosine-5'-monophosphate dehydrogenase. In cultured macrophages (RAW264.7), ribavirin also inhibited iNOS-derived NO, suggesting its anti-inflammatory activity [96]. However, use of ribavirin may need caution. Given an important role of eNOS-derived NO in liver homeostasis (Figure 1A), decreased eNOS-derived NO by ribavirin may cause LSEC dysfunction. In addition, decreased BH4 may induce eNOS uncoupling, which generates oxidative stress in the liver.

Interestingly, iNOS was also described as a main effector of IFN- $\gamma$ -mediated anti-HCV activity in hepatocytes [97]. It was speculated that iNOS might be beneficial in a very early event of the viral replication cycle, but less protective in later persistent stages.



The role of NO in the other types of hepatitis virus infection such as hepatitis A virus (HAV) or E virus (HEV) is largely unknown. It was recently reported that the iNOS C150T polymorphism, the eNOS G894T polymorphism, and high levels of iNOS and eNOS were associated with the risk of HEV-related acute hepatitis and acute liver failure [98]. However, further studies are needed to understand the role of these polymorphisms in iNOS and eNOS in HEV pathogenesis.

### Hepatocellular carcinoma

HCC development is also associated with elevated expression of iNOS and increased nitrosative stress [41] (Figure 3). GSNOR is a denitrosylase key in controlling protein S-nitrosylation. By preventing excessive protein S-nitrosylation, GSNOR appears to have a beneficial effect on health (Figure 1B). The human GSNOR gene (ADH5) is located approximately in the 4q23 region, where chromosomal deletion frequently occurs in HCC [99, 100]. Approximately 50% of liver samples from patients with HCC showed decreased GSNOR level and activity. In a diethylnitrosamine-induced HCC model, deletion of GSNOR gene substantially increased S-nitrosylation and degradation of O<sup>6</sup>-alkylguanine-DNA alkyltransferase (AGT), a key DNA repair protein, as well as phosphorylation of histone H2AX, a well-established marker of DNA double-strand breaks. These impairments were attenuated in GSNOR/iNOS double knockout mice [40, 101] as well as by administration of an iNOS inhibitor (1400W) [41]. Interestingly, gene-expression profiling of fixed tissues from patients with HCC correlated GSNOR and iNOS to good and poor survival, respectively [102].

GSNOR is also known as alcohol dehydrogenase class III and was originally recognized as glutathione-dependent formaldehyde dehydrogenase [103, 104]. Thus, its deletion may also cause accumulation of endogenous formaldehyde, which in turn may induce DNA injury, accelerating cancer progression. It is possible that dysregulated S-nitrosylation may not be the only mechanism through which GSNOR deficiency contributes to the development of HCC [41].

HCC frequently develops in patients with HBV and HCV. HBx has been implicated in hepatitis B-related HCC development through iNOS induction [105, 106]. iNOS induction by HBx was related to NF $\kappa$ -B signaling [107]. Interestingly, iNOS also upregulated HBx expression and activated HBx-mediated c-jun n-terminal protein kinase (JNK) signaling, thereby facilitating HCC development.

Likewise, HCV core protein is known to facilitate oncogenic events by causing double-strand DNA breaks, which potentiate the mutation of proto-oncogenes and tumor suppressors. iNOS and RNS/ROS induced by HCV core protein were indicated to play a role [93, 108]. In transgenic mice expressing HCV core protein, treatment with butylated hydroxyanisole, an antioxidant scavenger of ROS and RNS, significantly decreased liver tumor size and number as well as mutation frequency of p53, a major tumor suppressor. Furthermore, HCV core-induced iNOS/NO was implicated in the impairment of DNA damage repair through the inhibition of DNA glycosylase activity [109]. This impairment of DNA glycosylase is likely mediated by tyrosine nitration or S-nitrosylation [110, 111].

## Hepatic fibrosis

Under normal conditions, NO is constitutively synthesized by eNOS in LSECs, keeping HSCs quiescent in a paracrine manner [112, 113] (Figure 1A). In pathological conditions, however, LSECs become dysfunctional, accompanied by decreased eNOS-derived NO, which activates quiescent HSCs, causing extracellular matrix deposition and vasoconstriction and leading to fibrosis and further cirrhosis (Figure 1B).

