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## FORUM REVIEW ARTICLE

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# Role of NADPH Oxidases in Liver Fibrosis

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### Abstract

**Significance:** Hepatic fibrosis is the common pathophysiologic process resulting from chronic liver injury, characterized by the accumulation of an excessive extracellular matrix. Multiple lines of evidence indicate that oxidative stress plays a pivotal role in the pathogenesis of liver fibrosis. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is a multicomponent enzyme complex that generates reactive oxygen species (ROS) in response to a wide range of stimuli. In addition to phagocytic NOX2, there are six non-phagocytic NOX proteins. **Recent Advances:** In the liver, NOX is functionally expressed both in the phagocytic form and in the nonphagocytic form. NOX-derived ROS contributes to various kinds of liver disease caused by alcohol, hepatitis C virus, and toxic bile acids. Recent evidence indicates that both phagocytic NOX2 and nonphagocytic NOX isoforms, including NOX1 and NOX4, mediate distinct profibrogenic actions in hepatic stellate cells, the main fibrogenic cell type in the liver. The critical role of NOX in hepatic fibrogenesis provides a rationale to assess pharmacological NOX inhibitors that treat hepatic fibrosis in patients with chronic liver disease. **Critical Issues:** Although there is compelling evidence indicating a crucial role for NOX-mediated ROS generation in hepatic fibrogenesis, little is known about the expression, subcellular localization, regulation, and redox signaling of NOX isoforms in specific cell types in the liver. Moreover, the exact mechanism of NOX-mediated fibrogenic signaling is still largely unknown. **Future Directions:** A better understanding through further research about NOX-mediated fibrogenic signaling may enable the development of novel anti-fibrotic therapy using NOX inhibition strategy. *Antioxid. Redox Signal.* 20, 2854–2872.

### Introduction

LIVER FIBROSIS is the accumulation of extracellular matrix (ECM) proteins, mainly type I collagen, which occurs in most types of chronic liver disease. The main causes of liver fibrosis in industrialized countries include chronic hepatitis C virus (HCV) or hepatitis B virus (HBV) infection, alcohol abuse, and nonalcoholic steatohepatitis (NASH) (14). The accumulation of ECM proteins distorts the hepatic architecture by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes defines cirrhosis (70). Major complications of cirrhosis are portal hypertension

and liver failure. Portal hypertension can lead to serious complications such as variceal bleeding, ascites, spontaneous bacterial peritonitis, and hepatic encephalopathy that are the major causes of mortality in cirrhotic patients. Cirrhosis also leads to the development of hepatocellular carcinoma (HCC) (204).

Liver fibrosis is a model of the wound-healing response to chronic liver injury. Hepatic stellate cells (HSCs), formerly known as lipocytes, Ito cells, or perisinusoidal cells, have been identified as the main collagen-producing cells in the liver (72). This cell type undergoes a dramatic phenotypic change in chronic liver diseases with the acquisition of fibrogenic

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properties. Quiescent HSCs are desmin-positive, perisinusoidal cells that are the primary cells in the body which are responsible for vitamin A storage (14). On activation by liver injury, quiescent HSCs transdifferentiate into myofibroblasts that produce inflammatory cytokines and several ECM proteins, including at least five collagen types, fibronectin, undulin, elastin, laminin, entactin, tenascin, and several proteoglycans (71). The molecular mechanisms leading to HSCs activation and increased ECM synthesis in liver fibrosis have been extensively investigated by using cultured HSCs and experimental models of chronic liver injury in rodents (14, 70, 153, 161). Besides HSCs, portal fibroblasts and cells of bone marrow origin have fibrogenic potential (106, 155, 167). Recent knowledge indicates that most fibrogenic myofibroblasts are endogenous to the liver, and activated HSCs and fibroblasts are the major endogenous fibrogenic cells in the liver (26).

The demonstration that even advanced liver fibrosis may be reversible has stimulated researchers to investigate anti-fibrotic therapies (83, 111). The only effective, available therapy to treat hepatic fibrosis to date is to remove the causative agent (13). For example, long-term administration of oral nucleos(t)ide analogues demonstrate the regression of liver fibrosis in patients with advanced liver fibrosis or even cirrhosis caused by chronic HBV infection (38, 126). Similarly, peginterferon  $\alpha$  significantly reduces hepatic fibrosis in patients with chronic hepatitis C with or without cirrhosis in whom sustained virologic response occurs (32). However, a number of drugs are able to reduce liver fibrosis in experimental models without affecting the etiological agent causing the injury. Several candidate drugs, including antioxidants and renin-angiotensin system (RAS) blockers, have been evaluated for their anti-fibrotic efficacy and safety; however, evidence-based therapies are not yet available (17).

Reactive oxygen species (ROS) are involved as key secondary messengers in numerous signaling pathways, including transcriptional regulation, differentiation, proliferation, oncogenic transformation, and activation of programmed cell death (28). ROS contributes to the hepatic fibrosis from various kinds of liver injuries, including alcohol abuse, HCV infection, iron overload, and chronic cholestasis

(150, 154). ROS can stimulate the production of the type I collagen, and may act as an intracellular signaling mediator of the fibrogenic action of TGF- $\beta$  (56, 124, 136). ROS may be generated in the liver by multiple sources, including the mitochondrial respiratory chain, cytochrome P450 (CYP) family members, peroxisomes, xanthine oxidase, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (55, 150). Accumulating evidence indicates that NADPH oxidases (NOXs)-mediated ROS has a critical role in HSCs activation and hepatic fibrogenesis. This review summarizes the current knowledge on the profibrogenic mechanisms of various NOX isoforms expressed in specific cell types in the liver, with particular focus on HSCs and discusses NOX isoforms as potential therapeutic targets for hepatic fibrosis.

**NADPH Oxidases**

NOX is a multimeric transmembrane enzyme complex that generates superoxide ( $O_2^{\bullet -}$ ) and hydrogen peroxide ( $H_2O_2$ ) from molecular oxygen using NADPH as an electron donor (21). The mammalian NOX family comprises seven isoforms: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2 (Table 1) (21, 113). Classic NOX is the phagocytic NOX found in neutrophils. The phagocytic NOX contains a heterodimeric membrane-bound flavocytochrome b558 complex, which consists of the catalytic subunit gp91<sup>phox</sup> (renamed NOX2) and the regulatory subunit p22<sup>phox</sup> located in the membrane (21, 162). Rac is a component required for the activation of NOX2. Rac is a member of the Rho family of small GTPase proteins that regulates cell proliferation and dynamic reorganization of the actin cytoskeleton (44). The NOX2 regulatory components, p47<sup>phox</sup>, p40<sup>phox</sup>, p67<sup>phox</sup>, and Rac, are usually located in the cytoplasm (21). On stimulation with agonists, the cytosolic subunits translocate to the membrane-bound flavocytochrome complex, leading to enzymatic activity.

NOX2-derived ROS acts in immune defense in neutrophils and phagocytes (21). The genetic defect of phagocytic NOX (NOX2) activity called chronic granulomatous disease (CGD) results in an inability to produce the superoxide anion that is necessary for killing bacteria and fungi by neutrophils and

TABLE 1. THE CHARACTERISTICS OF SELECTED NOX ISOFORMS

<i>Catalytic subunit</i>	<i>Components</i>	<i>Major tissue/cell distribution</i>	<i>Major agonists</i>	<i>Subcellular localization</i>
NOX1	NOX1, p22 <sup>phox</sup> , NOXO1/p47 <sup>phox</sup> , NOXA1/p67 <sup>phox</sup> , Rac1	Colon, vessel, heart, uterus, prostate, liver	Ang II, TNF- $\alpha$ , IL-1 $\beta$ , LPS, PDGF/PGF2 $\alpha$ , EGF, bFGF IFN- $\gamma$ , ET-1	Plasma membrane, caveolae, perinuclear endosome
NOX2	NOX2, p22 <sup>phox</sup> , p40 <sup>phox</sup> , p47 <sup>phox</sup> , p67 <sup>phox</sup> , Rac1/Rac2	Neutrophil, phagocyte, vessel, heart, liver	LPS, IFN- $\gamma$ , Ang II	Plasma membrane, perinuclear, nucleus
NOX3	NOX3, p22 <sup>phox</sup> , NOXO1/p47 <sup>phox</sup> , NOXA1/p67 <sup>phox</sup> , Rac1	Fetal tissue, inner ear		Plasma membrane
NOX4	NOX4, p22 <sup>phox</sup> , POLDIP2	Kidney, vessel, heart, lung, liver	TGF- $\beta$ , TNF- $\alpha$ , IFN- $\gamma$	Endoplasmic reticulum, focal adhesion
NOX5	NOX5	Testis, spleen, lymph node, heart, vessel	Ang II, ET-1, TNF- $\alpha$	Plasma membrane
DUOX1/ DUOX2	DUOX1/DUOX2	Thyroid tissue		

Ang II, angiotensin II; EGF, epidermal growth factor; NOX, NADPH oxidase; IFN, interferon.

phagocytes. Patients with CGD are predisposed to serious bacterial and fungal infections as well as granulomatous complications (170). In contrast with CGD that are defective in NOX-mediated ROS generation, hereditary amyotrophic lateral sclerosis (ALS), a progressive motor neuron degenerative disease, may result from excessive ROS production by an overactive NOX (36).

