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NADPH Oxidases in Chronic Liver Diseases

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Abstract

Oxidative stress is a common feature observed in a wide spectrum of chronic liver diseases including viral hepatitis, alcoholic, and nonalcoholic steatohepatitis. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) are emerging as major sources of reactive oxygen species (ROS). Several major isoforms are expressed in the liver, including NOX1, NOX2, and NOX4. While the phagocytic NOX2 has been known to play an important role in Kupffer cell and neutrophil phagocytic activity and inflammation, the nonphagocytic NOX homologues are increasingly recognized as key enzymes in oxidative injury and wound healing. In this review, we will summarize the current advances in knowledge on the regulatory pathways of NOX activation, their cellular distribution, and their role in the modulation of redox signaling in liver diseases.

1. Introduction

In chronic liver diseases, hepatocyte injury triggers Kupffer cell activation and hepatic stellate cell (HSC) transdifferentiation to matrix-producing myofibroblasts, and the accumulation of extracellular matrix leads to fibrosis and cirrhosis [1, 2]. Most if not all pathogenic insults in the liver can cause oxidative stress, inducing lipid peroxidation, protein oxidation, and DNA damage, leading to hepatocyte mitochondrial dysfunction, amplifying inflammation and initiating fibrosis [3]. As important second messengers, ROS can have an impact on cell death/survival pathways [4]. In the liver there are several important sources of ROS production. While the role of cytochrome P4502E1 (CYP2E1), the mitochondrial respiratory chain, arachidonic acid oxidation, and the xanthine oxidase system have been extensively studied in the past [5–7], recently the group of NOX enzymes have been emerging as major sources of ROS production. H_2O_2 is one of the main oxidative radicals during liver diseases and it is generated by the NADPH oxidases or complex III of the mitochondrial respiratory chain. In physiological situations the amount of H_2O_2 is under a tight control by the peroxiredoxins and glutathione peroxidases as well as by catalase [8]. During chronic liver injury however this balance is perturbed and parenchymal cells are

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Conflict of Interests

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exposed to increasing concentrations of ROS. Among the seven NOX homologues found in mammals (NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2), the main ROS-producing NOXs in the liver are NOX1, NOX2, and NOX4 [9] (Table 1). NOX1 and 2 are mainly producing superoxide whereas NOX4 directly produces H_2O_2. To form active complexes, NOX1 and NOX2 bind with their structural subunits: NOX1 associates with p22phox, p47phox, p67phox, and active Rac (or NOXO1 and NOXA1, the homologues of p47phox and p67phox); besides these subunits, NOX2 activation also requires p40phox [10]. NOX4 is a constitutively active membrane-bound isoform and according to current knowledge associates with p22phox, only [10] (Figure 1). Thus interaction between the subunits is an important determinant of enzyme activity [11].

In general, to maintain homeostasis free radicals are scavenged by a powerful antioxidant system in the liver composed of enzymatic and nonenzymatic molecules. The former group consists of superoxide dismutases (SOD), catalase, and enzymes regulating glutathione (GSH) synthesis. The nonenzymatic antioxidants include the coenzyme Q10, GSH, and ROS binding proteins such as thioredoxin (Trx) [12–14]. In response to oxidative stress initially there is a rapid induction of antioxidant signaling. The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is a master regulator inducing the transcription of a spectrum of genes related to GSH metabolism *via* the antioxidant responsive element (ARE) on the target genes and is also playing a role in xenobiotic detoxification and proteome maintenance [15]. Another group of transcription factors the Forkhead box O (FOXO) regulate the SOD and catalase transcripts [16, 17]. However, after prolonged chronic injury, the antioxidant responses gradually fail leading to the unopposed actions of ROS inflammation and fibrogenesis.

