



Mini Review

Role of NADPH oxidases in the redox biology of liver fibrosis

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ABSTRACT

Liver fibrosis is the pathological consequence of chronic liver diseases, where an excessive deposition of extracellular matrix (ECM) proteins occurs, concomitantly with the processes of repair and regeneration. It is characterized by increased production of matrix proteins, in particular collagens, and decreased matrix remodelling. The principal source of ECM accumulation is myofibroblasts (MFB). Most fibrogenic MFB are endogenous to the liver, coming from hepatic stellate cells (HSC) and portal fibroblasts. Dys-regulated inflammatory responses have been associated with most (if not all) hepatotoxic insults and chronic oxidative stress play a role during the initial liver inflammatory phase and its progression to fibrosis. Redox-regulated processes are responsible for activation of HSC to MFB, as well as maintenance of the MFB function. Increased oxidative stress also induces hepatocyte apoptosis, which contributes to increase the liver injury and to transdifferentiate HSC to MFB, favouring the fibrogenic process. Mitochondria and other redox-active enzymes can generate superoxide and hydrogen peroxide as a by-product in liver cells. Moreover, accumulating evidence indicates that NADPH oxidases (NOXs), which play a critical role in the inflammatory response, may contribute to reactive oxygen species (ROS) production during liver fibrosis, being important players in HSC activation and hepatocyte apoptosis. Based on the knowledge of the pathogenic role of ROS, different strategies to prevent or reverse the oxidative damage have been developed to be used as therapeutic tools in liver fibrosis. This review will update all these concepts, highlighting the relevance of redox biology in chronic fibrogenic liver pathologies.

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Contents

1. Introduction	106
2. Inflammation, oxidative stress and liver fibrosis	107
3. NOXs and liver fibrosis	108
4. Targeting liver fibrosis by hampering ROS/NOX and/or modulating redox-related intracellular signals	109
5. Conclusions	110
Acknowledgements	110
References	110

Abbreviations: 4-HNE, 4-hydroxi-2'-nonenal; CTGF, Connective Tissue Growth Factor; COX, cyclooxygenase; EMT, epithelial-to-mesenchymal transition; ECM, extracellular matrix; GSH, glutathione; HCC, hepatocellular carcinoma; IR, ischaemia/reperfusion; MSCs, mesenchymal stem cells; MMP, metalloproteases; MGS, mitochondrial reduced glutathione; MFB, myofibroblasts; NOX, NADPH oxidase; NO, nitric oxide; NOS, nitric oxide synthase; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NFκB, Nuclear Factor kappa-B; PDGF, Platelet-Derived Growth Factor; RNS, reactive nitrogen species; ROS, reactive oxygen species; HSC, stellate cell; SOD, superoxide dismutase; TIMP, tissue inhibitors of metalloproteases; TLR4, toll-like receptor 4; TGF-β, Transforming Growth Factor-beta; TNF-α, Tumour Necrosis Factor-alpha; UPR, unfolding protein response; UDCA, ursodeoxycholic acid

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1. Introduction

In all organ systems, the normal mammalian response to injury occurs in three overlapping but distinct stages: inflammation, new tissue formation, and tissue remodelling. A critical step is when repair and regeneration take place, because excessive deposition of extracellular matrix (ECM) proteins leads to hypertrophic scars, which provoke tissue dysfunction [1]. Liver fibrosis is the pathological consequence of chronic liver diseases, resulting from the progressive accumulation of ECM, which is mainly enriched in types I and III fibrillar collagens. Scar deposition is a consequence of an altered wound healing response to prolonged parenchymal cell injury and/or inflammation. It is characterized by increased production of matrix proteins and decreased matrix remodelling. In advanced stages, fibrosis leads to cirrhosis, a condition defined by an abnormal liver architecture, failure in liver function, portal hypertension and high susceptibility to infection and to develop HCC [2,3].