Compared with NOX2 that is expressed in phagocytes, the nonphagocytic NOXs are expressed at low levels in complex tissue compartments composed of multiple cell types and different NOX isoforms (135). Among the seven isoforms of the NOX family, NOX1, NOX2, NOX3, and NOX4 form heterodimers with p22<sup>phox</sup>, whereas NOX5, DUOX1, and DUOX2 function without an association with p22<sup>phox</sup>. Instead, NOX5, DUOX1, and DUOX2 contain EF-hand calcium-binding motif (135). While NOX1 is also p22<sup>phox</sup> dependent, NOX1 may use NOXO1 (homologue of p47<sup>phox</sup>, an organizing cofactor) to organize the enzyme assembly and NOXA1 (homologue of p67<sup>phox</sup>, an activating cofactor) for enzyme activation (12). In many cases, neither NOXO1 nor NOXA1 has been identified in tissues where the implicated NOX protein will require these co-factors for activity (135). In contrast to other NOX isoforms, NOX4 requires only p22<sup>phox</sup> without recruitment of cytosolic regulatory components to exert its enzymatic activity (21, 76). All NOX enzymes are able to catalyze the reduction of molecular oxygen to superoxide, but there are key differences in their expression, subcellular localization, activation, and intracellular signaling.

#### Cellular distribution of NOXs in the liver

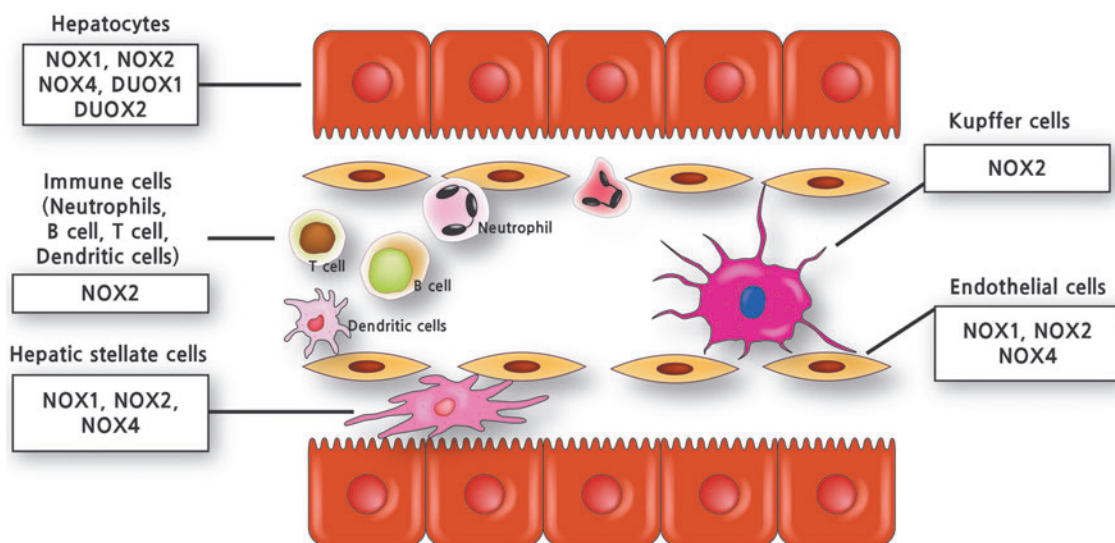
In the liver, NOX is functionally expressed both in the phagocytic form and in the nonphagocytic form (18, 192). Various NOX isoforms such as NOX1, NOX2, and NOX4 are distinctively expressed in the specific cell types, including Kupffer cells (KCs), HSCs, endothelial cells (ECs), hepatocytes, and infiltrating leukocytes in the liver (Fig. 1) (59, 143). KCs, the resident macrophage in the liver, express phagocytic NOX2 and its regulatory components such as p40<sup>phox</sup>,

p47<sup>phox</sup>, and p67<sup>phox</sup>. There is no detectable mRNA expression for other NOX forms such as NOX1, NOX4 in KCs (143). In HSCs, both phagocytic NOX2 and nonphagocytic NOXs such as NOX1 and NOX4 are expressed (143). In ECs, NOX1, NOX2, and NOX4 are expressed (99, 134, 143). NOXO1 and NOXA1 are predominant regulatory components expressed in ECs and HSCs (143). Hepatocytes express mRNAs for NOX1, NOX2, NOX4, DUOX1, and DUOX2 as well as regulatory subunit p47<sup>phox</sup> (35, 60, 143, 157).

#### Subcellular localization of NOX characterizes signal transduction

NOX-generated superoxide anion has a short half life and is rapidly dismutated to H<sub>2</sub>O<sub>2</sub> at a rate constant of  $8 \times 10^4 M^{-1}s^{-1}$  spontaneously and  $2 \times 10^9 M^{-1}s^{-1}$  by superoxide dismutase (SOD) (69). H<sub>2</sub>O<sub>2</sub> is more stable than superoxide and is able to cross the cell membrane. ROS can react with nearby cellular proteins reversibly (40, 193). These properties of diffusibility, short half life, and reversible reactivity make ROS function as signal messengers. Complexity of NOX signaling arises from the multiple NOX isoforms that are expressed within the same cell and mediate distinctive signaling pathways in response to diverse stimuli. The specific subcellular localization of NOX isoforms and their regulatory subunits within specific subcellular compartments may modulate the specificity of ROS-mediated signal transduction (152, 186).

NOX2 is predominantly expressed in the plasma membrane. Stimulation of ECs with Fas ligand or TNF- $\alpha$  induces translocation of NOX2, p47<sup>phox</sup>, and Rac1 to the lamellipodial leading edge of the cell, where the focal complexes undergo rapid turnover and ROS production promotes cytoskeleton rearrangement for directional cell migration (94, 133, 194, 196). NOX2 also targets to phagosomes in neutrophils and to endosomes in nonphagocytic cells, dubbed as "redoxosomes" (119, 138, 139). Redoxosomal NOX2 transduces several stimuli, such as angiopoietin-1, vascular endothelial growth factor, thrombin, and TNF- $\alpha$ , into necrosis and angiogenesis *via* ROS production (63, 125, 187). p22<sup>phox</sup> is essential for optimal



**FIG. 1. Cellular distribution of NOX isoforms in the liver.** Various kinds of cell types in the liver, including hepatocytes, KCs, ECs, HSCs, and infiltrating leukocytes express multiple NOX isoforms, including both phagocytic and nonphagocytic NOX. ECs, endothelial cells; HSCs, hepatic stellate cells; KCs, Kupffer cells; NOX, NADPH oxidase. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

activity of NOX2. In myeloid cells, coexpression and heterodimerization of NOX2 and p22<sup>phox</sup> are required for the migration from the endoplasmic reticulum (ER) and proper localization in the plasma membrane (61). A mouse model of hepatic fibrosis shows that NOX2 level is elevated in activated HSCs (98, 143). However, the subcellular localization of NOX2 in HSCs has not been identified.

NOX1 has been found at the plasma membrane, in early endosomes, and in the nucleus in vascular smooth muscle cells (VSMCs) (92, 131). Recently, it was reported that NOX1 is also found in caveolin 1-containing lipid rafts in VSMCs (92, 186, 206). Binding of angiotensin II (Ang II) stimulates the angiotensin type I receptor (AT1R) to translocate to caveola, where Rac1 is also recruited to locally produce ROS with subsequent activation of epidermal growth factor receptor (EGFR). NOX1 is also involved in endosomal ROS production after hypoxia-reoxygenation injury, which requires both Rac-1 and c-Src recruitment (120). In addition, chloride channel 3 is co-localized for TNF- $\alpha$ -induced NOX1-mediated ROS production, leading to NF- $\kappa$ B activation, at early endosomes in vascular cells (131). In Huh7 cells, a human hepatoma cell line, NOX1 predominantly localizes in the cytoplasm, with apparent punctuate staining at the plasma membrane, even though the identity of these punctuate stains was not specifically addressed (60). Although NOX1 is expressed in HSCs, ECs, and hepatocytes in the liver (143, 157), its subcellular localization in these cells has not yet been investigated.

NOX4 is ubiquitously expressed and inducible (27). Unlike other NOX, NOX4 does not require regulatory cellular binding partners and is constitutively active. However, p22<sup>phox</sup> is required for the enzymatic activity (5, 21, 39, 123). NOX4 is not only mainly localized in the perinuclear, ER area, but it is also detected at the plasma membrane, at focal adhesions, and within the nucleus (21). NOX4 is localized to the ER in VSMCs and ECs (41, 89, 195), at both ER and nucleus in renal cortical cells (151, 164), in the nucleus in monocytes and ECs, at the plasma membrane of lung epithelial cells (188, 189), and only in mitochondria in breast tumors and adipose-derived stem cells (78, 103). There are several possibilities for these conflicting reports for NOX4 localization. Different cell types with various functions might require local ROS production at various subcellular locations. It is also possible that NOX4 localization might be transient; different stimulation might lead NOX4 to translocate from one place to others for the necessary local ROS. Indeed, HCV infection in a hepatocyte cell line increases NOX4 expression level and also increases nuclear localization (60). In experiments with primary VSMCs, NOX4 relocates from actin-based stress fibers to focal adhesions on de-differentiation (47). NOX4 has several splicing variants, which might determine localization: In the A549 lung cell line, some splicing variants are detected in the ER and others are detected at the plasma membrane (77). Alternatively, different antibodies might recognize different isoforms of the NOX4. For example, two antibodies discovered through phage-display screening share similar epitopes and neutralize NOX4 enzymatic activity, but only one antibody detects NOX4 at the plasma membrane (205).