2. Hepatitis C

The role of NOXs in the pathogenesis of HCV has been increasingly recognized involving both the dysregulation of T-cell responsiveness and hepatocyte injury. The NS3 viral protein was shown to induce a significant ROS production in monocytes which coincided with p47phox phosphorylation and translocation [31]. The NS3-activated phagocytes, in turn, could induce the dysfunction of CD3+/56− T cells, CD3−/56+ natural killer (NK) cells, and CD3+/56+ NKT cells [32]. HCV could also promote the recruitment of CD33+ myeloid-derived suppressor cells leading to the NOX2/ROS-mediated suppression of T-cell responsiveness thus leading to persistent HCV infection [26]. The nonphagocytic NOXs were shown to be instrumental in HCV-induced oxidative stress. In hepatocytes, HCV proteins directly induced NOX4 expression through an autocrine TGFβ-dependent mechanism [21]. In a study by de Mochel et al., both NOX1 and 4 were localized in the nucleus of HCV-infected hepatocytes and were responsible for nuclear ROS production and nitrotyrosine generation [33]. The induction of NOX4 was believed to be mediated by TGFβ/Smad signaling [21, 33]. In patients with HCV, using losartan “an AT1 receptor blocker” the expression of NOX activator 1 (NOXA-1) and organizer 1 (NOXO-1), Rac-1 and fibrogenic genes were downregulated [34].

3. Alcoholic and Nonalcoholic Steatohepatitis (ASH and NASH)

Oxidative stress plays a key role in both ASH and NASH [35]. The phagocytic NOX in Kupffer cells is thought to be essential in the pathogenesis of early alcohol-induced hepatitis by activating NF-κB, which in turn activates the production of cytotoxic TNα [36, 37]. This was later also confirmed in the studies of Cubero and Nieto where chronic ethanol administration and arachidonic acid (AA) synergistically mediated Kupffer cell activation *via* an NADPH oxidase-dependent mechanism [38]. In a different study, chronic ethanol feeding increased the LPS-stimulated NADPH oxidase-dependent production of ROS in Kupffer cells, and ERK1/2 was an important target of ROS leading to an enhanced LPS-stimulated TNα production [39]. In hepatocytes the p47phox subunit containing NOXs seemed to play an important role in alcoholic steatohepatitis with a mechanism involving the lipid droplet-stabilizing protein, adipocyte differentiation-related protein (ADRP), and the fatty acid synthesis-associated genes, fatty acid synthase (FASN), and acetyl-CoA carboxylase (ACACA) [40]. NOXs and HIF-1α were shown to be involved in the alcohol-mediated induction of endothelin-1 (ET-1) in the liver sinusoidal endothelial cells (LSEC). This effect was attenuated by transfection of the p47phox siRNA suggestive of the activation of either NOX1 or 2 in these cells [41].

There are several lines of evidence suggesting that NOXs are also significant sources of oxidative radicals in NASH [42, 43]. Metabolic syndrome and diabetes mellitus are amongst the strongest risk factors for progressive NASH leading to liver fibrosis and cirrhosis. Persistent hyperglycemia can stimulate HSC to proliferate and produce extracellular matrix through activation of NOXs [43–45]. Increased NOX activity was observed in *fa/fa* rats on high fat diet and this was correlated to an increase in lipid peroxidation [46]. However, it has not been fully understood as to which NOX homologue is crucial to disease progression in ASH/NASH. Activation of the phagocytic NOX2 in Kupffer cells, for example, is an important determinant of leptin-mediated production of oxidative radicals and macrophage activation [47]. Leptin-mediated induction of NADPH oxidase activity in HSC is also an important factor in fibrosis progression in NASH [48, 49], and leptin was shown to induce collagen 1α(I) by a NOX and Sp1- and Sp3-mediated pathway [50]. Advanced glycation end-products that accumulate in patients with diabetes were described to have a major contribution to tissue injury by the activation of the receptor for AGEs (RAGE) and subsequent release of ROS [23, 51]. We recently showed that RAGE activation is directly linked to the activation of NOX2 in the liver causing a downregulation of TIMP3 levels and hence an unopposed increase in TACE and TNα activity leading to increased injury and fibrosis in two different dietary models of NASH [24]. The $NOX2^{-/-}$ mice in this study developed attenuated fibrosis but steatosis was unchanged consistent with the findings in the $p47phox^{-/-}$ and $NOX2^{-/-}$ mice on MCD diet [25, 52], suggesting that NOX2 regulates fibrosis but not steatosis. The dominant NOX homologue in hepatocytes responsible for redox stress signaling in NASH remains to be identified. As hepatocyte cell death is a major propelling factor for fibrogenesis in NASH [53], it is very plausible that one or several NOX homologues play a major role in hepatocyte ER stress. For example, CD95L was shown to induce oxidative stress in hepatocytes *via* NOX1 or 2 involving ceramide and PKCζ [27]. At the same time NOX4 activation in hepatocytes was shown to be proapoptotic in different conditions [28, 29, 54] and thus likely to contribute to NASH progression. It is also