It was reported that, *in vitro*, HSC quiescence required both LSECs (or eNOS) and VEGF [113] (Figure 1A). VEGF was thought to maintain LSEC integrity, partly by stimulating activation of eNOS and sGC. In rats with cirrhosis, an sGC activator, BAY 60-2770, restored LSEC structure, followed by increased quiescent HSCs and regressed fibrosis [112]. Statin (Simvastatin) was also reported to revert activated HSCs to quiescence by upregulating eNOS in LSECs via the transcription factor Krüppel-like factor 2 (KLF2) [114] (Figure 1B).

NO may also limit activated HSCs through apoptosis. Apoptosis of activated HSCs is one of the key events that facilitate regression of liver fibrosis. NO was reported to trigger HSC apoptosis via nitrosative stress-mediated mitochondrial membrane depolarization [115].

## Portal hypertension

Portal hypertension, which is defined as an increase in pressure in the portal vein that carries blood to the liver, is a detrimental complication of liver disease. The most common cause of portal hypertension is liver cirrhosis due to increased intrahepatic vascular resistance (Figure 3). LSEC is a key player in the regulation of intrahepatic vascular resistance through production of eNOS-derived NO [116, 117] (Figure 1).

eNOS is regulated by complex post-translational modifications and protein-protein interactions in LSECs [117, 118]. Those regulators include phosphatidylinositol 3-kinase / Akt, caveolin-1, Hsp-90, G-protein-coupled receptor kinase 2 (GRK2), G-protein-coupled receptor kinase-interacting protein 1 (GIT1) [119, 120], dimethylarginine dimethylaminohydrolase-1 (DDAH-1) [121], and a cofactor BH<sub>4</sub> [122]. In cirrhotic conditions, all these eNOS regulators, by suppression or activation, work toward the inhibition of eNOS activity and favor increased vasoconstriction by activated HSCs, leading to increased intrahepatic vascular resistance.

For example, in the livers of cirrhotic rats, the levels of guanosine triphosphate (GTP)-cyclohydrolase (BH<sub>4</sub> rate-limiting enzyme) were significantly decreased, leading to decreased BH<sub>4</sub> levels and eNOS uncoupling. eNOS uncoupling was associated with LSEC dysfunction, increased intrahepatic resistance and subsequent development of portal hypertension [123]. However, BH<sub>4</sub> levels were significantly increased in the extrahepatic territory (i.e., mesenteric lymph nodes and mesenteric arteries) as a result of increased endotoxin levels via bacterial translocation, a pathological feature of liver cirrhosis [124, 125]. This resulted in increased NO production in the mesenteric arteries of cirrhotic rats. Although local BH<sub>4</sub> production is an important determinant of NO bioavailability derived from eNOS in endothelial cells, the relationship between BH<sub>4</sub> levels, iNOS uncoupling and NO production is largely unknown.

As mentioned above, in portal hypertension, regulation of NO is opposite inside and outside of the liver. Contrary to decreased NO availability in the intrahepatic circulation, NO production in splanchnic and systemic arteries increases and causes excessive vasodilation, resulting in increased blood flow to the portal vein and thereby worsening portal hypertension. Therefore, for a therapeutic purpose, liver-specific NO donors that do not cause the additional vasodilative effects on the splanchnic and systemic circulation are needed. Examples of NO donors can be found in Table 1 [117]. Some of them have demonstrated positive outcomes in experimental models. For example, NCX-1000, a hepatocyte-specific NO donor, ameliorated portal hypertension in cirrhotic rats [126], but failed in a clinical trial [127]. Although this discrepancy may come from differences between animals and humans, treatment of portal hypertension could benefit more from NO donor delivery to LSECs than to hepatocytes.

Recently, relaxin, a peptide hormone, was reported to increase intraheptic NO levels and decrease portal pressure without changing systemic NO levels and pressure. This selective effect of relaxin was attributed to the upregulation of relaxin receptor restricted to HSCs in fibrotic livers [128].