### Role of NOX-Derived ROS in Liver Diseases

Oxidative stress contributes to a variety of liver diseases, including alcohol abuse, acetaminophen, HCV, iron overload,

chronic cholestasis, and ischemia-reperfusion (55). Small amounts of ROS are usually released by the mitochondrial respiratory chain in the liver. Liver injury can develop when excessive amounts of ROS are generated from multiple potential sources, including the mitochondrial respiratory chain, CYP family members, peroxisomes, xanthine oxidase, and NOX (55, 150). It has been reported that NOX-mediated ROS contributes to various liver diseases, including alcoholic hepatitis (107, 109), chronic hepatitis C (24, 29, 60, 183), cholestatic liver injury (157, 158, 177, 180), and hepatic fibrosis (16, 18, 58, 59, 98, 143).

The phagocytic NOX2 releases large amounts of superoxide anion in bursts in response to agonists, exhibiting no detectable constitutive activity. While the nonphagocytic NOX produce low levels of superoxide intracellularly in the basal state, which can be further stimulated by various agonists (68). NOX generates ROS in response to a wide range of stimuli, including TNF- $\alpha$ , IL-1 $\beta$ , Ang II, and leptin (42, 57). All NOX isoforms generate superoxide from molecular oxygen, and superoxide is converted to H<sub>2</sub>O<sub>2</sub> by SOD. However, the kind of ROS may vary according to specific NOX isoforms that are expressed in specific cells. For example, NOX4 generates predominantly H<sub>2</sub>O<sub>2</sub> instead of superoxide (128, 173). ROS derived from specific NOX isoforms in specific cell types in the liver may serve as diverse signaling function by inducing specific biochemical changes in their molecular targets.

### Alcoholic hepatitis

There is strong evidence that ROS play a pivotal role in alcohol-induced liver injury. Ethanol-induced oxidative stress results in a hepatocellular injury by promoting hepatocyte apoptosis or necrosis and also contributes to hepatic fibrosis by triggering the release of profibrogenic cytokines and HSCs activation (3). Ethanol induces ROS production by several enzymatic systems, including the CYP2E microsomal monooxygenase system, mitochondrial respiratory chain, and NOX (9). Both intracellular and extracellular sources of ROS contribute to alcohol-induced oxidative stress (3). Oxidative DNA damage as an index of intracellular oxidative stress is decreased in CYP2E1-deficient mice but not in p47<sup>phox</sup>-deficient mice (25). However, CYP2E1-deficient mice are not protected from early alcohol-induced liver injury (107). Four-week intragastric alcohol administration to p47<sup>phox</sup>-deficient mice results in less hepatic-free radical production and liver pathology compared with wild-type (WT) mice (109). KCs highly express phagocytic NOX2 and generate ROS during early ethanol-induced liver injury (192). Collectively, these data indicate that ROS from NOX in hepatic KCs plays a critical role in the pathogenesis of early alcohol-induced hepatitis.

The important role of NOX in the pathogenesis of alcoholic liver disease is corroborated by microarray analysis of hepatic gene expression in patients with alcoholic hepatitis (48). As expected, the expression of genes encoding ECMs such as procollagen  $\alpha$ 1(I),  $\alpha$ 4(I), fibrogenesis mediators, and inflammatory cytokines are markedly elevated in human livers with alcoholic hepatitis. In addition, various NOX components, including DUOX2 (23-fold), NOX4 (12.7-fold), DUOX1 (6.7-fold), p22<sup>phox</sup> (2.0-fold), and Rac1 (4.1-fold), are upregulated in the livers with alcoholic hepatitis compared with normal livers (48). An important finding to note is that nonphagocytic NOX components (NOX4, DUOX1, and DUOX2) are barely

detected in normal livers but are *de novo* expressed in alcoholic hepatitis (48). Expression of NOX2, NOXO1, NOXA1 are unchanged and other NOX isoforms such as NOX1, NOX3, and NOX5 are undetected. By contrast, another important ROS generator CYP2E1 is down-regulated in the livers with alcoholic hepatitis (48). Taken together, these results suggest that nonphagocytic NOX isoforms such as NOX4 and DUOXs as well as phagocytic NOX2 mediate alcohol-induced liver injury.

#### *HCV-induced hepatocellular damage*

HCV is the leading cause of mortality from chronic liver disease, including cirrhosis and HCC in many countries including Japan, Southern Europe, and the United States (66). Accumulating evidence suggests that ROS play an important role in the development and progression of chronic hepatitis C (43). HCV is a 9.6-kb positive strand RNA virus consisting of 10 genes that encode 4 structural and 6 nonstructural proteins (24). Among 10 HCV proteins, the core, NS3, and NS5a protein are associated with increased oxidative stress (16, 29, 74, 141). Recombinant NS3 protein phosphorylates p47<sup>phox</sup> and activates NOX to produce ROS, mainly superoxide, in human monocytes (29). A strong HCV-specific T-cell response, especially CD4<sup>+</sup> T-cell response, is critical for the clearance of HCV in patients with acute HCV infection (101, 182). NS3 protein induces a prolonged release of ROS *via* NOX2 in mononuclear and polymorphonuclear phagocytes, which, in turn, induces dysfunction and/or apoptosis of lymphocytes to relevance (183).

Expression of HCV proteins in hepatocytes results in increased ROS generation, steatosis, and cell transformation developing liver cancer (118, 141). HCV NS5A and core protein induce ROS and reactive nitrogen species in a human hepatocyte-derived cell line (74). Expression of HCV (genotype 1a) cDNA constructs, core protein, viral RNA, or replicating HCV (JFH-AM2) induces NOX4 mRNA expression and ROS production in human hepatocytes cell lines (24). Dominant-negative NOX4 or knockdown of NOX4 using NOX4 short hairpin RNA decreases HCV-induced superoxide production in a human hepatocyte cell line. Full-length HCV as well as structural and nonstructural HCV constructs increases ROS generation in an NOX4-dependent manner. Human NOX4 promoter/reporter assay demonstrates that HCV positively regulates both human and murine NOX4 at the transcriptional level (24). TGF- $\beta$ 1 is a major fibrogenic cytokine that activates HSCs. TGF- $\beta$  induces NOX4 in various cell types, including HSCs, hepatocytes, cardiac fibroblasts, and VSMCs (35, 53, 97, 163, 179). HCV core protein up-regulates TGF- $\beta$  mRNA in HepG2 cells, and treatment of cells with TGF- $\beta$  blocking antibody prevents the HCV core-mediated induction of NOX4 and reduces superoxide production (24). Taken together, these data indicate that HCV induces NOX4-mediated ROS generation *via* an autocrine TGF- $\beta$  dependent mechanism (24).

Huh7 cells transfected with virus producing JFH1 HCV RNA induces extracellular H<sub>2</sub>O<sub>2</sub> secretion and intracellular superoxide production (60). Both genotype 2a and 1b HCV induce NOX1 and its regulators such as NOXO1 and NOXA1 as well as NOX4 in the Huh7 human hepatocyte cell line (60). Furthermore, the increased expression of NOX1 and NOX4 proteins is demonstrated in HCV-infected human liver (60). Knockdown of either NOX1 or NOX4 using siRNA signifi-

cantly decreases H<sub>2</sub>O<sub>2</sub> and intracellular superoxide production in a hepatocyte cell line producing JFH1 HCV RNA (60). Interestingly, subcellular localization of NOX1 and NOX4 varies. NOX1 is predominantly located in the cytoplasm, and this location does not change significantly with HCV expression in Huh7 cells. While NOX4 is localized in the cytoplasm as well as in the nucleus in control Huh7 cells, HCV increases localization of NOX4 in the ER and nuclear membrane (60). Further investigation is required to understand the distinct contributory roles of NOX1 and NOX4 in HCV-induced hepatocyte injury that may result from different compartmentalization and signaling.

HCV core and NS3 proteins induce a marked increase in ROS production in human HSCs (16). These effects are attenuated by the nonspecific NOX inhibitor diphenylene iodonium (DPI). HCV core protein increases HSC proliferation in an Ras/extracellular signal-regulated kinase (ERK) and PI3K/protein kinase B (Akt)-dependent manner. In contrast, HCV NS3-NS5 protein preferentially induces proinflammatory actions such as chemokine secretion and expression of cell adhesion molecules through NF- $\kappa$ B and c-Jun-N-terminal kinase (JNK) pathways. Both HCV core and NS3-NS5 proteins increase TGF- $\beta$ 1 secretion and type I collagen expression in HSCs (16). Therefore, HCV induces liver inflammation and fibrosis, at least in part, *via* NOX-generated ROS. The NOX isoforms that are involved in HCV-induced fibrogenic responses in HSCs are not yet identified.

#### *Hydrophobic bile salts-induced liver injury*

Hydrophobic bile salts can induce hepatocytes apoptosis through a ligand-independent activation of the CD95 death receptor (132, 176). Tauroolithocholate-3-sulfate, a hydrophobic proapoptotic bile salt, stimulates ceramide formation through chloride-dependent acidification of endosomes, with subsequent activation of acidic sphingomyelinase, which, in turn, activates protein kinase C (PKC) $\zeta$  (19). Activated PKC $\zeta$  rapidly induces serine phosphorylation of p47<sup>phox</sup>, which is followed by ROS generation in hepatocytes (158). NOX-derived ROS activates JNK and an Src family kinase Yes, which then associates with and activates EGFR, followed by EGFR-catalyzed CD95 tyrosine phosphorylation, formation of the death-inducing signaling complex, and apoptosis of hepatocytes (157, 158).