important to note that NOXs could potentially confer an individual sensitivity to metabolic syndrome and NASH by polymorphisms associated with ROS generation. Recently it was shown that a single nucleotide polymorphism, rs1836882 in the NOX4 gene's promoter region, was associated higher caloric intake and ROS levels [22].

4. Liver Fibrosis

Liver fibrosis is a result of a complex interplay between several cell types during chronic liver injury leading to HSC activation and deposition of scar tissue. It is more and more recognized that NOXs play a significant role in this process. The key role of NOX2 activation in macrophages has been explored by Paik et al. [18]. Using NOX2 bone marrow (BM) chimeric mice, they showed that the $NOX2^{-/-}$ BM cells significantly reduced fibrosis in wt. recipient mice treated with CCl4 [18]. HSC also express functional NOX2, and the enzyme could be activated by the phagocytosis of apoptotic hepatocytes triggering the upregulation of αSMA, collagen I, and TGFβ as shown by our group [20]. NOX2-derived ROS could directly activate collagen I transcription in HSC as the promoter activity was significantly suppressed in $NOX2^{-/-}$ cells or cells transfected with truncated promoter constructs, lacking the ROS-responsive area. Consistently, $NOX2^{-/-}$ mice showed attenuated fibrosis in response to bile duct ligation (BDL) [20] or CCl4 injection [18].

Hepatocytes and sinusoidal endothelial also express all NOX2 subunits; however, the mechanism of enzyme activation or its functional relevance in these cells is yet to be determined.

NOX1 and NOX4 are widely expressed in the liver, mainly by hepatocytes, HSC, and endothelial cells [55]. NOX1 was upregulated in fibrotic livers and in active HSC, and $NOX1^{-/-}$ mice developed attenuated fibrosis after CCl4 injection or BDL [18, 19]. NOX1 could be induced by PDGF leading to HSC proliferation [19]. Angiotensin II is also a potent inducer of ROS production *via* NOXs, and liver fibrosis and the expression of procollagen α1(I), TGFβ, and Timp1 were attenuated in the $p47phox^{-/-}$ mice [56]. Later, it was shown that both NOX1 and NOX2 were involved in angiotensin II signaling as HSC deficient in these NOXs to upregulate collagen α1(I) and TGFβ in response to angiotensin II [18]. Interestingly, recently it was found that CCN1, a matricellular protein secreted by hepatocytes could engage integrin α6d835defd1 to induce ROS accumulation in HSC and portal fibroblasts through the Rac1-NOX1 complex regulating HSC senescence [30]. Thus the role of NOX1 may be context or disease stage dependent, and further studies would be required to determine the regulation of its function during disease progression.

Different from the other NOXs, NOX4 is constitutively active associated with p22phox while other regulatory subunits are not known to be required [57]. NOX4 is induced in patients with HCV [28] and NASH (unpublished observations). TGFβ-mediated NOX4 activation in hepatocytes is proapoptotic [58] and is required for FasL or TNα-mediated apoptosis [29], therefore indirectly contributing to fibrogenesis. NOX4 is increasingly induced in HSC during culture-activation, and $NOX4^{-/-}$ cells have blunted collagen I production [28, 29].

