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NOX IN LIVER FIBROSIS

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Abstract

NADPH oxidase is a multi-protein complex producing reactive oxygen species (ROS) both in phagocytic cells, being essential in host defense, and in non-phagocytic cells, regulating intracellular signalling. In the liver, NADPH oxidase plays a central role in fibrogenesis. A functionally active form of the NADPH oxidase is expressed not only in Kupffer cells (phagocytic cell type) but also in hepatic stellate cells (HSCs) (non-phagocytic cell type), suggesting a role of the non-phagocytic NADPH oxidase in HSC activation. Consistent with this concept, profibrogenic agonists such as Angiotensin II (Ang II) and platelet derived growth factor (PDGF), or apoptotic bodies exert their activity through NADPH oxidase-activation in HSCs. Both pharmacological inhibition with DPI and genetic studies using p47^{phox} knockout mice provided evidence for a central role of NADPH oxidase in the regulation of HSC-activity and liver fibrosis. In addition to the p47^{phox} component, only rac1 has been identified as a functional active component of the NADPH oxidase complex in HSCs.

Keywords

Liver fibrosis; Hepatic stellate cells; NADPH oxidase (NOX); Reactive Oxygen Species (ROS); Angiotensin II; PDGF; Apoptotic bodies; p47^{phox} component; Rac1 component

NADPH oxidase is a multi-protein complex that generates reactive oxygen species (ROS) in response to a wide range of stimuli[1,2]. In the liver, NADPH oxidase (NOX) is functionally expressed both in the phagocytic form[3] and in the non-phagocytic form[4]. It has previously been demonstrated that chronic liver diseases are characterized by increased ROS production as well as decreased activity of antioxidant systems, resulting in oxidative stress[5-9]. This feature is commonly detected in patients with alcohol abuse, hepatitis C virus infection, iron overload, and chronic cholestasis[10-12], as well as in most types of experimental liver fibrogenesis[13]. In these conditions, oxidative stress is not only a consequence of chronic liver injury but also significantly contributes to excessive tissue remodelling and fibrogenesis[14]. Among the different molecules involved in ROS production during liver damage [14-17], a critical role is played by the NADPH oxidase complex.

While Kupffer cells in the liver mainly produce ROS through the phagocytic form of NADPH oxidase, which exerts an important role in host defence[18] and inflammation[3], hepatic stellate cells (HSCs) express the non-phagocytic form of NADPH oxidase which plays an important role in regulating cell signalling[4,19]. Kupffer cells are the resident macrophage

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in the liver[20], and HSCs are the main fibrogenic cell[21,22]. Thus, if ROS from phagocytic NADPH oxidase expressed in Kupffer cells are important in mediating liver injury and fibrosis through a paracrine mechanism leading to the activation of HSCs[3,23,24], the finding that HSCs express their own form of non-phagocytic NADPH oxidase[4,19,25] led researchers to study whether the direct effect of this complex in HSCs might play a key role in hepatic fibrosis.

BACKGROUND

Liver fibrosis represents the normal response of the liver to chronic injury caused by viral, toxic, metabolic or autoimmune disease and is associated with significant morbidity and mortality worldwide[21,22]. After an acute liver injury (e.g. viral hepatitis), parenchymal cells regenerate and replace necrotic or apoptotic cells. This process is associated with an inflammatory response and a limited deposition of extracellular matrix (ECM). In specific pathological conditions the hepatic injury occurring in the organism might persist, determining a chronic reparative process which leads to uncontrolled deposition of collagen and alteration of normal structure of the liver[26]. Subsequently liver regeneration fails and hepatocytes are replaced by abundant ECM, including fibrillar collagen[27].

In advanced stages of fibrosis, the ECM in the liver increases six-fold, including collagens (I, III and IV), fibronectin, undulin, elastin, laminin, hyaluronan and proteoglycans. The excessive accumulation of extracellular matrix in fibrotic diseases is a dynamic process largely regulated by hepatic stellate cells (HSCs), recognizing this cell population as the major effector of fibrogenesis[28] [29]. Following liver injury, HSCs transdifferentiate from quiescent to an activated myofibroblast-like phenotype, resulting in increased proliferation and ECM synthesis [30,31].