Bile acids also activate EGFR in activated HSCs. In contrast to hepatocytes, bile acid-induced EGFR activation in HSCs does not induce apoptosis, but leads to cell proliferation (180). Hydrophobic bile acids induce phosphorylation of p47<sup>phox</sup> and ROS generation followed by a Yes-mediated EGFR activation, which subsequently phosphorylates ERK1/2 but not JNK and stimulates cell proliferation in quiescent HSCs (177). Treatment of cycloheximide or H<sub>2</sub>O<sub>2</sub> in addition to hydrophobic bile acid was required for the activation of JNK signal and for the shift from proliferation to apoptosis in HSCs (177).

#### **NOX-Derived ROS Play a Key Role in Hepatic Fibrogenesis**

Liver fibrosis is a common pathway resulting from chronic liver injury. Although the pathogenesis of liver fibrosis varies according to etiology of the injury, oxidative stress seems to play a crucial role in most types of hepatic fibrogenesis (14). ROS has diverse effects with regard to different kinds,

concentrations, and cell types. ROS tend to be compartmentalized in the cell and form a concentration gradient that originates from their sites of generation (20).  $H_2O_2$  can diffuse across plasma membrane and throughout the cell; however, superoxide diffuses poorly across cell membranes (30). High concentrations of ROS can be cytotoxic, whereas low concentrations of ROS may serve as second messengers during cellular responses to a variety of physiologic stimuli. A low concentration of  $H_2O_2$  has mitogenic effects and can mimic the function of growth factors (159). Regarding the effects of ROS on HSCs, contradictory results have been reported: both mitogenic and cell-death inducing properties. Nontoxic levels of ROS or lipid peroxidation products stimulate the activation, proliferation, and collagen production of HSCs, but high concentrations of ROS induce HSCs death (117, 136, 137).

Expression of various NOX isoforms is increased in patients with heart (91), lung (4), kidney (64), and liver fibrosis (48, 60). Moreover, NOX-mediated ROS is required for experimental animal models of fibrosis in various organs, including heart (53), lung (87), kidney (169), and liver (143). In HSCs, NOX mediates the fibrogenic responses in response to various agonists, including Ang II (18), PDGF (2), leptin (58), TGF- $\beta$  (97), phagocytosis of apoptotic body (203), and advanced glycation end products (81). Taken together, NOX-derived ROS is a key mediator in hepatic fibrogenesis as well as in other organs such as the heart, lung, and kidney.

#### *Upregulation of NOX components in activated HSCs*

HSCs, the major fibrogenic cell type in the liver, express multiple NOX isoforms and their regulatory components. Analysis of mRNA expression of NOX components in isolated liver cell fractions from WT C57BL/6 mice demonstrated that WT HSCs express the mRNAs for both phagocytic NOX2 and nonphagocytic NOX, including NOX1 and NOX4. HSCs also express other NOX regulatory components, including p22<sup>phox</sup>, p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, NOXO1, NOXA1, and Rac1 (143). These NOX components increase when quiescent HSCs activate into myofibroblasts. After bile duct ligation (BDL)-induced fibrosis, the mRNA expression of p47<sup>phox</sup> is strongly upregulated in HSCs to a similar degree as in KCs, whereas less upregulation occurs in other cell types such as ECs, hepatocytes in the liver (59). The mRNAs for both phagocytic and nonphagocytic NOX catalytic subunits, including NOX1, NOX2, and NOX4, are upregulated in activated HSCs compared with quiescent HSCs (7, 143).

In human quiescent HSCs, the mRNA for the cytosolic regulatory factor p47<sup>phox</sup> and the catalytic subunits NOX2 and NOX1 at cell membrane are detected at low levels, while they are highly upregulated in culture-activated HSCs and *in vivo*-activated HSCs isolated from patients with liver fibrosis (18). Although NOX plays an important role in HSCs activation, the exact structure of this complex is still unknown. All components and functionalities of the phagocytic NOX are already well established, but the interactions between the nonphagocytic NOX components in HSCs are unknown.

#### *Contribution of various NOX isoforms in hepatic fibrogenesis*

Expression of both NOX2 and NOX1 proteins is increased in the fibrotic liver in mice (143). BDL-induced liver fibrosis is attenuated in p47<sup>phox</sup>-deficient mice compared with WT mice

(18). Although the degree of protection was less, metabolically induced hepatic fibrosis by the methionine choline-deficient diet is also reduced in p47<sup>phox</sup>-deficient mice (59). Direct evidence of the contribution of NOX2 in hepatic fibrogenesis is demonstrated by the attenuated hepatic fibrosis in NOX2-deficient mice compared with WT mice after CCL<sub>4</sub> treatment or BDL (8, 98). BDL-induced hepatic fibrosis is attenuated in NOX2-deficient mice regardless of eradication of NOX2-expressing KCs by GdCl<sub>3</sub>, demonstrating that NOX2 activity in HSCs contributes to hepatic fibrosis (98). Furthermore, NOX2 is involved in the activation of HSCs by phagocytosis of apoptotic hepatocytes (33, 203). Phagocytosis of apoptotic hepatocytes induces HSC activation and collagen production *via* NOX2, and NOX2-derived ROS induces collagen  $\alpha 1$  promoter activity in HSCs (98). In addition, impaired translocation of Rac1 to the cell membrane is observed in NOX2-deficient HSCs (98). Collectively, these data indicate that phagocytic NOX2 is an important mediator of HSCs activation and hepatic fibrosis.

It is important to note that nonphagocytic NOXs, in addition to phagocytic NOX2, contributes to hepatic fibrogenesis. Mice deficient for either NOX1 or NOX2 display significantly less hepatic fibrosis compared with WT mice after CCL<sub>4</sub> injection or BDL (143). Experiments of hepatic fibrosis induction in NOX1 or NOX2 bone marrow chimeric mice demonstrated that both NOX1 and NOX2 mediate hepatic fibrosis in endogenous liver cells, including HSCs, whereas NOX2 in KCs has a lesser contribution (143). Moreover, Ang II-induced ROS generation is more severely attenuated in NOX1-deficient HSCs than in NOX2-deficient HSCs. The contribution of NOX1 in hepatic fibrogenesis was corroborated by Cui *et al.* (54). Cell proliferation is attenuated in HSCs isolated from NOX1-deficient mice. NOX1-derived ROS oxidize and inactivate phosphatase and tensin homologue (PTEN), which, in turn, phosphorylates Akt and forkhead box O 4, resulting in down-regulation of p27<sup>kip1</sup>, a cell cycle suppressor, to promote HSCs proliferation (54).

SOD1 is an enzyme that converts superoxide to  $H_2O_2$ . SOD1 also interacts with Rac1 in the active NOX complex to stimulate NOX activity. Specifically, SOD1 binds to Rac1 and stabilizes the GTP-bound active form of Rac1 (85). Mutations in SOD1, such as G93A and G37R, that are associated with hereditary ALS enhance Rac1 activation and NOX-dependent superoxide production in the spinal cord as well as in the liver (85, 129). Hepatic ROS production and Rac1 activity are increased in SOD1 G37R-mutant mice. CCL<sub>4</sub>-induced liver fibrosis is more pronounced in SOD1 G37R-mutant mice compared with WT mice (7).

GKT137831 is a potent dual NOX1/NOX4 inhibitor with an affinity similar to the irreversible, unspecific flavocytochrome inhibitor, DPI. GKT137831 has 15-fold less potency in NOX2 and 3-fold less potency in NOX5, and it has no significant inhibition in the NOX2-driven oxidative burst in neutrophils (7, 112). Inhibition of NOX1 and NOX4 by GKT137831 attenuates both CCL<sub>4</sub>-induced and BDL-induced ROS production and hepatic fibrosis in WT and SOD1 G37R-mutant mice, as well as mRNA expression of fibrogenic and NOX genes (7). GKT137831 inhibits collagen  $\alpha 1(I)$  promoter activity and ROS production induced by Ang II in WT and SOD1 G37R-mutant HSCs. The specific contribution of NOX4 in hepatic fibrogenesis is demonstrated by the attenuation of hepatic fibrosis in NOX4-deficient mice compared with WT mice (97).

Taken together, nonphagocytic NOX, including NOX1 and NOX4 as well as phagocytic NOX2, represent fundamental mediators in experimental hepatic fibrogenesis.

#### *NOX-mediated redox signaling contributing to hepatic fibrosis*

**Angiotensin II.** The RAS plays a key role in maintaining blood pressure homeostasis, as well as fluid and salt balance through coordinated effects on the heart, blood vessels, and kidneys (95). In the classic pathway of RAS, renin cleaves the liver-derived precursor peptide angiotensinogen into the decapeptide Ang I. Ang I is further hydrolyzed into the octapeptide Ang II by the angiotensin-converting enzyme (ACE), which represents the main bioactive peptide of the RAS. Angiotensinogen is the only component of the RAS that is expressed in the normal liver. However, RAS, including ACE and AT1R, are markedly upregulated in the fibrotic liver (17, 146). Inhibition of Ang II synthesis and/or the blockade of AT1R decreases hepatic inflammation and fibrosis in experimental models of chronic liver injury (52, 147, 191). Consistently CCl<sub>4</sub>- or BDL-induced hepatic fibrosis is reduced in AT1R-deficient mice compared with WT mice (100, 199). These data strongly indicate that RAS is an important mediator of hepatic fibrosis. Many of the biological effects of Ang II involve the NOX-mediated production of ROS (17, 80). Ang II is functionally linked to NOX1, NOX2, and variably to NOX4 in the vasculature, NOX2 and NOX4 in the kidney, and NOX2 in the brain (75). p47<sup>phox</sup><sup>-/-</sup> mice were the first used as a genetic model to study NOX inhibition (73, 109, 114), and p47<sup>phox</sup><sup>-/-</sup> mice show attenuated liver injury and fibrosis compared with WT counterparts after BDL (18).