So far, all examined NOXs displayed profibrogenic activity both *in vitro* and *in vivo*. Further studies are required, however, to determine whether there is a functional redundancy among the different NOX homologues, disease stage-specific function, or whether there is a compensatory increase (or decrease) in their function in the different knockout models. These data will be required to design adequate antifibrotic therapies that have no significant off-target effects. To date, a dual NOX4/1 inhibitor GKT137831 has shown promise in several studies [29, 59]. Using this inhibitor Aoyama and colleagues demonstrated a protective effect in CCl4-treated SOD1 active mutant transgenic mice that developed exacerbated fibrosis [59]. In the BDL model of fibrosis mice in both the preventive and treatment arm displayed significantly improved liver injury and fibrosis [29]. Our unpublished data also showed that GKT137831 improved fibrosis in mice fed with NASH-inducing diets.

5. HCC

The role of different NOX homologues in the development of hepatocellular carcinoma (HCC) is still under investigation. Recent evidence suggests that NOX4 may be protective in HCC, as TGF-β-induced liver tumor cell senescence was NOX4-dependent [60]. NOX4 expression was suppressed in human and mouse HCC. Silencing NOX4 in immortalized HCC cells promoted proliferation and xenograft experiments in athymic mice indicating that NOX4 silencing conferred an advantage to HCC growth, resulting in earlier onset of tumor formation and increase in tumor size [61]. In a different study the tumor suppressor $STAT5^{-/-}$ mice developed severe hepatic steatosis as well as hepatocellular carcinomas by 17 months of age, and the authors showed that one of the target genes of STAT5 was NOX4. STAT5-induced activation of the proapoptotic proteins Puma and Bim was dependent on NOX4 [54].

On the other hand, NOX1 is considered as a survival signal in tumor cells. The epidermal growth factor receptor-(EGFR-) mediated HCC cell growth was mediated by NOX1 and p38/Akt activation. NOX1 siRNA inhibited the expression of EGFR, TGFα, and consequently cell proliferation in an autocrine manner [62]. The tumor suppressor HACE1 (HECT domain and Ankyrin repeat Containing E3 ubiquitin-protein ligase 1) ubiquitinates and degrades active Rac1 thereby controlling Rac1-dependent NADPH oxidase activation. HACE1 deletion caused chronic oxidative stress, DNA damage, and enhanced tumor growth, and the effects of HACE1 deficiency were reversed in $NOX1^{-/-}$ cells [63]. Overall, it is still early to postulate whether different NOXs have differential effects on HCC tumor initiation, proliferation, or metastatic capacity. Further *in vivo* studies using inducible knockouts may be needed to advance our knowledge on the role of NOXs in hepatocarcinogenesis. The prolonged use of potential fibrosis inhibitors such as NO1/4, however, may require caution in late-stage liver diseases.

6. Antioxidant Signals

Glutathione (GSH), the nonprotein ROS scavenger, plays a major protective role in liver injury [13]. The amount of GSH is tightly related to the cellular oxidative homeostasis, which is mediated by a transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). As a part of defensive response to liver injury, Nrf2 dissociates from its inhibitory regulator