In this line, a recent study explored if vitamin A-coupled polymeric nanoparticles encapsulated with an NO donor could be specifically delivered to HSCs and ameliorate liver fibrosis and portal hypertension [129]. Although this study showed decreased portal pressure without changing mean arterial pressure in cirrhotic rats treated with vitamin A-conjugated NO-nanoparticles, intake of vitamin A-coupled nanoparticles by each type of hepatic cells was not examined *in vivo*. Whether NO was delivered specifically to HSCs is not certain, so it is difficult to attribute the observed decrease in portal pressure in cirrhotic rats to the targeted delivery of NO to HSCs. To our best knowledge, there are no *in vivo* studies that have demonstrated cell-specific delivery of NO in the liver.

While decreased eNOS-derived NO in LSECs causes hepatic microvascular dysfunction and facilitates the development of fibrosis/cirrhosis and portal hypertension, iNOS-derived NO generally increases in this development. iNOS upregulation was shown to contribute to liver sinusoidal endothelial dysfunction in endotoxemia [130]. Unlike eNOS-derived NO, iNOS-derived NO does not appear to cause vasodilation. In addition, a paradoxical role of increased NO by iNOS in endothelial dysfunction, which is otherwise characterized by decreased NO by eNOS, may indicate that the effects of NO are highly dependent on its sources and surrounding microenvironments.

NO is also thought to regulate lymphatic flows by affecting the contractility of lymphatic smooth muscle cells. Increased levels of eNOS and NO were observed in endothelial cells isolated from mesenteric lymphatic vessels of cirrhotic rats, which was also attributed to decreased smooth muscle cell coverage of mesenteric lymphatic vessels in those rats [131]. Excessive NO-mediated relaxation of mesenteric lymphatic vessels may have implications in the formation of ascites (fluid accumulation in the peritoneal cavity), which is commonly associated with cirrhosis with portal hypertension [132].

### Drug-induced liver injury (acetaminophen-induced liver injury)

Drug-induced liver injury causes acute hepatocellular injury with jaundice in patients, which showed a fatality rate of 10–50% depending on the drug involved (Figure 3). In severe cases, acute liver failure (ALF) with concomitant coagulopathy (blood clotting disorders) and encephalopathy (global brain dysfunction) occurs. In most Western countries, acetaminophen (paracetamol) overdose is the most frequent identified cause of ALF [133].

CYP2E1 enzyme plays a critical role in acetaminophen-induced liver injury. CYP2E1 metabolizes acetaminophen to N-acetyl-p-benzoquinone imine (NAPQI). This toxic compound is normally detoxified through conjugation with GSH. However, in the presence of large amounts of NAPQI, GSH is depleted and NAPQI remains unconjugated, which causes liver damage by binding to other proteins [25]. Oxidative stress resulting from depleted GSH is also thought to contribute to liver damage. NO produced from Kupffer cells, cytokines, ROS, and RNS are all involved in this process [134]. For example, the formation of nitrotyrosine protein adducts (3-NT), a hallmark of nitrosative stress, was observed in the centrilobular regions where acetaminophen-induced necrosis occurred. The formation of 3-NT was related to decreased levels/activities of nitrated proteins including superoxide dismutase 1 (SOD1), an important O<sub>2</sub><sup>-</sup> scavenger, through ubiquitin-mediated degradation. The formation of 3-NT and liver necrosis was dependent on the presence of CYP2E1 [135].

Acetaminophen was also reported to inhibit the activity of various mitochondrial enzymes through nitration, including aldehyde dehydrogenase, Mn-SOD (SOD2), glutathione peroxidase, ATP synthase, and 3-ketoacyl-CoA thiolase, which are involved in antioxidant defense, energy supply, or fatty acid metabolism [136].

### Ischemia/reperfusion injury

Prolonged organ ischemia characterized by reduced tissue oxygenation induces the activation of anaerobic metabolic pathways and causes cell death (Figure 3). However, reperfusion after ischemia can also lead to cellular injury because the increase in oxygen delivery can generate ROS and RNS [137]. Hepatic ischemia/reperfusion (I/R) injury is a major problem in liver resection and transplantation.

Like in other liver diseases, NO has been known to have both protective and harmful effects on hepatic I/R injury. In general, eNOS-derived NO is beneficial [95-98], while iNOS-derived NO may be either protective or detrimental [5].