Activated HSCs highly express AT1R, which mediates cell proliferation, migration, and cell contraction (15). Stimulation of HSCs with Ang II results in TGF- $\beta$  and type I collagen expression (18, 201). Ang II increases the intracellular calcium concentration and induces production of ROS, stimulating several key intracellular pathways, including PKC, PI3K/Akt, and MAPKs (18). NOX mediates the intracellular pathways induced by Ang II in HSCs (17, 18). Ang II-induced ROS formation in HSCs is inhibited by treatment with either an NOX inhibitor, DPI (108) or with the AT1R antagonist, losartan (52). Furthermore, Ang II induces the phosphorylation of p47<sup>phox</sup> through AT1R in activated HSCs. In particular, Ang II leads to an increase of intracellular ROS in HSCs isolated from WT mice, but not in p47<sup>phox</sup><sup>-/-</sup> HSCs. In addition, Ang II induces DNA synthesis and cell migration in WT HSCs, as well as phosphorylation of ERK and c-Jun, while these effects are blunted in p47<sup>phox</sup>-deficient HSCs (18). Taken together, Ang II exerts direct profibrogenic actions on HSCs through the activation of the NOX complex and the subsequent production of ROS (Fig. 2).

The molecular mechanisms of NOX activation by Ang II are complex and still incompletely understood. In VSMCs, Ang II-stimulated ROS generation is biphasic (174). The first phase occurs rapidly (peak at 30 s) and is dependent on PKC activation, which phosphorylates p47<sup>phox</sup>, and then phosphorylated p47<sup>phox</sup> translocates to the membrane where it activates the NOX catalytic subunit NOX1 and/or NOX2. The larger second phase of ROS generation (peak at 30 min) requires Rac activation by upstream c-Src, EGFR transactivation, and PI3K activation (174). Ang II may stimulate PKC-dependent NOX

activity through three different phospholipases (PLs), PLC, PLD, and PLA<sub>2</sub> (75). The prolonged production of NOX-mediated ROS by Ang II requires upregulation of NOX catalytic subunits and components (184). Indeed, Ang II induces upregulation of both NOX1 and NOX4 mRNA in HSCs (7).

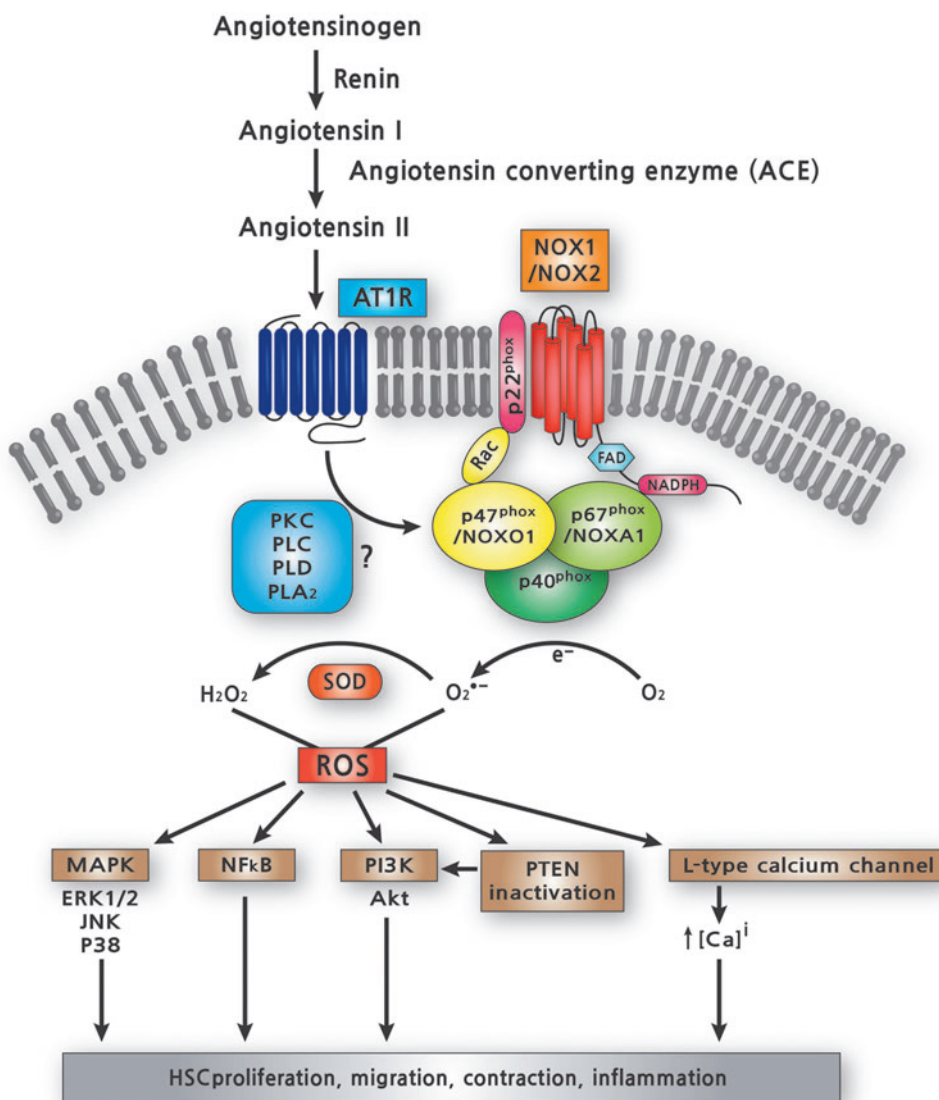
**PDGF and leptin.** PDGF, the most potent mitogen of HSCs (153), exerts its activity, in part, through NOX (2). PDGF-induced increase in DNA synthesis of HSC is reduced by pre-treatment with the anti-oxidant Mn-TBAP, and also with two different inhibitors of the NOX complex, DPI or apocynin. PDGF-induced HSCs proliferation is suppressed by an inhibitor of MEK or p38 MAPK, but the extent of suppression is greater with the p38 MAPK inhibitor (2). The experimental model of liver fibrosis induced in mice by parenteral DMN provides additional *in vivo* evidence for the role of NOX in liver fibrosis. The use of the NADPH inhibitor DPI or antioxidant drug Mn-TBAP reduces collagen deposition and liver fibrosis in DMN-treated mice (2). Similarly, PDGF increases NOX activity and DNA synthesis in pancreatic stellate cells (93). PDGF causes hypertrophy of VSMCs by induction of NOX1 through activation of PKC $\delta$  and activating transcription factor (ATF)-1 (67, 102). PDGF upregulates NOX1 in VSMCs, and an adenovirus expressing antisense NOX1 mRNA completely inhibits the early phase of superoxide production (115). A consensus activator protein (AP)-1 site was found at -98/-92 in the 5'-flanking region of the rat NOX1 gene (37). The NOX isoform that is responsible for PDGF-induced mitogenic signaling in HSCs is not yet identified. NOX is also a crucial mediator of the profibrogenic action of leptin (2, 58). Leptin-induced NOX acts downstream of Janus kinase activation but is independent of STAT3 (58). Thus, NOX is a core mediator of the fibrogenic signaling response in HSCs and demonstrates its potential as a pharmacological target for antifibrotic therapy.

**Interaction of TGF- $\beta$ /Smads signaling with NOX4.** TGF- $\beta$ 1 induces the conversion of fibroblasts to  $\alpha$ -SMA-expressing myofibroblasts in multiple organs, including the heart, lung, kidney, and liver (62, 84, 110). TGF- $\beta$ 1 is the strongest agonist for HSCs activation, and it is released from KCs and recruited macrophages with hepatic injury (14, 88). After being modified post-translationally, TGF- $\beta$ 1 binds to and activates TGF- $\beta$ 1 receptor (TGF- $\beta$ R I and II), transmembrane receptor serine/threonine kinases (50, 175). Phosphorylation of the receptors is accompanied by phosphorylation of Smad2 and Smad3 that are then complexed with Smad4. Then, activated Smad complexes enter the nucleus and initiate the transcription of TGF- $\beta$ 1 target genes. Liver fibrosis is strongly reduced in TGF- $\beta$ 1-deficient and Smad3-deficient mice, while overexpression of TGF- $\beta$ 1 induces fibrosis in several organs, including the liver (14, 88, 116, 166, 185).