The principal source of ECM accumulation and prominent mediators of fibrogenesis are “activated fibroblasts” or myofibroblasts (MFB). Different origins for activated fibroblasts have been proposed: resident fibroblasts, bone marrow-derived fibrocytes, epithelial cells that undergo epithelial-to-mesenchymal transition (EMT), vascular smooth muscle cells and pericytes. However, recent knowledge indicates that most fibrogenic MFB are endogenous to the liver, coming from hepatic stellate cells (HSC) and portal fibroblasts. HSC are considered to be the major source of fibrogenic cells in response to chronic liver injury, while portal fibroblasts play an important role during cholestatic liver diseases [4]. A complex network of autocrine/paracrine fibrogenic signals promotes the activation, usually called transdifferentiation, of quiescent HSC to a myofibroblastic phenotype. This fibrogenic inputs include cytokines, chemokines, growth factors, lipid mediators and reactive oxygen species (ROS) that are produced by epithelial cells (hepatocytes and cholangiocytes), endothelial cells and cells of the immune system (macrophages, dendritic cells, and B and T lymphocytes). Apoptotic bodies derived from damaged hepatocytes can also transdifferentiate HSC to MFB favouring the fibrogenic process [2,5]. A number of growth factors are pro-fibrotic in the liver, including Platelet-Derived Growth Factor (PDGF), angiotensin II, Connective Tissue Growth Factor (CTGF), and Transforming Growth Factor-beta (TGF- β) [6]. TGF- β has a pivotal role in fibrogenesis, and some of the other growth factors involved exert their effects by directly stimulating TGF- β production [7].

2. Inflammation, oxidative stress and liver fibrosis

Inflammation plays an essential role in the development of liver fibrosis. When a chronic injury takes place, a large infiltration of mononuclear cells, which include macrophages, lymphocytes and eosinophils, occur. Mobilization of lymphocytes produces lymphokines that activate macrophages, which, in turn, stimulate lymphocytes, fibroblasts, and other inflammatory cells, thus setting the stage for persistence of an inflammatory response. Dysregulated inflammatory responses have been associated with most (if not all) hepatotoxic insults, including ischaemia/reperfusion (IR) injuries; alcohol overconsumption; intoxications by xenobiotics or heavy metals; bacterial, viral and parasitic infections; as well as systemic metabolic conditions, such as non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), obesity, diabetes, and the metabolic syndrome [8] (Fig. 1). Macrophages produce pro-fibrotic mediators, including TGF- β and PDGF, and control ECM turnover by regulating the balance of various matrix metalloproteinases (MMP) and tissue inhibitors of

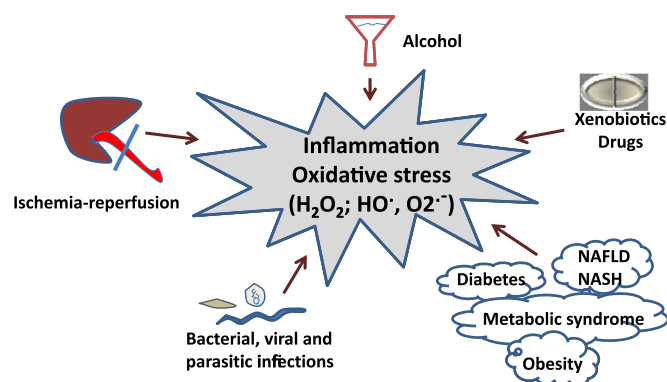


Fig. 1. Different causes of chronic liver injury concur with inflammatory processes and oxidative stress.

metalloproteinases (TIMP). Examples of knock-out mice that are resistant to fibrosis because they have less inflammation include those with gene deletions of Tumour Necrosis Factor-alpha (TNF- α) or Toll-like receptor 4 (TLR4), among others [9,10].

ROS are short-lived, highly electrophilic molecules, generated by the partial reduction of oxygen; reactive nitrogen species (RNS) such as nitric oxide (NO) are sometimes included [11]. Different types of ROS have different intrinsic chemical properties, which dictate their reactivity, subcellular localization and preferred biological targets. ROS are critical intermediates in both the normal physiology and pathological conditions of liver cells. Over-produced ROS may directly deplete antioxidant molecules, such as glutathione (GSH) and inhibit the activities of antioxidant enzymes, such as superoxide dismutase (SOD), but they may also induce the expression of antioxidant genes to counteract oxidative stress effects [12]. When the balance between ROS generation and the antioxidant defence of cells is disrupted, it results in oxidative stress. Importantly, it has been recently reported that activated HSC have increased ROS-detoxifying capacity compared to quiescent HSC that protects them from ROS-induced apoptosis and necrosis [13].