The protective effect of eNOS-derived NO is exerted at least in part through the sGC-cGMP-protein kinase G (PKG) pathway [138, 139]. Circulating adiponectin also showed protective property against hepatic I/R injury by reducing inflammatory response and apoptosis through activation of adenosine monophosphate-activated protein kinase (AMPK)/eNOS pathway [140].

The role of iNOS-derived NO in I/R injury is not straightforward because studies reported on its protective [141], harmful [142] or no [141, 143] effects. These studies differ in the

length of ischemia or reperfusion, which may account for the observed different roles of iNOS in liver I/R injury.

iNOS-derived NO also contributed to hepatic I/R injury through S-nitrosylation or nitration of mitochondrial proteins. I/R-induced liver damage was associated with an increase in S-nitrosylated or oxidized mitochondrial proteins, including aldehyde dehydrogenase, 3-ketoacyl-CoA thiolases, and ATP synthase, and their activities were suppressed [144].

Many therapeutic and preventive trials have been conducted on hepatic I/R injury. NO inhalation to patients during the operative period of liver transplantation showed significant protection of hepatocytes from apoptotic death, quicker restoration of liver graft function, and reduced hospital length of stay [145]. Administration of sodium nitrite also reduced liver I/R injury with decreased hepatocyte apoptosis [146, 147].

## Concluding remarks

NO has differential effects on the liver depending on its source. In general, eNOS-derived NO is protective against liver diseases, whereas iNOS-derived NO is deleterious because it mainly acts as a pro-inflammatory mediator. NO-mediated protein modifications such as tyrosine nitration and S-nitrosylation are significantly involved in liver biology. The new modifications, NO<sub>2</sub>-FA and S-guanylation, have increasingly been recognized and should be explored in the liver as well.

Due to its regulation of Nrf2/Keap1 and NFκB, which play important roles in the development of liver diseases [56, 59], NO<sub>2</sub>-FA [46] could be used as a potential therapeutic agent. For example, NO<sub>2</sub>-FA could block progression of NAFLD to NASH, in which inflammatory responses are involved. Given its vasoprotective role through induction of eNOS expression and activation, NO<sub>2</sub>-FA may also have an anti-fibrotic property. Because there is currently no effective anti-fibrotic drug, testing the potential of NO<sub>2</sub>-FA to ameliorate fibrosis/cirrhosis may have significant clinical implications. Lastly, given its high stability in the blood and urine [148, 149], a potential of NO<sub>2</sub>-FA as a biomarker of NAFLD, NASH, or fibrosis could be explored in combination with other non-invasive imaging systems.

Similarly, 8-nitro-cGMP exhibited an anti-inflammatory property through activation of Nrf2/Keap1 signaling to induce antioxidant enzymes [65, 66, 150]. Therefore, 8-nitro-cGMP could also be used as an anti-inflammatory agent for a wide range of liver diseases in which inflammation plays a central role.

The roles of NO in the liver have not been examined in relation to some important biological events such as autophagy and epigenetics. Since there are studies showing both its inductive [151] and inhibitory role [152], unraveling the effect of NO on autophagy is complicated. Autophagy has been implicated in a variety of liver diseases [153], including NAFLD [154], viral hepatitis [155], fibrosis [156], and HCC [157]. Given the importance of NO as a signaling molecule and its relation to ROS/RNS, examining the role of NO in the axis of autophagy and liver is interesting. Similarly, despite that NO is an epigenetic modulator in

tumor cells [158], NO's epigenetic regulation has not been explored in HCC and other liver diseases. This is also an important area to be investigated.

As for NO-related therapy, NO's differential roles require liver/cell specificity. For reference, Table 1 lists NO donors that have been used in experimental models or clinical settings. Currently, there is no cell-specific NO donor delivery system. The development of such delivery systems will warrant effective treatments for liver diseases. At last, the role of nNOS in the liver is little known and should also be explored. There is much to do in NO-related studies in the liver.