Kelch-like ECH associating protein 1 (Keap1) in response to oxidative stress and translocates to nucleus to induce the transcription of antioxidant defense genes GSH synthetases (Gclc and Gclm), glutathione-S-transferase (Gst), GSH peroxidase (GPx), GSH reductase (GR), and NAD(P)H quinone oxidoreductase 1 (Nqo1), [15, 64]. Nrf2 has been shown to be protective against liver injury induced by multiple pathogenic stimuli [65]. Cell line studies demonstrated that Nrf2 was induced by hepatitis B and hepatitis C; and knockdown Nrf2 by siRNA lowered the cell survival rate [66, 67]. Studies on Nrf2 null mice and Keap1 knockout mice proved that Nrf2 was activated in chronic alcohol consumption by CYP2E1, and that it played a protective role [68, 69]. Nrf2 was shown to be in acute liver injury induced by various hepatotoxicants including CCl4, ethanol, and acetaminophen [70]. Nrf2 knockout mice were more sensitive to diet-induced steatosis, inflammation, and fibrosis [71, 72]; and Nrf2 pharmaceutical activators were beneficial to rats on high fat diet [73]. However, in transgenic mice expressing constitutively activated Nrf2, no protective effects were found against CCl4-induced liver injury and fibrosis and regeneration was impaired, too. This was thought to be due to the induction of cyclin-dependent kinase inhibitor P15 and proapoptotic protein Bim [74]. A recent study in lung fibrosis demonstrated the importance of NOX4-Nrf2 balance in redox homeostasis. Comparing young and aged mice NOX4 in aging mice had an impaired ability to induce Nrf2 responses and were more prone to develop persistent fibrosis [75].

The Forkhead box gene, group O (FOXO) family of transcription factors are important metabolic and antioxidant regulators and are known to play a complex role in liver diseases [76]. Their activity is tightly regulated by posttranslational modifications, including phosphorylation or acetylation [77]. It is well established that activation of FOXO transcription factors reduces the level of oxidative stress by the induction of enzymes that breakdown ROS such as MnSOD and catalase [78]. FOXO1 can directly induce MnSOD in HSC and inhibit HSC proliferation, and the FoxO1 (+/−) mice were more susceptible to the BDL-induced fibrosis [79]. FOXO1 is also a direct transcriptional regulator of gluconeogenesis by inducing the phosphoenolpyruvate carboxykinase (PEPCK)/glucose 6-phosphate pathway and disrupting mitochondrial metabolism and lipid metabolism *via* heme oxygenase 1/sirtuin 1/Pparγclα pathway, thereby likely playing a significant role in NASH [76, 80, 81]. FOXO3 were shown to regulate the innate immune signaling pathway in HCV directly suppressing TLR signaling [82]. It is also important to note that in certain context FoxO3 could mediate oxidative stress-induced apoptosis of hepatocytes. In the hepatotoxin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet model Wnt/β-catenin signaling was required for hepatocyte protection against oxidative stress-induced apoptosis through the inhibition of FoxO3 [83].

7. Conclusion

Oxidative stress is an important driving force in almost all chronic liver diseases. Although hepatocytes are equipped with potent adaptive antioxidant mechanisms to maintain homeostasis the persistent imbalance between the generation of ROS and the antioxidant defense capacity of hepatocytes can cause cell death, inflammation, and fibrosis. Phagocytic and nonphagocytic NADPH oxidases are major sources of ROS in liver diseases and play key roles in hepatocyte damage and HSC/KC activation; therefore they have become

attractive therapeutic targets against chronic liver diseases and fibrosis. Further studies are, however, required to carefully analyze the cell and stage specific regulation of NOX activity in liver disease to be able to translate the data for human trials and to design adequate and rational treatments.