High concentrations of ROS induce HSC death, however, non-toxic levels of ROS stimulate the activation, proliferation, and collagen production of HSC [14]. Furthermore, intracellular generation of ROS, through activation of specific signalling pathways, is a critical event for directional migration of HSC/MFB, as well as mesenchymal stem cells (MSCs) [15]. Indeed, in the liver, HSC transdifferentiation was shown to be inhibited by antioxidants [16,17]. Oxidative stress markers have been detected in the serum and in biopsy samples from liver cirrhosis patients, as well as in experimental liver fibrosis/cirrhosis animals [18,19]. In liver biopsies, areas of fibrosis were localized to areas with increased 4-hydroxy-2'-nonenal (4-HNE), a marker of lipid peroxidation [20,21]. ROS production associated to steatosis affects nuclear redox state and induces modifications of nuclear proteins [22]. Furthermore, recent results indicate that hepatic stromal cells promote leucocyte migration through catalytic generation of ROS, which indicates that a clear cross-talk exists among liver inflammation, oxidative stress and fibrogenesis [23]. Indeed, chronic oxidative stress plays a role during both the initial inflammatory phase and its progression to fibrosis.

There are numerous potential sources of ROS within the cell. ROS can be produced as by-products of enzymatic processes, like in the case of mitochondrial ROS. Mitochondria generate ATP in an oxygen-dependent manner during which the flow of electrons down the respiratory chain eventually culminates at complex IV with the reduction of molecular oxygen to water. Throughout this process, superoxide is generated, predominantly at complex I and complex III of the cytochrome chain, when electrons initially

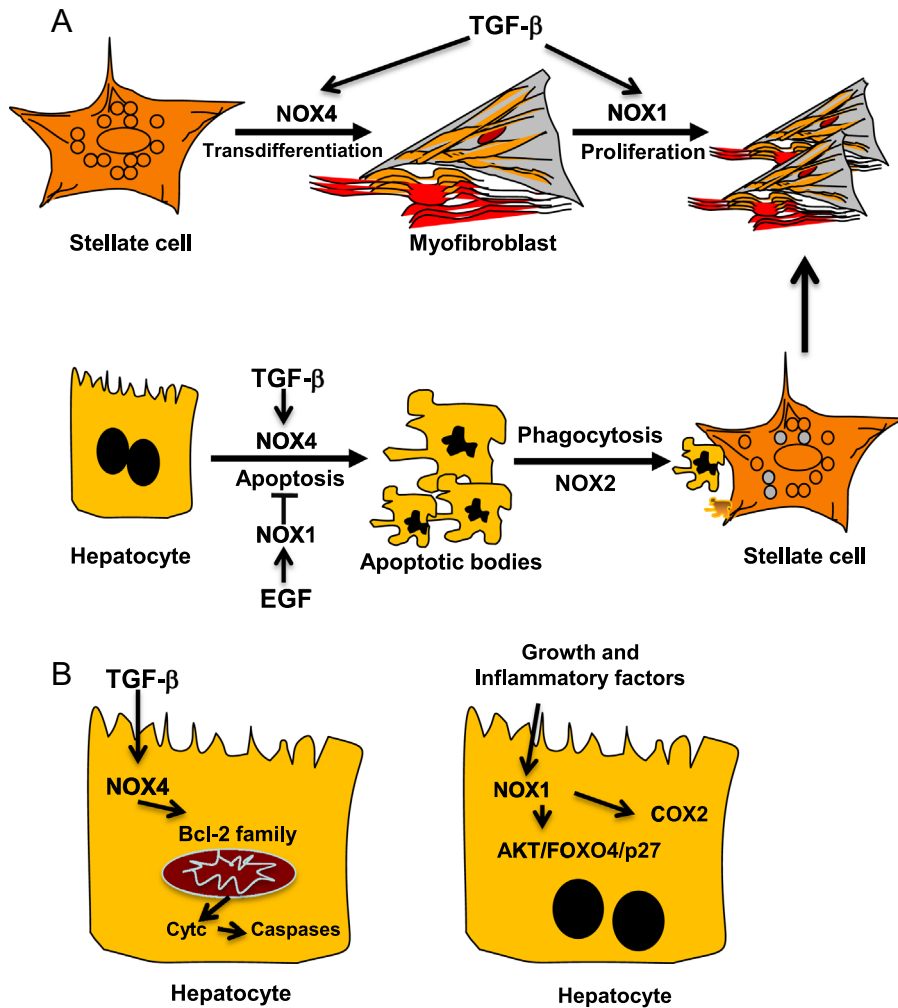


Fig. 2. Role of NOXs on HSC activation and regulation of hepatocyte apoptosis. Role of TGF- β , growth and inflammatory factors in this process. [See text for further details.]