Among the mechanisms mainly involved in mediating the process of liver fibrosis, an important role is played by ROS[10,14]. Reactive oxygen species (ROS) include superoxide, hydrogen peroxide, hydroxyl radicals and a variety of reaction products.

Several differentially localized and expressed enzymatic systems contribute to ROS formation in the liver, including endothelial NO synthetases, mitochondrial uncoupling, cytochrome P450 monooxygenases (CYP2E1) and NADPH oxidase. In the normal liver, antioxidant systems such as superoxide dismutase and catalase efficiently remove excess of ROS to maintain the normal cell homeostasis. In contrast, during chronic liver diseases, there is increased ROS production, as well as decreased activity of antioxidant systems, resulting in oxidative stress.

It has been proposed that ROS derived from damaged hepatocytes through cytochrome P450 monooxygenases (CYP 2E1) can induce phenotypic activation, proliferation, and increased collagen synthesis in HSCs and thus contribute to liver fibrosis[15-17]. In particular, ROS produced by CYP 2E1, expressed in hepatocytes, are important in HSCs activation, as assessed by studies in which HSC were co-cultured with HepG2 cells over-expressing cytochrome P450 CYP2E1 [32]. CYP 2E1 may produce diffusible mediators, most likely stable ROS such as H₂O₂ and lipid peroxidation metabolites, which up-regulate important matrix-synthesizing genes and consequently play a role in liver injury.

NADPH oxidase complex has been recently recognized as a critical mediator of liver fibrosis [3,4]. Specifically, the phagocytic form of NADPH oxidase expressed in Kupffer cells has been proposed to indirectly activate HSCs contributing to liver fibrosis[3,24], while the non-phagocytic form of NADPH oxidase complex is directly expressed and functionally active in HSC[4,25].

NADPH OXIDASE COMPLEX

Until recently, the single example of deliberate generation of ROS in mammalian cells was phagocytic NOX (PHOX) that catalyzes the respiratory burst[33,34]. The NADPH oxidase complex (originally named flavocytochrome B) consists of the catalytic subunit gp91^{phox} (renamed NOX2) together with the regulatory subunit p22^{phox} located in the membrane[35, 36]. The other regulatory components p47^{phox}, p40^{phox}, p67^{phox} and the small GTPase Rac are normally located in the cytoplasm[37-40]. Following stimulation, the cytosolic proteins translocate to the membrane, where they interact with the flavocytochrome B, increasing the activity of NADPH oxidase. The activated oxidase produces large amount of extracellular superoxide that plays a pivotal role in host defence against microbial infections[41,42].

The non-phagocytic NADPH oxidase complex is structurally and functionally similar to the phagocytic NADPH, resulting in reduction of molecular oxygen to generate superoxide[43, 44]. ROS levels in the non-phagocytic form of NADPH oxidase are typically a few percent of ROS levels in activated phagocytic cells. However, unlike the phagocytic type, the NADPH oxidase complex is constitutively active, producing relatively low levels of ROS under basal conditions and generating higher levels of oxidants in response to agonists such as Angiotensin II (AngII)[4,45,46]. ROS produced in response to specific agonists are known to stimulate several intracellular pathway in a redox-sensitive manner[47]. In addition to the membrane component gp91^{phox} (NOX2) there are six additional homologous NOX proteins that may function in non-phagocytic NADPH oxidases[44] (NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2). NOX1 is primarily expressed in epithelial cells of the digestive tract and could be important in replacing NOX2 function[48-50] [51], while NOX4, mainly expressed in the kidney, seems to produce constant amount of ROS independent of the activation of other inducible NADPH components[52]. On the other hand NOX3 is expressed in the inner ear and is important for otocoma formation[53]. NOX1,2,3,and 4 are characterized as calcium independent[44]. In contrast, the activity of NOX5, which is mostly expressed in spermatozoae and lymphocytes, is increased by calcium[54]. Two more homologues of NOX2 has been recently identified, DUOX 1 and 2[55]. These components are mainly expressed in lung, intestine, thyroid and are characterized by the presence of an additional peroxidase domain [56,57]. Similarly, NOXO1 (NOX organizer 1) is homologous to p47^{phox} except that it lacks the auto-inhibitory region [58]. NOXA1 (NOX activated 1) is a homologue of p67^{phox} and shares the similar domain organization [58] (TABLE I). Unlike the well-characterized regulation of phagocytic NOX, the regulation of NOX is largely unknown in non-phagocytic cells [59]. In fact each cell type may have a unique group of components that are regulated and assembled in a unique way to perform the required cell function [46]. The exact structure of non-phagocytic NADPH oxidase expressed in HSCs in the liver is incompletely known (Fig. 1). Although it is clear that p47^{phox} and Rac1 are crucial components for NADPH oxidase functionality in HSCs, complete data are currently available regarding all the other components. [2].