A study using NOX4-deficient HSCs shows that TGF- $\beta$ 1-mediated HSCs activation is dependent on NOX4-generating ROS (97). NOX4 siRNA inhibits ROS production in HSCs. Moreover, the activation of cultured HSCs is inhibited in NOX4-deficient HSCs compared with WT HSCs (97). TGF- $\beta$ 1 increases the level of NOX4 mRNA and protein in an Smad3-dependent manner, thereby increasing NOX4-derived ROS (87, 97). NOX4 knockdown decreases HSCs activation without affecting the expression of TGF- $\beta$ 1 and its receptor and phosphorylation of Smad2, suggesting that NOX4-mediated



**FIG. 2. Ang II/AT1R/NOX signaling in HSCs.** Ang II binds to AT1R, which stimulates NOX-mediated ROS generation. The molecular mechanism of NOX activation by Ang II on HSCs is still poorly understood. Ang II may stimulate PKC-dependent NOX activity through three different PLs such as PLC, PLD, and PLA<sub>2</sub>. NOX-derived ROS activate MAPK, NF- $\kappa$ B, and PI3K, and increase intracellular calcium levels *via* L-type calcium channel in HSCs. ROS also inactivates PTEN, which, in turn, further activates PI3K/Akt pathway. As a result, NOX-derived ROS mediates cell proliferation, migration, contraction, and inflammatory response in HSCs. PLs, phospholipases; Akt, protein kinase B; Ang II, angiotensin II; AT1R, angiotensin type I receptor; PKC, protein kinase C; PTEN, phosphatase and tensin homologue; ROS, reactive oxygen species. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

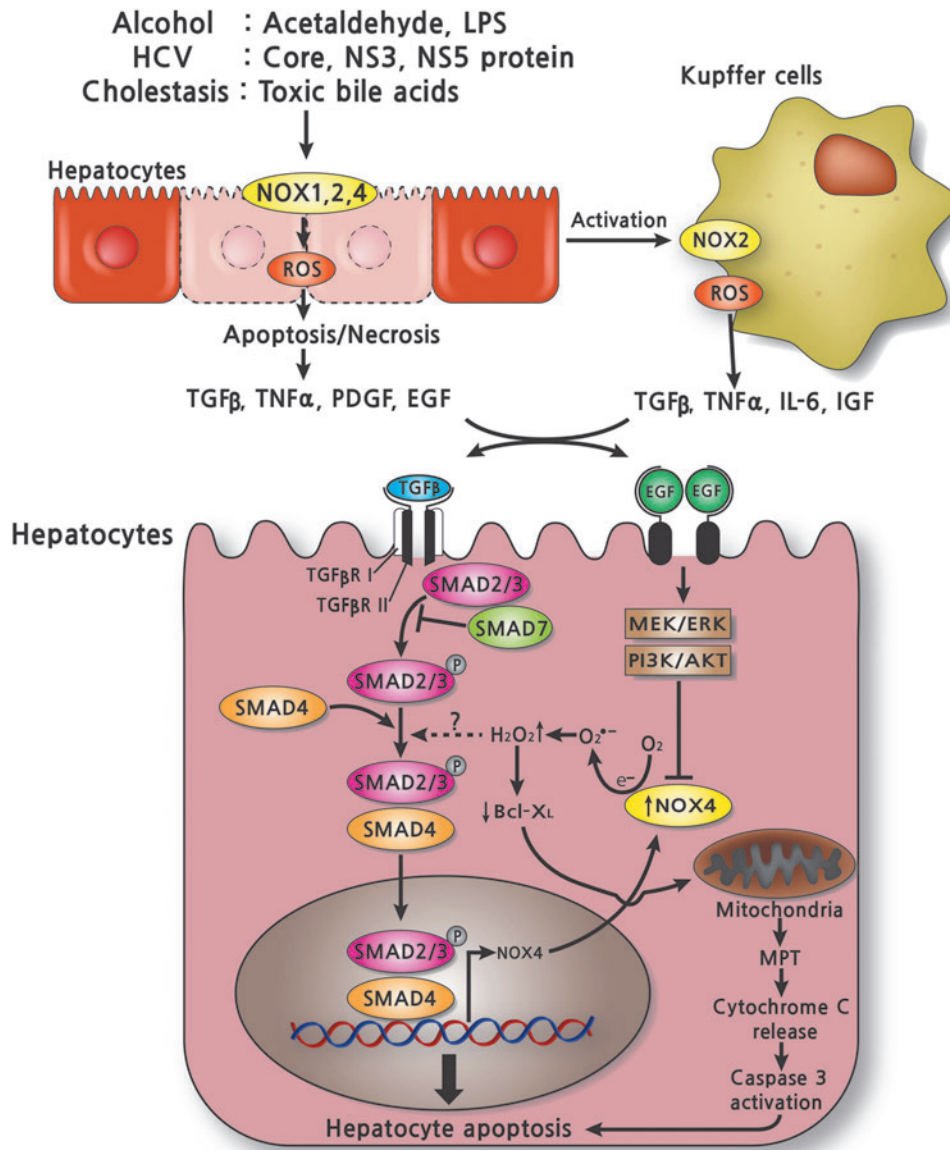


ROS is downstream of both TGF- $\beta$ 1 and the Smad complex (163). It has been reported that NOX4 acts downstream of Smad3 in kidney and lung myofibroblasts (23, 87), but upstream of Smad2/3 in cardiac myofibroblasts (53). Ang II induces upregulation of both NOX1 and NOX4 mRNA in HSCs (7). Interestingly, Ang-II-induced NOX4 mRNA up-regulation is suppressed in NOX1KO HSCs; however, NOX4 induction by TGF- $\beta$  is not suppressed in NOX1 KO HSCs. This finding suggests that NOX1 is required for NOX4 up-regulation in HSCs stimulated with Ang II, but that TGF- $\beta$  induces NOX4 independent of NOX1 in HSCs (7).

TGF- $\beta$ 1 induces apoptosis of hepatocytes (140). TGF- $\beta$  binds to and activates the TGF- $\beta$  receptor (TGF- $\beta$ R I and II), which induces phosphorylation of Smad2 and Smad3 that are then complexed with Smad4. Activated Smad complexes enter the nucleus and initiate the transcription of TGF- $\beta$  target genes, including NOX4 (35). TGF- $\beta$  induces NOX4 expression in rat and human hepatocytes. Knockdown of NOX4 using siRNA attenuates NOX activity, caspase activation, and cell death in hepatocytes, indicating that NOX4 mediates TGF- $\beta$ -induced hepatocyte apoptosis (35). NOX4-generated H<sub>2</sub>O<sub>2</sub> down-regulates Bcl-X<sub>L</sub>, a member of

the Bcl-2 family of pro-survival proteins, resulting in mitochondrial permeability transition followed by cytochrome C release, caspase 3 activation, and apoptosis of hepatocytes (90). In addition, NOX4-derived ROS upregulates two pro-apoptotic genes, Bmf and Bim (31, 156). Counteracting this effect, EGF binds to the EGFR, which, in turn, activates MEK/ERK and PI3K/Akt, which prevents TGF- $\beta$ -induced NOX4 upregulation and hepatocyte death (34) (Fig 3.). Furthermore, FasL or TNF $\alpha$ /actinomycin D-induced apoptosis is significantly reduced in NOX4-deficient hepatocytes, suggesting that NOX4 contributes to death ligand-induced apoptosis of hepatocytes (97). Since hepatocytes apoptosis may trigger KCs and HSCs activation by secreting cytokines, chemokines, and microparticles, NOX4-mediated apoptosis of hepatocytes is another mechanism contributing to hepatic fibrosis. Taken together, NOX4 contributes to hepatic fibrogenesis by the mechanism of inducing HSCs activation and mediating TGF- $\beta$ - or death ligand-induced hepatocytes apoptosis (7, 97).

NOX4 generates predominantly H<sub>2</sub>O<sub>2</sub> instead of superoxide (128, 173). Since H<sub>2</sub>O<sub>2</sub> activates protein tyrosine phosphorylation by inhibiting phosphatases and by activating



**FIG. 3. TGF- $\beta$  mediates apoptosis of hepatocytes via NOX4 upregulation.** After various kinds of liver injury, including alcohol, HCV, and toxic bile acids, ROS is produced via NOXs in hepatocytes, some hepatocytes undergo apoptosis/necrosis, and KCs get activated, releasing proinflammatory and profibrogenic cytokines such as TGF- $\beta$ . TGF- $\beta$  binds to and activates TGF- $\beta$  receptor (TGF- $\beta$ R I and II), which is accompanied by phosphorylation of Smad2 and Smad3 that are then complexed with Smad4. Then, activated Smad complexes enter the nucleus and initiate the transcription of TGF- $\beta$  target genes, including NOX4. NOX4-generated H<sub>2</sub>O<sub>2</sub> down-regulates Bcl-X<sub>L</sub>, a Bcl-2 family of pro-survival protein, resulting in mitochondrial permeability transition in mitochondria followed by cytochrome C release, caspase 3 activation, and apoptosis of hepatocytes. However, EGF binds to EGF receptor, which, in turn, activates MEK/ERK and PI3K/Akt and blocks TGF- $\beta$ -induced NOX4 upregulation and hepatocytes cell death. EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HCV, hepatitis C virus. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

kinases, regulation of H<sub>2</sub>O<sub>2</sub>-generating NOX4 might play a central role in mediating several TGF- $\beta$ 1 signaling events (11). However, the exact mechanisms of NOX4/TGF- $\beta$ -mediated fibrogenic signaling, especially what are the targets of NOX4-generating H<sub>2</sub>O<sub>2</sub>, are largely unknown.