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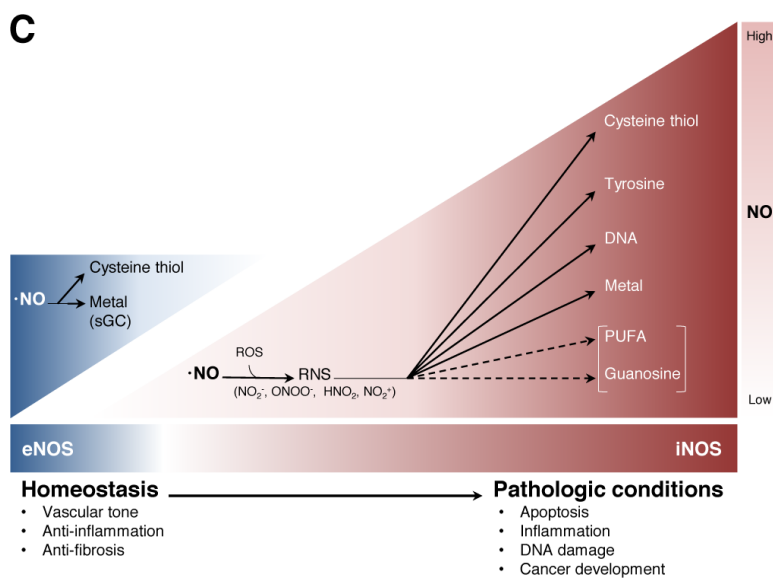
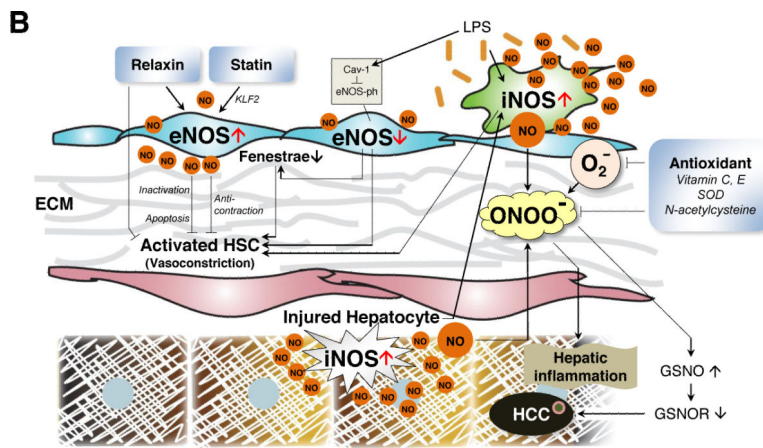
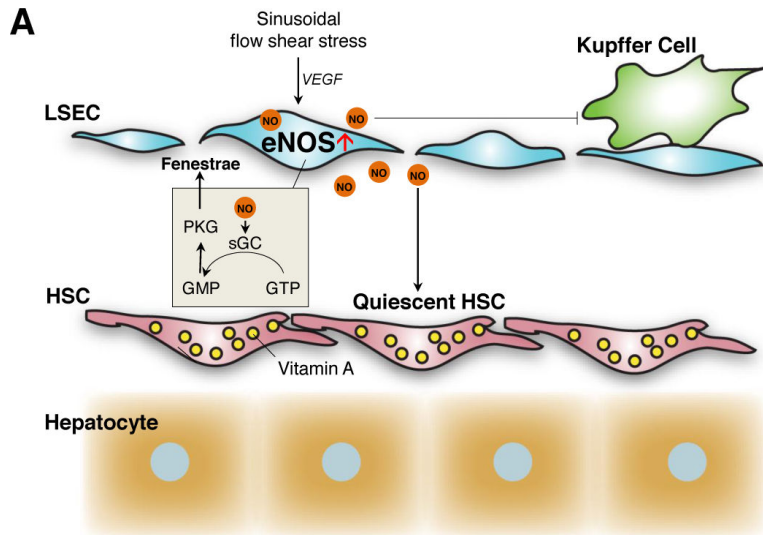
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### Highlights

- eNOS-derived NO is protective against liver diseases, whereas iNOS-derived NO is deleterious.
- In liver biology, eNOS and iNOS are major players, whereas the role of nNOS is little known.
- NO regulates a wide range of cellular activities by binding to metal ions, proteins, lipids, and guanine nucleotides.
- In liver biology, NO signaling through protein modifications by S-guanlylation or NO<sub>2</sub>-FA S-alkylation should be explored.