NADPH OXIDASE ACTIVITY IN HSCs AND ITS CONTRIBUTION TO FIBROSIS

Although the best defined activity of ROS is extracellular killing in response to exogenous insults[18,60], a more recent view proposes that NADPH oxidase exerts a second and important action in the liver by regulating cell biology[4,61,62]. Thus, if high levels of ROS, from phagocytic cells such as Kupffer cells mainly protect the organism from external pathogens, a lower amount of ROS, mainly from non-phagocytic cells such as HSCs, actively participates in the regulation of intracellular signalling[4,25]. This interpretation led researchers to investigate the role of ROS in liver diseases and evaluate the potential role of NADPH oxidase as a mediator of different profibrogenic agonists involved in liver fibrosis. Based on this hypothesis, we (Ramon et al.) showed that HSCs express components of NADPH oxidase[4].

Moreover, the functional activity of this complex in HSCs was also demonstrated from the same group. Ang II-induced HSC activation was mediated by NADPH oxidase function, introducing for the first time the new role of non-phagocytic NADPH oxidase in HSCs.

The importance of NADPH oxidase and the role of this molecule complex during liver injury were recently confirmed by microarray analysis in human liver tissues. The gene expression pattern of liver samples obtained from patients with alcoholic hepatitis was characterized by a significant up-regulation of several components of the NADPH oxidase and related molecules. These findings clearly demonstrated that NADPH oxidase is involved in the process of liver fibrosis, as stated from in vivo study in rodents and in humans as well, and is also significantly associated to human liver diseases such as alcoholic hepatitis [63,64].

NADPH OXIDASE MEDIATES ANG II PROFIBROGENIC ACTIVITY IN THE LIVER

As shown previously in the kidney[65], Ang II exerts its activity for the regulation of intracellular pathways using NADPH oxidase as a mediator in HSCs[4,19]. This finding is consistent with older studies which demonstrated that inhibition of Ang II synthesis and/or the blockade of Ang II type 1 (AT1) receptors markedly attenuate inflammation and fibrosis in experimental models of chronic liver injury[66-68]. Based on these findings, we (Ramon et al.) showed that the profibrogenic activity of Ang II in the liver[69-71] directly acts on HSCs through the activation of NADPH oxidase complex and the subsequent production of ROS [4].

Several experiments provide evidence that Ang II is able to induce ROS production in human activated HSCs. Dichlorodihydrofluorescein diacetate (DCFDA) fluorescence experiments show that Ang II (10^{-8} M) induces a marked increase in ROS formation in activated human HSCs. Moreover, Ang II-induced ROS formation in HSCs is significantly inhibited when HSCs are treated both with the flavinoid inhibitor diphenyleneiodonium chloride (DPI) [72] and with the AT1 inhibitor losartan [66]. The response of HSCs to Ang II via activating NADPH oxidase demonstrates that AT1 receptor expressed on HSCs directly correlates to this complex in order to regulate intracellular signalling[4].