derived from NADH or FADH₂ react with oxygen. Normal mitochondria provide a low basal level of ROS in most of cells, but this level may increase during aging or mitochondrial damage. Mitochondrial oxidants have been historically viewed as purely toxic, however, recent evidence suggests that they can be also regulators of intracellular signalling pathways [11]. In addition to mitochondria, other redox-active enzymes can generate superoxide and hydrogen peroxide as a by-product. These include xanthine oxidase, cytochrome p450, cyclooxygenase, lipoxygenase, and nitric oxide synthase (NOS) [11,24]. Moreover, accumulating evidence indicates that NADPH oxidases (NOXs), which play a critical role in the inflammatory response, may contribute to reactive oxygen species (ROS) production during liver fibrosis, being important players in HSC activation and hepatocyte apoptosis [14]. Recent findings also implicate the tumour suppressor p53 as a critical co-factor for several key fibrotic and cell cycle effectors. Increased oxidative stress associated with the fibrotic process is both a likely initiator and an upstream mediator of p53 signalling in the injured tissue [25].

3. NOXs and liver fibrosis

NOXs are a family of enzymes that generate ROS (either superoxide or hydrogen peroxide) as the primary species during the catalytic metabolism of oxygen for a range of host defence and signalling functions. Seven isoforms of NOX are expressed in

mammalian cells (NOX1–5, DUOX1 and DUOX2). All NOX isoforms are membrane bound enzymes that rely on NADPH for their activity and the major source of ROS is generated when the flavin- and haem-containing protein complex transfer electrons from cytosolic NADPH to molecular oxygen to produce O₂^{•-} or H₂O₂ [11,26]. Both parenchymal and non-parenchymal hepatic cells express different members of the NOX family. Hepatocytes and HSC express NOX1, NOX2, NOX4, DUOX1 and DUOX2; endothelial cells express mainly NOX1, NOX2 and NOX4; and Kupffer cells, which are hepatic-resident macrophages, express the phagocyte NOX2 [14,27].

NOX-derived ROS have been previously related to fibrosis in several organs such as lung [28], pancreas [29], kidney [30,31] and heart [32]. Recent evidence also suggests a key role for NOX proteins in the progression of hepatic fibrosis [33–35]. NOX mediates liver fibrogenic responses induced by different agonists [14], as well as phagocytosis of apoptotic bodies [36]. But, of relevance, NOX mediates TGF- β -induced MFB activation in different organs [28,31,32,37–39]. HSC responds to TGF- β inducing NOX4-derived ROS [40], which play a key role in hepatic MFB activation both *in vivo* and *in vitro* [41, 42]. Indeed, TGF- β -induced HSC activation is attenuated either by NOX4 down regulation or in a *Nox4*^{-/-} genetic background, and, interestingly, the MFB activated state could also be reversed by NOX4 down regulation [42]. Levels of NOX4 are elevated in patients with hepatitis C virus (HCV)-derived liver fibrosis, increasing along the fibrosis degree, as well as in patients with NASH [43]. Hepatocyte specific deletion of NOX4

reduced oxidative stress, lipid peroxidation and liver fibrosis in mice with diet-induced steatohepatitis [43]. All these data strongly suggest the essential role played by NOX4 in the development of liver fibrosis. However, studies performed in *Nox1^{-/-}*, *Nox2^{-/-}*, *p47phox^{-/-}* have demonstrated also the importance of other NOX proteins, concretely NOX1 and NOX2 in fibrogenesis [35,44,45]. NOX1 seems to mediate the pro-fibrogenic effects in endogenous liver cells, through PTEN inactivation and positive regulation of the AKT/FOXO4/p27 signalling pathway [34] and it may further contribute to the inflammatory process, promoting cyclooxygenase (COX)-2 expression and prostaglandin synthesis in hepatocytes [46]. NOX2 could be implicated in both endogenous liver cells and bone marrow-derived cells [35], possibly acting in the process of phagocytosis of dead hepatocytes [47] (Fig. 2).

Hepatocyte apoptosis is another crucial event during fibrogenesis since it triggers Kupffer cells and HSC activation by secreting cytokines, chemokines and microparticles. TGF- β induces hepatocyte apoptosis through ROS that are derived from both mitochondria and NOX activity [48]. In addition, NOX4 also mediates apoptosis induced by other stimuli since *NOX4^{-/-}* hepatocytes are resistant to apoptosis induced by CD95L and TNF- α /actinomycin D [41]. Hepatocyte apoptosis during fibrosis might be relevant to blunt regeneration and create a pro-fibrogenic micro-environment. In agreement with these results, it has been proposed a role for NOX4 in epithelial cell death during development of bleomycin-induced lung fibrosis. Using a model of NOX4 deficient mice, authors demonstrated that these animals were resistant to fibrosis due to the abrogation of TGF- β -induced apoptosis in epithelial cells [49]. Of note, hepatocytes express not only NOX4 but also other NOX proteins, and they play opposite roles in the control of hepatocyte survival and death. Indeed, whereas NOX4 is necessary to mediate apoptosis induced by TGF- β [50,51], this pro-apoptotic effect of the cytokine can be attenuated after NOX1 activation by EGF or other growth factors [52,53]. Phagocytosis of hepatocyte apoptotic bodies by HSC induces their activation to MFB, a process that involves NOX2 activation, thereby contributing to exacerbate the fibrotic response [36,47] (Fig. 2).

4. Targeting liver fibrosis by hampering ROS/NOX and/or modulating redox-related intracellular signals

Regression of fibrosis can be achieved by the successful control of chronic liver injury, with the consequent termination of the fibrogenic reaction followed by the clearance of hepatic MFB and restoration of full liver function. However, this regression is only possible at early stages, since when advanced fibrosis or cirrhosis is established, all therapeutic approaches seem non-efficient. MFB can be eliminated by apoptosis, senescence or reversion to a quiescent (HSC) phenotype. Indeed, the first line treatment is, when possible, to counteract the underlying liver disease to stop fibrosis progression. For instance, patients with viral hepatitis should be treated with anti-viral therapies while corticosteroids are useful for autoimmune hepatitis. However, inactivated HSC remain primed for re-transdifferentiation, and may be even more responsive to recurrent fibrogenic stimuli than its original quiescent state. In advanced stages of fibrosis and cirrhosis the potential for reversibility declines [2,5]. MFB and their products are primary targets for antifibrotic therapies, which in principle would address all types of fibrosis, including advanced fibrosis. Nevertheless, additional cellular elements that are either upstream of MFB or tightly linked to fibrogenic activation may provide a basis for complementary and more disease-specific antifibrotic approaches. A combined therapy may be a more effective approach, given the crosstalk between different cell types that generally underlies the fibrogenic activation [2,3,5,54].

At present, there is no FDA approved drug for liver fibrosis treatment. Based on the knowledge of the pathogenic role of ROS, different strategies to prevent or reverse the oxidative damage are being developed in pre-clinical experiments to be used as therapeutic tools in liver fibrosis. Indeed, natural antioxidants, such as pyrroquinoline-quinone has been demonstrated to suppress oxidative stress and liver fibrogenesis in mice [55]. In the same line of evidence, natural compounds with antioxidant and anti-inflammatory properties have proved to be hepatoprotective in liver of rat with secondary biliary cirrhosis [56]. Mice lacking Methionine Adenosyltransferase 1A (MAT1A), which catalyses the production of S-Adenosylmethionine (AdoMet), a precursor of the glutathione (GSH) synthesis, spontaneously develop oxidative stress and non-alcoholic steatohepatitis [57], emphasizing the relevant role that maintaining GSH levels has in counteracting oxidative stress. In this same line of evidence, recent findings underscore a critical role for mitochondrial reduced glutathione (mGSH) in the therapeutic potential of superoxide scavengers and suggest that the combined approach of these agents with mGSH replenishment may be important in steatohepatitis and liver fibrosis [58]. A recent study has emphasized the role of chloride channels in the activation of HSC, allowing the entry of superoxide anion radicals [59]. Indeed, chloride channels may constitute a potential target for new anti-fibrotic drugs (Fig. 3).

The experimental use of agents that prevent oxidative stress is contributing to a better understanding about the intracellular pathways that play essential roles in mediating or protecting against the consequences of an intracellular ROS increase in liver cells. Indeed, the use of ROS inhibitors allowed proving that HIV and HCV cooperatively promote hepatic fibrogenesis via induction of ROS and Nuclear Factor kappa-B (NF κ B) [60]. Ursodeoxycholic acid (UDCA) plays a cytoprotective effect in primary biliary cirrhosis through activating Nuclear factor Erythroid-2-related transcription factor 2 (Nrf2), which plays a critical role in protecting against oxidative stress [61]. Indeed, UDCA improves circulating redox changes in primary biliary cirrhotic patients [62]. Ursolic acid, via LKB1-AMP-activated protein kinase signalling offers also protective effects on bile duct ligation-induced liver injury in mice, which is related to inhibition of oxidative stress [63]. Blockage of the IRE α pathway, the branch of the unfolding protein response (UPR) mainly affected during liver fibrosis, in stellate cells significantly decreased both their activation and autophagic activity in a p38 MAPK-dependent manner, leading to a reduced fibrogenic response [64] (Fig. 3).