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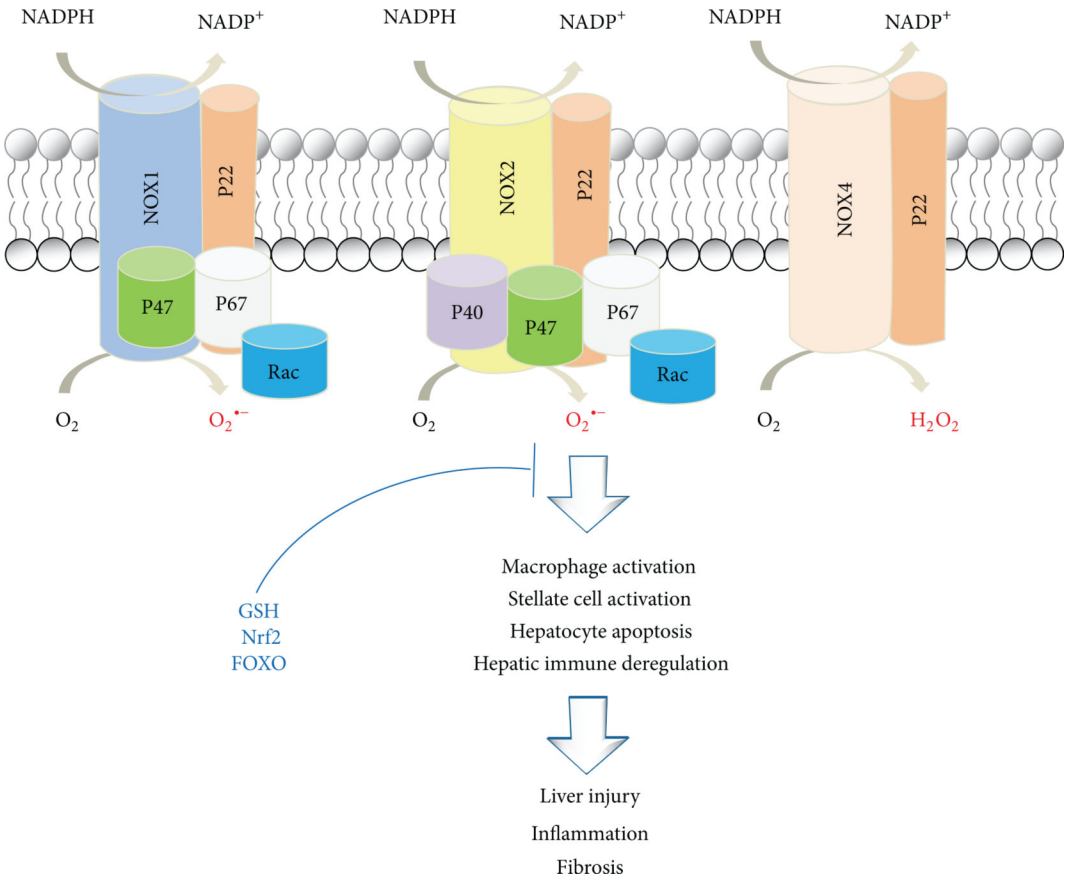

Figure 1.

NADPH oxidases in the liver. The NOX isoforms expressed in the liver are NOX1, NOX2, and NOX4. NOX1 and NOX2 form multimeric complexes on the plasma membrane with their regulatory components and catalyze the production of superoxide ($O_2^{\bullet-}$) whereas NOX4 is constitutively active and mainly produces H_2O_2. The NOX-derived reactive oxygen species (ROS) mediate hepatocyte apoptosis, macrophage, and stellate cell activation in a spectrum of liver diseases.

Table 1

The major NOX homologues expressed in the liver and their function.

NOX homologue	Expressing cells in the liver	Function
NOX1	HSC, hepatocytes, sinusoidal endothelial cells (SECs), and macrophages	HSC: liver fibrogenesis [18,19], proliferation [20], senescence [19] Hepatocyte: HCV-mediated oxidative stress [21] Macrophage: oxidative stress, liver fibrogenesis [18] SECs: unknown
NOX2	Macrophages, HSC, SECs, and hepatocytes	Macrophage: oxidative burst [10], liver fibrosis [22] HSC: phagocytosis [18], liver fibrosis [18, 22–25] Hepatocyte: unknown SECs: unknown
NOX3	HepG2 Unknown in primary liver cells	Unknown
NOX4	Hepatocytes, HSC, and SECs	Hepatocyte: oxidative stress [21, 26], apoptosis [27–30] HSC: liver fibrogenesis [28, 29] SECs: unknown
NOX5	Unknown	
Duox1	Unknown	
Duox2	Unknown	