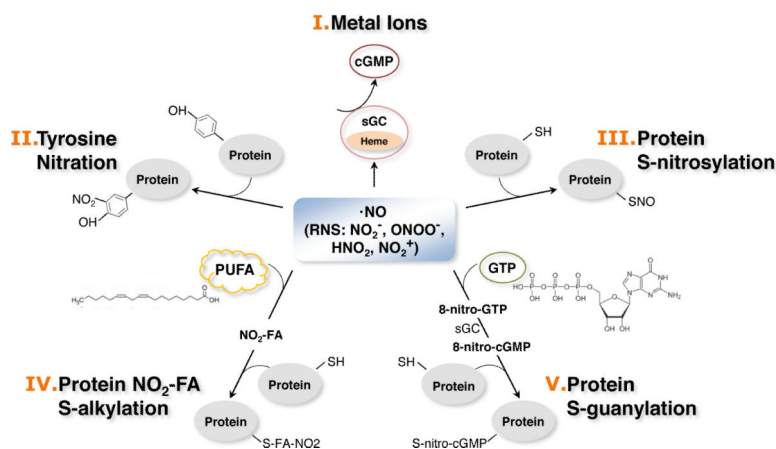
**Figure 1. The role of nitric oxide (NO) in the hepatic sinusoids in normal and pathologic liver conditions**

**A. Homeostatic roles.** Small amounts of NO constantly produced by eNOS in liver sinusoidal endothelial cells (LSECs) are essential for controlling intrahepatic sinusoidal vascular tone and blood flow. eNOS is activated by stimuli such as sinusoidal blood flow shear stress and vascular endothelial growth factor (VEGF) [116, 117]. NO keeps hepatic stellate cells (HSCs) [63, 64] and Kupffer cells quiescent [73]. NO maintains fenestrae of LSECs through activation of soluble guanylyl cyclase (sGC) that converts GTP to cGMP, which then stimulates protein kinase G (PKG) [112].

**B. Pathological roles.** Pro-inflammatory cytokines and bacterial endotoxin induce iNOS to produce large amounts of NO, facilitating liver injury. In pathological conditions, eNOS activity decreases, whereas iNOS is upregulated. Decreased NO production in LSECs causes endothelial cell capillarization and activation of HSCs, which is accompanied with extracellular matrix (ECM) deposition, HSC contraction and proliferation, and finally an increase in intrahepatic resistance and sinusoidal flow disturbance. Bacterial endotoxin lipopolysaccharide (LPS) secondary to gut bacterial translocation induces iNOS expression and NO production [130], which combines with superoxide to generate peroxynitrite. LPS also increases caveolin-1 and decreases eNOS phosphorylation (an active form of eNOS). These oxidative and nitrosative stresses also facilitate HSC activation and hepatocyte injury with decreased S-nitrosoglutathione reductase (GSNOR) and increased S-nitrosoglutathione (GSNO), which is related to the development of hepatocellular carcinoma (HCC) [41]. Several anti-oxidants such as superoxide dismutase (SOD) are important controllers of nitrooxidative stress. Recently, statin and relaxin were shown to restore LSEC function. Statin upregulated eNOS in LSECs via the transcription factor Krüppel-like factor 2 (KLF2) and reverted activated HSCs to the quiescent state [114]. Relaxin increased intraheptic NO levels and decreased portal pressure due to upregulation of relaxin receptor restricted to HSCs in fibrotic livers [128].