Additional proof that human HSCs express a functionally active form of NADPH oxidase is provided by the analysis of the genetic expression of key enzymatic components. Indeed, the mRNA for the cytoplasmic factor p47^{phox} and the cell membrane proteins gp91^{phox} and NOX1 are not detected in quiescent HSCs by real time RT-PCR, while they are highly expressed following HSC activation in culture and in cells freshly isolated from patients with liver fibrosis. In contrast, gp91^{phox} is only expressed in culture-activated HSCs [4]. These data indicate that after HSCs are activated in vivo, there is activation of non-phagocytic NADPH oxidase components. Furthermore, Ang II induces the phosphorylation of p47^{phox} through AT1 receptors in activated HSCs. Finally, Ang II induces the expression of heme oxygenase-1, a sensitive marker of cellular oxidative stress[73]. This effect is inhibited by losartan or DPI, indicating that oxidative stress is due to an AT1-induced NADPH oxidase activation.

The p47^{phox} component of NADPH oxidase is increased in activated HSC. p47^{phox} $-/-$ mice have been widely used as a genetic model to study NADPH oxidase inhibition both in phagocytic[20,74,75] and in non-phagocytic cells[2,4,76,77]. In particular, Ang II leads to an increase of intracellular ROS in HSCs isolated from wild type (WT) C57BL/6 mice, but not in p47^{phox} $-/-$ HSCs. Also, Ang II used at the concentration of 10^{-8} M 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^{phox} $-/-$ HSCs. Collectively, these results demonstrate that NADPH oxidase-derived ROS play a major role in Ang II-induced activation of HSCs. Moreover, after

2 weeks of bile duct ligation (BDL), the degree of liver injury is attenuated in $p47^{\text{phox}}/-$ mice. Specifically, $p47^{\text{phox}}/-$ mice show only small amounts of collagen deposition without formation of bridging fibrosis and a reduced amount of hydroxyproline content in comparison to the respective WT mice. These results, in addition to the immunostaining and western blot for alpha smooth muscle actin (α -SMA) (a marker of activated myofibroblasts), demonstrate that NADPH oxidase inhibition, either pharmacological with DPI or genetical with $p47^{\text{phox}}/-$ mice, leads to a reduced degree of fibrosis in the liver[4]. The reduction correlates with the reduced profibrogenic activity operated by Ang II on HSC. Thus, non-phagocytic NADPH oxidase represents a fundamental mediator in experimental liver fibrosis.

PDGF AND NADPH OXIDASE

The knowledge that NADPH oxidase plays an important activity in HSC activation was further supported by experiments showing that platelet derived growth factor (PDGF), the most potent mitogen of HSCs[78,79], exerts its activity through the use of this complex[25].

Immunohistochemistry and in situ hybridization studies have demonstrated that PDGF and PDGF-receptors are overexpressed at both the messenger RNA (mRNA) and protein levels in liver tissue from patients with chronic hepatitis or cirrhosis, and are positively correlated with the severity of histological lesions and collagen deposition[80,81]. These reports strongly suggest that PDGF facilitates the progression of hepatic fibrosis in human chronic liver diseases. Specifically PDGF-induced increase in DNA synthesis of HSCs is markedly reduced by pre-treatment with the anti-oxidant drug Mn-TBAP (100 nM/L), and also with two different inhibitors of NADPH oxidase complex, DPI (25 μ M/L), and apocynin (100 μ M/L). The interaction between PDGF and NADPH oxidase supports the critical role of NADPH oxidase in HSCs.

The experimental model of liver fibrosis induced in mice by parenteral DMN provides additional in vivo evidence for the role of NADPH oxidase 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[25].

APOPTOTIC BODIES ACTIVATE NADPH OXIDASE

In addition to receptor mediated agonists, NADPH oxidase may be activated in HSCs by the engulfment of apoptotic bodies from dead hepatocytes leading to the up-regulation of collagen $\alpha 1(I)$ in HSCs[82]. Apoptosis is a common feature of chronic liver disease. The engulfment of apoptotic bodies not only acts on macrophages stimulating production of $TGF\beta_1$ [83,84..]but also on HSC promoting their activation[82]. In support of this model, Zhan et al. showed that apoptotic bodies activate NADPH oxidase in HSCs, thereby up-regulating HSC activation and fibrosis[85]. Double staining for α -SMA and E-cadherine[86] demonstrates that intracellular apoptotic bodies are really expressed in HSCs. Specifically, apoptotic bodies in HSCs increase ROS production in a NADPH-dependent manner. In fact, the increased production of oxidative stress in response to apoptotic bodies is blocked by the NADPH inhibitor DPI at the concentration of 10 μ M/L. Subsequently, up-regulation of collagen $\alpha 1(I)$, observed in HSC after treatment with apoptotic bodies, is also inhibited by the use of the NADPH inhibitor DPI. Although the interpretation of these experiments is limited by the non-specificity of a single pharmacological inhibitor, the results are again consistent with an important role of NADPH oxidase in HSC activation.