Inflammation, LPS, and toll-like receptor signaling in hepatic fibrosis. Patients with liver fibrosis have increased intestinal permeability, which allows entry of bacterial LPS and CpG-containing DNAs into the liver where they stimulate inflammatory responses through toll-like receptors (TLRs) (171, 198). All TLRs consist of an N-terminal extracellular domain containing leucine-rich repeats and a C-terminal conserved cytosolic domain. While the extracellular domain of TLRs recognizes the corresponding ligands having either pathogen-associated molecular pattern or damage-associated molecular pattern, the cytosolic domain mediates downstream signal transduction to produce interferon (IFN)- $\alpha$ / $\beta$  or proinflammatory cytokines (6, 171). The inflammatory

cytokines recruit and activate KCs to release TGF- $\beta$ 1, which activate HSCs.

Both quiescent and activated HSCs express the mRNAs of multiple TLRs (190). However, HSCs respond weakly to TLR2 ligands (144, 172). TLR3 ligands are able to stimulate HSCs for IFN- $\beta$  production, but not TLR4 ligands, while macrophages are responsive to both ligands for IFN- $\beta$  production (190). Instead, TLR4 ligands on HSCs induce NF- $\kappa$ B and JNK/AP-1 activation to produce proinflammatory cytokines (145). On binding of LPS to TLR4 with the aid of CD14 and MD2, TLR4 homodimerizes and transmits signals through MyD88 and TIRAP. Then, MyD88 recruits IL-1R-associated kinase (IRAK)-1/-4, and activates NF- $\kappa$ B through the TRAF6/TAK1/TAB2 complex (6, 171). Activated NF- $\kappa$ B enters the nucleus and drives the transcription of various chemokines (MCP-1, MIP-1 $\alpha$ , RANTES, and IL-8) and adhesion molecules (ICAM-1 and VCAM-1). Then, those molecules facilitate the recruitment of KCs and circulating macrophages to the site of HSCs, where KCs and recruited macrophages release TGF- $\beta$ 1

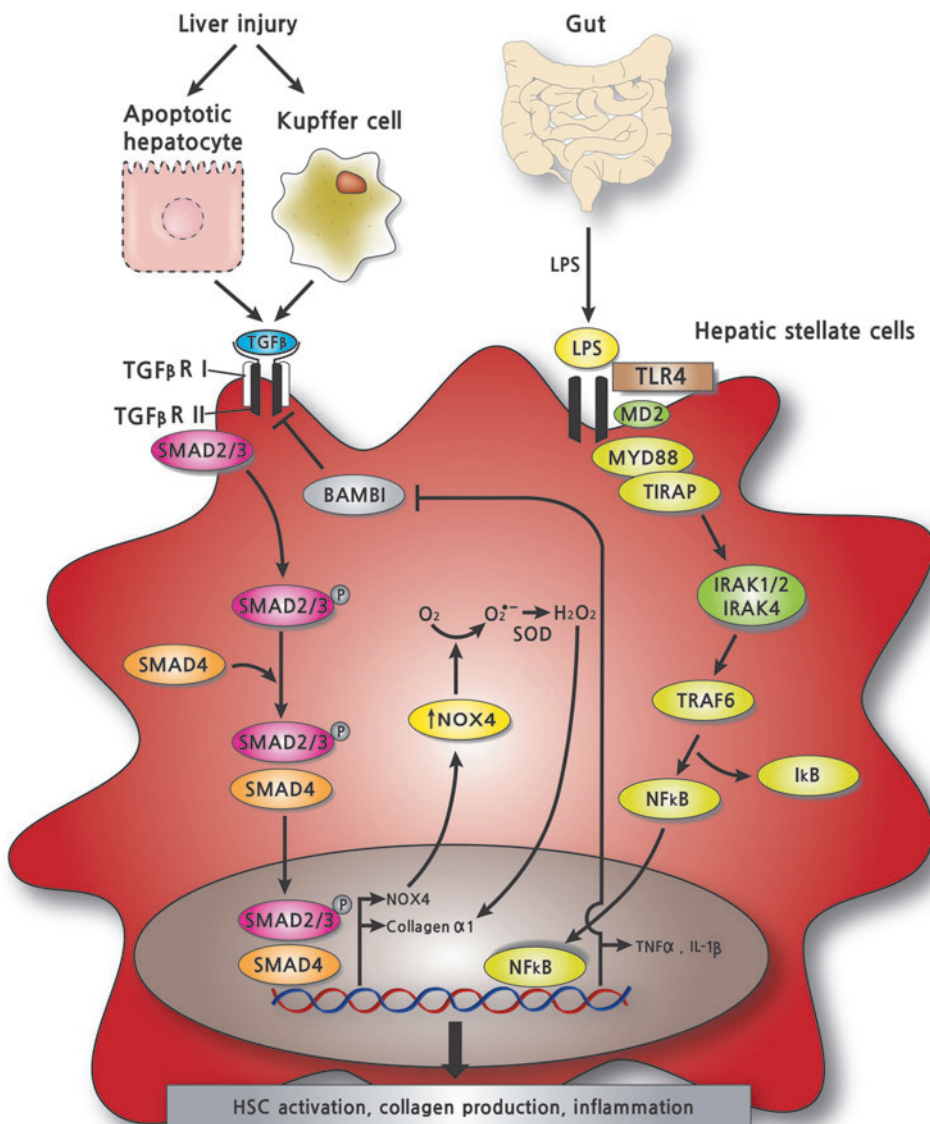
to activate HSCs (6, 198). In fact, antibiotic treatment to reduce the gut microflora attenuates liver fibrosis, indicating the essential role of bacterial products in hepatic fibrogenesis, and TLR4-mutant mice and MyD88-deficient mice are resistant to fibrosis (172).

HSCs containing a collagen promoter-driven GFP reporter (Col1-GFP) show weak reporter activity on TGF- $\beta$ 1 stimulation, but much better reporter activity on pretreatment with LPS, suggesting that LPS-TLR4 signaling primes HSCs for TGF- $\beta$ 1 signaling (172). In addition, coculture studies of TLR4-mutant KCs with Col1-GFP containing HSCs show that LPS stimulation does not enhance TGF- $\beta$ 1-mediated reporter activity, suggesting that KCs are not responsible for LPS-TLR4-mediated HSCs activation. Indeed, two TLR4 SNP mutants, T399I and D299G, reduce not only NF- $\kappa$ B activity and chemokine production, but also HSCs activation (82). Gene expression analysis identified bone morphogenetic protein and activin membrane-bound inhibitor (Bambi) as a key factor for LPS-TLR4-mediated enhancement of HSCs activation by TGF- $\beta$ 1 (172). HSCs, but not KCs and hepatocytes,

express high levels of Bambi that binds TGF- $\beta$ 1 receptor and inhibits Smad3 phosphorylation on TGF- $\beta$ 1 stimuli (142, 172, 197). LPS-TLR4 in HSCs activates NF- $\kappa$ B in an MyD88-TIRAP-dependent manner, activates NF- $\kappa$ B, and down-regulates Bambi, thereby rendering HSCs ready for TGF- $\beta$ 1-mediated activation (Fig. 4) (172).

Little work has been reported on the role that NOX or ROS might play in LPS-TLR4 mediated inflammatory or fibrogenic signaling in HSCs. LPS induces the expression of NOX1 through TLR4 in macrophages, suggesting that the interaction between LPS/TLR4 signaling and NOX1 might modulate fibrogenic response in the liver (104). In neutrophils, antioxidant treatment reduces LPS-stimulated nuclear localization of NF- $\kappa$ B (10). In macrophages, NOX inhibitors (DPI and apocynin) or siRNA against p22<sup>phox</sup> greatly diminish LPS-TLR4-mediated X-box-binding protein 1 splicing, a marker of ER stress, suggesting that ROS plays an important role in LPS-TLR4 signaling (127). Moreover, yeast-two hybrid assay and GST-pull down assay in HEK 293 cells demonstrates that the C-terminal domain of NOX4 interacts with the C-terminal

**FIG. 4. Interactions between TGF- $\beta$ -NOX4 and LPS-TLR4-mediated profibrogenic signaling in HSCs.** TGF- $\beta$  released from injured hepatocytes and activated KCs binds to TGF- $\beta$  receptor, which, in turn, upregulates NOX4 in an Smad2/3-dependent manner in HSCs. NOX4-generated H<sub>2</sub>O<sub>2</sub> activates collagen  $\alpha$ 1 promoter to produce type I collagen in HSCs promoting hepatic fibrosis. Gut-derived LPS binds TLR4 and activates NF- $\kappa$ B via MyD88/TIRAP/IRAKs/TRAF6 pathway, which, in turn, suppresses BAMBI, a TGF- $\beta$  inhibitor, in HSCs. LPS/TLR4/MyD88/NF- $\kappa$ B signaling further propagates TGF- $\beta$ /NOX4-mediated profibrogenic redox signaling via down-regulation of BAMBI in HSCs. TLR, toll-like receptor; IRAK, IL-1R-associated kinase. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)



cytosolic domain of TLR4 (149). In human aortic ECs, overexpression of the C-terminal region of NOX4 inhibits LPS-mediated NF- $\kappa$ B activation, *via* reduced ROS production (148). Moreover, siRNA of NOX4 decreases adhesion molecule (ICAM-1) and chemokines (IL-8 and MCP-1) by LPS stimulation, suggesting that LPS-TLR4 signaling induces NF- $\kappa$ B activation and inflammatory responses through NOX4-derived ROS in human aortic ECs (148). Interestingly, mouse renal tubule epithelial cells express a splicing variant of NOX4 (28 kDa NOX4) more than full-length 65 kDa NOX4, and the 28-kDa NOX4 interacts with TLR4 in co-immunoprecipitation (22). Therefore, future studies are needed to determine the role of NOX4-derived ROS in mediating the LPS-TLR4-NF- $\kappa$ B cascade in HSCs activation, perhaps through a direct interaction between NOX4 and TLR4.

#### *Therapeutic strategies and future direction of NOX-mediated hepatic fibrosis*

Based on the knowledge of the pathogenic role of ROS in hepatic fibrogenesis, clinical trials of antifibrotic therapy targeting oxidative stress using antioxidants have been attempted in several kinds of chronic liver disease. However, treatment using agents with nonspecific antioxidant actions such as vitamin E, vitamin C, polyenyl phosphatidyl choline, or UDCA failed to demonstrate antifibrotic efficacy in patients with various kinds of chronic liver disease, including alcoholic hepatitis (121), NASH (122, 165), and chronic hepatitis C (202). Moreover, high doses of vitamin E supplementation ( $\geq 400$  IU/day) may increase all-cause mortality (130). Perhaps more potent and specific inhibitors of oxidative stress may be required for the development of antifibrotic agents.

The multiple lines of evidence demonstrating the critical role of NOX in hepatic fibrogenesis provide a strong rationale for the use of pharmacological inhibitors of NOX to treat patients with chronic liver disease. Ang II blocking agents are the first such drugs that have been assessed by several investigators. Antifibrotic effects of angiotensin type 1 receptor (AT1-R) blocker (ARB) are reported in several retrospective or small pilot studies in patients with chronic hepatitis C (51, 160, 178) and NASH (200). The effects of losartan, an ARB, on liver fibrosis in patients with chronic hepatitis C were prospectively investigated (49). Baseline biopsies showed that the degree of fibrosis was F2 in seven patients (50%), F3 in four patients (29%), and F4 in three patients (21%). After 18 months of oral losartan 50 mg/day treatment, the degree of fibrosis decreased in seven patients (50%), remained unchanged in three patients (21%), and progressed in four patients (29%). Furthermore, losartan treatment was associated with a significant decrease in the expression of several fibrogenic genes, including procollagen  $\alpha 1(I)$  and  $\alpha 1(IV)$ , urokinase type plasminogen activator, metalloproteinase type 2, and NOX genes, including NOXO1, NOXA1, and Rac1 (49). The demonstration that AT1R blockade is associated with a decrease in procollagen  $\alpha 1(I)$  suggests that losartan inhibits collagen synthesis in patients with chronic hepatitis C. Since prolonged hepatic necroinflammation results in fibrosis, the anti-inflammatory effect of losartan may also contribute to the reduction of hepatic fibrosis. Recently, a randomized controlled study by Kim *et al.* reported that candesartan, an ARB, decreases hepatic fibrosis as well as hepatic expression of

NOX components such as p22<sup>phox</sup> and Rac1 in patients with alcoholic liver disease (105).

In contrast, long-term administration of angiotensin-converting enzyme inhibitor (ACEi) or ARB did not retard the progression of hepatic fibrosis in patients with chronic hepatitis C who were enrolled in the HALT-C cohort (1). Specifically, fibrosis progression occurred in 33.3% of the ACEi/ARB group, 32.5% of the other antihypertensive medications group, and in 25.7% of subjects taking no antihypertensive medications among the HALT-C cohort. Cholongitas *et al.* investigated the effect of ARB use in a cohort of 102 consecutive hypertensive patients with recurrent HCV after liver transplantation. Administration of an ARB was associated with less progression in inflammation but not in fibrosis (45).

These conflicting results suggest the possibility that ARB's antifibrotic efficacy may vary according to the etiology of the chronic liver disease or that alone are insufficient to block the multiple fibrogenic pathways. The extent of hepatic fibrosis may be an important factor that affects anti-fibrotic effects of ARB. ARB may exert the maximal anti-fibrogenic effects early in the fibrogenic process, and this effect may diminish at a later stage of fibrosis (1, 181). Further well-designed, large-scale clinical studies are required to determine the antifibrotic efficacy of ARB in the specific setting of chronic liver disease. A clinical study is underway to examine the efficacy of irbesartan on the progression of liver fibrosis in adult patients with chronic hepatitis C (ClinicalTrials.gov NCT00265642).

The development of novel pharmacological NOX inhibitors is being assessed as potential anti-fibrotic therapeutics for hepatic fibrosis. Detailed information of these NOX inhibitors has been provided in comprehensive review articles (46, 65, 96). Recently, a small molecule NOX1/NOX4 dual inhibitor was developed by GenKyoTex (Geneva, Switzerland) (112). As previously mentioned, the inhibition of both NOX1 and NOX4 by GKT137831 attenuates CCl<sub>4</sub>- or BDL-induced ROS production and hepatic fibrosis in mice (7, 97). GKT137831 inhibits collagen  $\alpha 1(I)$  promoter activity, and it suppresses TGF- $\beta$ ,  $\alpha$ SMA, and ROS production in HSCs. NOX4 also mediates lung myofibroblasts activation and fibrogenic responses in lung (87). GKT137831 attenuates hypoxia-induced H<sub>2</sub>O<sub>2</sub> release, proliferation, and TGF- $\beta 1$  expression in human pulmonary artery endothelial or smooth muscle cells (79). In 2010, GKT137831 was granted orphan drug status for the treatment of idiopathic pulmonary fibrosis by the European Commission (86). In phase I clinical study, GKT137831 was found to be safe and well tolerated when administered orally to a total of 72 healthy adult men, at single doses of approximately 1800 mg OD, and at multiple doses of approximately 900 mg OD for 10 days. The results of animal study and phase I clinical study warrant a clinical trial of GKT137831 to investigate antifibrotic efficacy in the treatment of hepatic fibrosis.

When considering NOX inhibition as a therapeutic strategy for hepatic fibrosis, the collateral inhibition of normal physiological function of NOX should be also considered. For example, the protective role of NOX4 in vasculature from ischemic or inflammatory injury was reported (168). Loss of NOX4 results in the reduction of endothelial nitric oxide synthase expression, nitric oxide production, and heme oxygenase-1 expression, which was associated with apoptosis and inflammatory activation in vasculature (168). Since the liver comprises multiple cell types that express multiple NOX

isoforms, inhibition of specific NOX isoforms may disturb the normal physiological role. A better understanding of the components and functions of specific NOX isoforms in specific cell types and interactions of NOX signaling between cell types in the liver may answer these unresolved questions.

### Conclusions

NOX-generated ROS is implicated in the pathogenesis of various kinds of chronic liver disease. A growing body of literature indicates that the excessive amount of ROS generated by NOX plays a pivotal role in hepatic fibrogenesis. Importantly, nonphagocytic NOXs, including NOX1 and NOX4 as well as phagocytic NOX2 in HSCs, mediate hepatic fibrosis. Of note, NOX4-mediated ROS generation is related to TGF- $\beta$ /Smads signaling, critical profibrogenic signaling pathways in the liver. Various NOX isoforms expressed in cell types comprising the liver such as KCs, ECs, hepatocytes, and infiltrating leukocytes also seem to be involved in hepatic fibrogenesis. However, identification and regulation of the NOX isoforms in specific cell types in the liver are still largely unknown. Targeting specific NOX isoforms such as NOX1 and/or NOX4 may be promising to treat hepatic fibrosis, while escaping the collateral suppression of host defence caused by NOX2 inhibition. A better understanding about NOX-mediated fibrogenic signaling may enable the development of novel anti-fibrotic therapy using NOX inhibition strategy.

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#### Abbreviations Used

ACE = angiotensin converting enzyme  
 ACEi = angiotensin-converting enzyme inhibitor  
 Akt = protein kinase B  
 ALS = amyotrophic lateral sclerosis  
 Ang II = angiotensin II  
 AP = activator protein

ARB = angiotensin receptor blocker  
 AT1R = angiotensin type I receptor  
 ATF = activating transcription factor  
 BDL = bile duct ligation  
 CGD = chronic granulomatous disease  
 CYP = cytochrome p450  
 DPI = diphenylene iodonium  
 ECM = extracellular matrix  
 ECs = endothelial cells  
 EGF = epidermal growth factor  
 EGFR = epidermal growth factor receptor  
 ER = endoplasmic reticulum  
 ERK = extracellular signal-regulated kinase  
 H<sub>2</sub>O<sub>2</sub> = hydrogen peroxide  
 HBV = hepatitis B virus  
 HCC = hepatocellular carcinoma  
 HCV = hepatitis C virus  
 HSCs = hepatic stellate cells  
 IFN = interferon  
 IRAK = IL-1R-associated kinase  
 JNK = c-Jun-N-terminal kinase  
 KCs = Kupffer cells  
 NADPH = nicotinamide adenine dinucleotide phosphate  
 NASH = nonalcoholic steatohepatitis  
 NOX = NADPH oxidase  
 PKC = protein kinase C  
 PLs = phospholipases  
 PTEN = phosphatase and tensin homologue  
 RAS = renin-angiotensin system  
 ROS = reactive oxygen species  
 SOD = superoxide dismutase  
 TLR = toll-like receptor  
 VSMCs = vascular smooth muscle cells  
 WT = wild-type