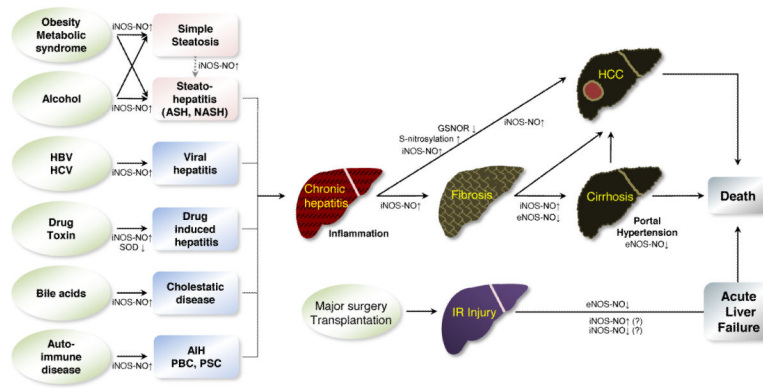
**C. Mode of action of NO in homeostatic and pathological conditions in the liver.** eNOS is constitutively expressed in endothelial cells and produces small amounts of NO, which play an important role in vascular homeostasis. In contrast, in pathological conditions, iNOS is upregulated and produces large amounts of NO, which cause cell apoptosis, inflammation, DNA damage, and cancer development. Nitration of polyunsaturated fatty acids (PUFA) and guanosines has not been studied in the liver, but may have protective roles in diseased liver. Abbreviations: reactive oxygen species (ROS), reactive nitrogen species (RNS).





**Figure 2. Mode of action of nitric oxide (NO)**

NO regulates a wide range of cellular activities by binding to metal ions, proteins, lipids, and guanine nucleotides. **I) Metal ions.** A primary target for NO is ferrous heme iron. The most known example is the binding of NO to ferrous heme iron of soluble guanylyl cyclase (sGC) to produce cGMP as the second messenger [11, 12]. **II) Tyrosine nitration.** Nitration of protein tyrosine residues results in irreversible and stable adducts and has been considered an indicator of nitrosative stress [35]. **III) S-nitrosylation.** S-nitrosylation occurs through the binding of oxidized NO to a specific cysteine thiol anion in proteins/peptides and is a reversible reaction [36]. **IV) Nitration of unsaturated fatty acids (S-alkylation).** NO or NO-derivatives can bind to unsaturated fatty acids, resulting in the formation of electrophilic compounds, nitrated fatty acids (NO<sub>2</sub>-FA). NO<sub>2</sub>-FA can readily react with nucleophilic amino acids, such as histidine and cysteine, in proteins and change protein function [148, 149]. **V) Nitration of guanine nucleotides (S-guanylation).** NO or NO-derivatives can also bind to guanine nucleotides and produce electrophilic NO derivatives (8-nitroguanosine), which conduct protein modification [65].



**Figure 3. The natural course of acute and chronic liver disease**

Chronic liver disease includes various heterogeneous disease conditions depending on etiology and time. Chronic exposures to different insults lead to various kinds of chronic hepatitis. If causal factors are not corrected and continue the harmful effects on the liver, hepatic inflammation progresses to hepatic fibrosis and further cirrhosis. In this stage, cirrhosis often accompanies portal hypertension, which is a main pathophysiologic cause of cirrhosis-related death and complications such as variceal hemorrhage and ascites formation. Hepatocellular carcinoma can develop at any stage of chronic liver disease even though advanced cirrhosis is the most risky stage. Ischemic-reperfusion injury after major liver surgery or transplantation can progress to sudden acute hepatic liver failure and death. The roles of NO derived from eNOS or iNOS vary, depending on disease etiologies and conditions. In general, NO produced by eNOS can be protective, but iNOS-derived NO or its derivatives such as peroxynitrite have been shown to promote inflammation.