NADPH-OXIDASE COMPONENTS IN HSCs

Although NADPH oxidase plays an important role in HSCs activation, the exact structure of this complex is still unknown. All components and functionalities of the phagocytic NADPH oxidase are already well established, but the respective interactions between the non-

phagocytic NADPH oxidase components are still under investigation. Thus, the role of the different members of the NOX family in HSCs is just starting to be investigated. The only well established component which is recognized to play a central role in the activity of NADPH oxidase in HSC is p47^{phox} (as discussed above) [4]. Another integral component required for the activation of NADPH oxidase is Rac1. Rac1 is a member of the Rho family of small GTPase proteins that regulates cell proliferation and dynamic reorganization of the actin cytoskeleton [87].

An *in vivo* study using transgenic mice that over-express Rac1 allowed Choi et al. to prove functional and biological regulation of Rac1 as an important component of NADPH oxidase in mediating liver injury[88]. Accordingly to these experiments, livers from CCl₄-treated Rac1-transgenic mice are more grossly nodular and demonstrate more acidophilic necrosis with greater numbers of TUNEL-positive liver cells than CCl₄-treated WT control mice. In addition, cell culture experiments with cultured HSCs demonstrated increased activation from transgenic mice compared to WT mice. Thus, Rac1 plays a role in HSC-NADPH oxidase activity and further defines the real structure and functionality of the non-phagocytic NADPH oxidase complex.

FUTURE DIRECTION

The demonstration that NADPH oxidase is expressed in HSC led researchers to evaluate the functional activity of this complex in liver fibrosis. The results demonstrate that NADPH oxidase in HSCs regulates their activation process, so that the NADPH oxidase complex regulates liver fibrogenesis in response to Ang II, PDGF or apoptotic bodies. Since NADPH oxidase mediates the effects of several other agonists in extra-hepatic cells [89-91] (leptin, endothelin, homocystein), these potentially interesting agonists need to be assessed in HSC activation. Secondly, two different NADPH oxidase forms, the phagocytic and the non-phagocytic form, are apparently both actively operating in liver fibrogenesis. However, the critical contribution of each of these forms to liver fibrosis is still unknown.

Moreover, while the structure and function of the phagocytic form of NADPH oxidase is well known, many questions are still remaining about the structure and interactions among the several components expressed in the non-phagocytic NADPH oxidase of HSC. The knowledge of NADPH oxidase structure and specific data on functional interactions in HSCs can provide new insights into developing treatments.

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Fig. 1. Composition of non-phagocytic NADPH oxidase

The classical components of phagocytic NADPH oxidase can be substituted by several potential homologues in non-phagocytic cells such as HSCs: nox family (NOX1, NOX3, NOX4, DUOX1, DUOX2) as a substitute for gp91^{phox}, NOXO1 for p47^{phox}, NOXA1 for p67^{phox}.

TABLE 1

NADPH oxidase components. Classical components of phagocytic NADPH oxidase and potential homologues in non-phagocytic NADPH oxidase: nox family (NOX1, NOX3, NOX4, NOX5, DUOX1 AND DUOX2) homologue of gp91^{phox}, NOXA1 homologue of p47^{phox}, NOXA1 homologue of p67^{phox}.

Classical components	NOX2 (gp91 ^{phox})	p22 ^{phox}	p40 ^{phox}	p47 ^{phox}	p67 ^{phox}	Rac1 Rac2
Potential Homologues	NOX1 NOX3 NOX4 NOX5 DUOX1 DUOX2			NOXA1	NOXA1	