Considered the more precise role of NOX-derived ROS in hepatic fibrogenesis, the development of novel pharmacological NOX inhibitors is being assessed as the most promising potential anti-

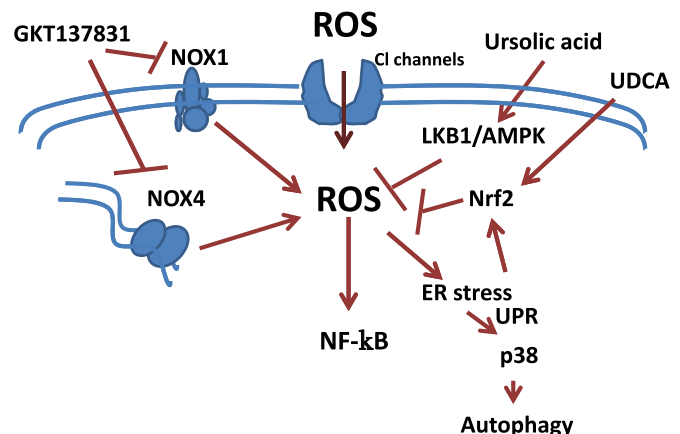


Fig. 3. Targeting liver fibrosis by hampering ROS/NOX and/or intracellular signals. [See text for further details.]

fibrotic therapeutics (Fig. 3). Historical NOX inhibitors (such as apocinin or diphenyliodonium-DPI) are unspecific and not isoform selective. Novel NOX inhibitors stemming from rational drug discovery approaches show improved specificity for NOX and even moderate isoform selectivity [65]. Interestingly, evidences for the role of dual NOX4/NOX1 pharmacological inhibitor GKT137831 in decreasing both the apparition of fibrogenic markers and hepatocyte apoptosis *in vivo*, upon bile duct ligation and CCl₄ treatment, are reported [41,66]; therefore, it is a promising therapeutic agent for future translational studies (Fig. 3). However, it is important to mention that NOX4 mediates suppressor effects of TGF- β in hepatocytes [50,67] and recent results indicate that NOX4 would inhibit hepatocyte growth and liver tumorigenesis [68]. Indeed, stable knockdown of NOX4 expression in human liver tumour cells increased their *in vitro* cell proliferation and conferred them higher *in vivo* tumorigenic capacity in xenograft experiments in nude mice, resulting in earlier onset of tumour formation and increase in tumour size [68]. *In vivo* analysis in mice revealed that NOX4 expression was downregulated under physiological proliferative situations of the liver, such as regeneration after partial hepatectomy, as well as during pathological proliferative conditions, such as diethylnitrosamine-induced hepatocarcinogenesis [68]. Considering that liver fibrosis and cirrhosis predispose to the development of HCC, the collateral effects of inhibition of some physiological functions of NOX must be considered in future studies about the clinical safety of these compounds.

5. Conclusions

Current knowledge about the molecular mechanisms of liver fibrosis places inflammation and oxidative stress as one of the main causes for the initiation and progression of this disease. Different agents that cause chronic liver injuries provoke the production of ROS by different mechanisms, among them, NOXs may play an essential role. Different NOXs have been involved in fibrogenic responses, such as HSC activation to MFB or regulation of hepatocyte cell death. The experimental use, both *in vitro* and *in vivo*, of agents that prevent oxidative stress is contributing to a better understanding about the intracellular pathways that play essential roles in mediating or protecting against the consequences of an intracellular ROS increase in liver cells. Future expectations are focused on the use of specific NOX inhibitors that prevent HSC activation and protect hepatocyte injury, although further work is necessary to fully confirm the clinical safety of these compounds. However, it cannot be forgotten that liver fibrosis has multiple etiologies and, consequently, multiple mechanisms. Indeed, much further experimental work is necessary for a better understanding of the efficacy of ROS-chelating agents as therapeutic tools in this complex disease.

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