Table 1

Nitric oxide delivery systems or pro-drugs for liver disease

Drug (Pro-drug or delivery system)	Experimental model	Route	Effects	Reference
	Murine model of autoimmune hepatitis, <i>in vivo</i> / HepG2 cell, <i>in vitro</i>	i.p.	Inhibition of apoptosis and inflammation via regulation of the caspase superfamily	Fiorucci. et al.[159]
NCX-1000 (2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester)	BDL cirrhotic rat model, <i>in vivo</i>	p.o.	Decrease of portal pressure and intrahepatic resistance without systemic effects along with increase of nitrite/nitrate and cGMP (cyclic guanosine monophosphate) concentrations in the liver	Fiorucci. et al.[126]
	Clinical, patients with cirrhosis and portal hypertension	p.o.	Systemic hypotensive effects and lack of reduction in portal pressure	Berzigotti. et al.[127]
O <sup>2</sup> -vinyl 1-(pyrrolidin-1-yl)diazene-1-ium-1,2-diolate (V-PYRRO/NO)	Adult female CD-1 mice with acetaminophen (600 mg/kg, dissolved in saline, 25 mL/kg, i.p.) model / Osmotic pump of V-PYRRO/NO, i.v.	i.v.	Blocking of acetaminophen-induced hepatotoxicity by reduction of oxidative stress and apoptosis	Liu. et al.[160]
	Male BALB/c mice with CCl <sub>4</sub> (by gavage) hepatic fibrosis and portal hypertension model / V-PYRRO/NO, i.v. bolus	i.v.	Decrease of portal pressure and systemic mean arterial pressure	Edwards. et al.[161]
	C57BL/6J mice with high fat diet NAFLD model	i.p.	Anti-steatotic effects and improvement of postprandial glucose tolerance	Maslak. et al.[162]
	Pig ischemia/reperfusion liver model	i.v.	Decrease of hepatic resistance and improvement of hemodynamics. Improvement of plasma clearance of bile acids	Ricciardi. et al.[163]
NO-releasing carbon-dot-based nanosystem (C-dot-triphenylphosphonium (TPP)-S-nitrosothiol)	Three cancer cell lines; HeLa (human cervical cancer cells), HepG2 (human liver carcinoma cells) and A549 (adenocarcinomic human alveolar basal epithelial cells), <i>in vitro</i>	-	Cytotoxic and apoptotic effects by mitochondria targeting, photo-enhanced NO release	Xu. et al.[164]
Gold and silica nanoparticles and NO donors conjugate *NO donors: S-nitroso-N-acetyl-DL-penicillamine (SNAP), glyco-SNAP, 3-morpholino-sydnominine (SIN-1), S-nitrosoglutathione (GSNO)	Primary human HSCs and LX2 cell-line, <i>in vitro</i>	-	Decrease of HSC proliferation and vascular tube formation	Das. et al.[165]
Nitro-aspirin (NO-aspirin) (Cyclooxygenase-inhibiting nitric oxide donors (CINODs))	Male Wistar albino rats with 2% cholesterol diet NAFLD model, <i>in vivo</i>	p.o. (gavage)	Decrease in expression of iNOS and cyclooxygenase-2 (COX-2). Blocking of NAFLD development	Ibrahim. et al.[166]
Furoxan-based NO releasing derivatives of farnesylthiosalicylic acid (FTS)	HCC SMMC-7721, Bel7402, LO2, HepG2 and Hep3B cells, <i>in vitro</i>	-	HCC cell apoptosis	Ling. et al.[167]

Drug (Pro-drug or delivery system)	Experimental model	Route	Effects	Reference
O <sup>2</sup> -(2,4-dinitrophenyl)-diazoniumdiolate and oleanolic acid (OA) activated by glutathione S-transferase $\pi$ (GST $\pi$ )	HepG2, <i>in vitro</i> / ICR mice inoculated with H22 (mice hepatoma cell line), <i>in vivo</i>	i.v.	anti-HCC via apoptosis and increase of intracellular ROS production	Fu. et al.[168]
Polymeric nanoparticles decorated with vitamin A conjugated with S-nitrosoglutathione (GSNO)	Primary rat HSCs and LX2 cell-line, <i>in vitro</i> / BDL rats, <i>in vivo</i>	i.v.	Inhibition of fibrogenic genes associated with HSC activation and attenuation of hemodynamic disorder	Duong. Et al.[129]
DETA NONOate [(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amino]diazene-1-ium-1,2-diolate]	Male C57BL/6J mice with MCD NASH model, CYP2E1 KO and iNOS KO mice with high fat diet NASH model, <i>in vivo</i> / BDCM-Induction of liver Injury	i.p.	Inhibition of CYP2E1-mediated oxidative stress	Seth. et al.[76]

BDCM, bromodichloromethane; BDL, bile duct ligation; cGMP, cyclic guanosine monophosphate; CYP2E1, cytochrome P450 2E1; GSNO, S-nitrosoglutathione; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; MCD, methionine and choline-deficient; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis