Exploring anti-fibrotic drugs

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Chapter A4

Targeting oxidative stress for the treatment of liver fibrosis

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Abstract

Oxidative stress is a reflection of the imbalance between the production of reactive oxygen species (ROS) and the scavenging capacity of the anti-oxidant system. ROS-derived oxidative stress generated from various endogenous oxidative biochemical enzymes interferes with the normal function of liver-specific cells and presumably plays a role in the pathogenesis of liver fibrosis. Once exposed to harmful stimuli, Kupffer cells are the main effectors responsible for the generation of ROS which consequently affect hepatic stellate cells and hepatocytes. ROS-activated hepatic stellate cells undergo a phenotypic switch and deposit an excessive amount of extracellular matrix that alters the normal liver architecture and negatively affects liver function. Additionally, ROS stimulate necrosis and apoptosis of hepatocytes, which ultimately causes liver injury and leads to the progression of end-stage liver disease. In this review, we provide an overview on the role of ROS in liver fibrosis, and discuss promising therapeutic interventions related to oxidative stress. In addition to anti-oxidant therapy, novel drugs that directly target the molecular pathways responsible for ROS generation such as mitochondrial dysfunction inhibitors, endoplasmic reticulum stress inhibitors, NADPH oxidase (NOX) inhibitors and Toll-like receptor (TLR)-affecting agents are emerging as promising new approaches to modulate oxidative stress. Nevertheless, for the treatment of liver fibrosis, the consequences of modulating oxidative pathways need to be further elucidated.

Key words: reactive oxygen species; oxidative stress; liver fibrosis; therapeutic target.

Abbreviations: ADP, adenosine diphosphate; ALD, alcoholic liver disease; BDL, bile duct-ligated; Ca^{2+}, calcium; CCl_4, carbon tetrachloride; CoA, coenzyme A; CoQ10, coenzyme Q10; CytC, cytochrome C; CYP, cytochrome P450; ECM, extracellular matrix; eIF2α, eukaryotic translation initiation factor 2 alpha; ER, endoplasmic reticulum; Ero1α, endoplasmic reticulum oxidoreductin 1 alpha; $FADH_2$, flavin adenine dinucleotide; GSH, glutathione; H_2O_2, hydrogen peroxide; HBV, hepatitis B virus; HCV, hepatitis C virus; HMGB, high mobility group box; HNE, 4-hydroxynonenal; HSC, hepatic stellate cells; IRAK-1, interleukin-1 receptor associated kinase-1; KC, Kupffer cells; Ldlr, low density lipoprotein receptor; LPS, lipopolysaccharide; MDA, malondialdehyde; NADH, nicotinamide adenine dinucleotide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NFE2L2, nuclear factor (erythroid-derived 2)-like 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, nucleotide-binding domain, leucine-rich repeat family, pyrin domain containing 3; NOX, NADPH oxidase; O_2, oxygen; O_2^-, superoxide anion; PDI, protein disulfide isomerase; ROS, reactive oxygen species; PDGF, platelet-derived growth factor; PERK, protein kinase R (PKR)-like endoplasmic reticulum kinase; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; TLR, Toll-like receptors; UPR, unfolded protein response.

Reactive oxygen species, oxidative stress and diseases

Reactive oxygen species (ROS): particularly, superoxide anion ($O2^{-}$), hydroxyl radical ($\bullet OH$), hydrogen peroxide (H_2O_2), are important signaling molecules generated by specific enzymes or as by-products during different molecular processes [1]. ROS play a role in various physiological functions such as signal transduction, cell cycle regulation and the defense against microorganisms [1]. However, when the generation of ROS surpasses the overall ROS scavenging capacity by anti-oxidant systems, the normal redox state is disturbed resulting in oxidative stress [1, 2].

ROS are highly reactive, and excessive free radicals can damage all components of the cell [3]. Toxic effects include DNA base damage and strand breaks, lipid peroxidation and modification of amino acid residues of numerous proteins [4]. If not controlled, continuous ROS-induced damage can instigate various pathophysiological conditions such as atherosclerosis, diabetes, neurodegenerative diseases and cancers [4]. In addition, proteins modified by ROS can act as secondary messengers that alter normal cell homeostasis leading to pathophysiological conditions as evidenced in the pathogenesis of allergic inflammation and asthma [5]. In the liver, ROS are continuously generated in various physiological processes, and changes in the redox state are an integral part of the development of liver fibrosis [6, 7].

Liver fibrosis is defined as an overproduction and deposition of extracellular matrix (ECM) in the liver due to repeated injury such as chronic viral infections, alcohol addiction and non-alcoholic steatohepatitis (NASH) [7-9]. To date, eradication of suspected causes is the only management to prevent disease progression to liver failure, cirrhosis and hepatocellular carcinoma [7-9]. In the case that the injury is not properly resolved, liver transplantation remains the sole therapy for the end-stage liver disease patients [7-9]. In this review, we provide an overview of the role of ROS in liver fibrosis, as well as promising therapeutic interventions related to oxidative stress.

Effects of ROS-derived oxidative stress on liver fibrogenesis

ROS affect the functionality of several liver-specific cells, and can therefore contribute to the development of fibrosis. Figure 1 illustrates the effect of oxidative stress on hepatocytes, Kupffer cells (KC) and hepatic stellate cells (HSC) during fibrogenesis.

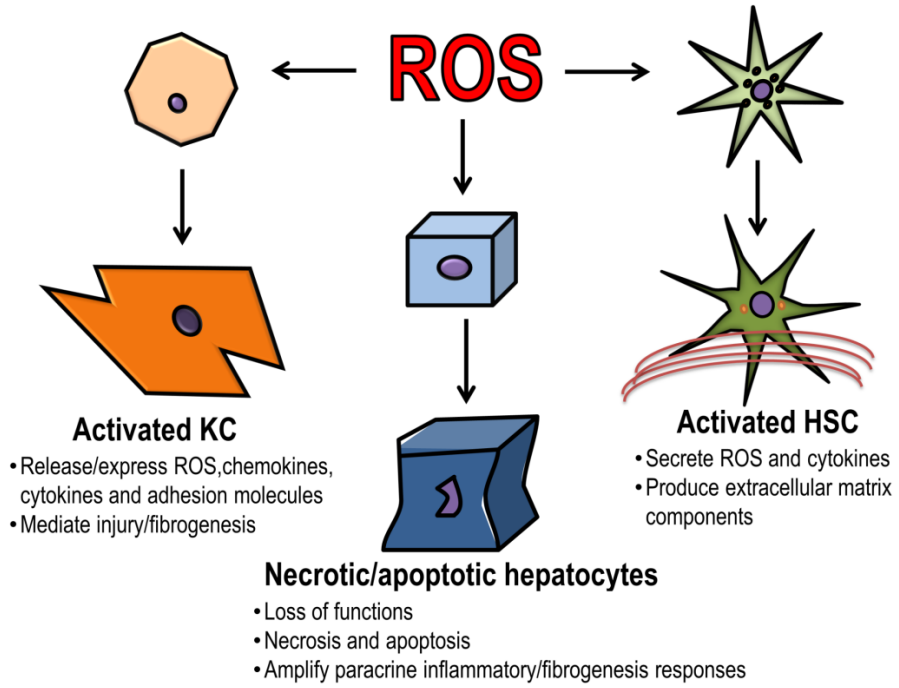

Figure 1: Effects of ROS-derived oxidative stress on liver fibrogenesis (HSC, hepatic stellate cells; KC, Kupffer cell; ROS, reactive oxygen species).

Hepatocytes

Hepatocytes are the central regulators controlling the systemic metabolic demand, electrolyte homeostasis and detoxification processes in the liver [10, 11]. In these parenchymal cells, organelles and enzymes that produce ROS are abundantly present. Once damaged by harmful stimuli, oxidative stress derived from both intracellular ROS sources and extracellular phagocytic cells including KC damages the hepatocytes and induces necrosis or apoptosis [12, 13]. Following hepatocytes' death, several mediators such as tumor necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β) are released to amplify inflammatory and fibrogenesis responses in adjacent hepatocytes, KC and HSC [6, 10]. The oxidative alterations that induce apoptosis and necrosis of hepatocytes have been studied extensively implicating a role of oxidative stress in regulating the cell cycle of hepatocytes [11]. Although hepatocytes are not considered to be the major effector cells responsible for the progression of fibrosis, persistent apoptosis of hepatocytes is sufficient to induce a fibrotic response. This was demonstrated in Bcl-xL-deficient mice, an experimental model of spontaneous apoptosis of hepatocytes, in which the rate of hepatocytes' apoptosis correlated with the progression of liver fibrosis [14]. In addition, treatment with a pan-caspase inhibitor IDN-6556 to inhibit the apoptosis of hepatocytes attenuated liver injury and fibrosis in bile-duct ligated (BDL) mice [15].

Kupffer cells (KC)

KC, liver-specific resident macrophages, not only play a central role in the response to injury but also act as a ROS-generator, mainly by activity of phagocytic NADPH oxidase (NOX) 2 in association with Toll-like receptor (TLR) signaling [16]. Upon activation by pro-fibrogenic stimuli such as alcohol and endotoxins, KC release/express biologically active mediators (chemokines, cytokines, adhesion molecules and ROS) to adjacent hepatocytes and HSC to mediate injury and fibrogenesis [17]. Intercellular communication via ROS was demonstrated in a co-culture model using primary rat KC and HSC where increased proliferation and activation of HSC was observed in HSC/KC co-culture, compared to HSC alone, which was mediated by the production of H_2O_2 by KC [18]. Similarly in a rabbit NASH model, it was found that KC play an important role in the generation of oxidative stress leading to liver steatosis [19]. Furthermore, measurements of oxidative stress in a rat post-ischemic liver model indicated that the paracrine connection between activated KC and hepatocytes was the key event in the induction of oxidative stress prior to the development of liver injury [13]. Additionally, KC were proven to be the central effectors responsible for oxidative stress induced by various pro-fibrogenic toxins such as iron, copper and dichlorobenzene [20, 21].

Hepatic stellate cells (HSC)

Activated HSC are the main producers of ECM during liver fibrogenesis. Although TGF-β, produced by KC and hepatocytes, is regarded as the main activator of HSC [22], ROS also significantly contribute to this process. To illustrate, primary rat HSC proliferation and collagen production could be stimulated by treatment with the culture medium of pro-oxidant ferric nitrilotriacetate complex-treated hepatocytes [23]. In addition, long-term administration of arsenic, which is a metalloid known to induce liver cancer, induced liver fibrosis in mice, where oxidative stress was recognized as the key event in HSC activation [24]. In carbon tetrachloride (CCl_4)-treated rats, expression of alpha smooth muscle actin, a marker of HSC activation, was effectively decreased after treatment with the anti-oxidant vitamin alpha-tocopherol, while it was increased after treatment with ferrous sulfate which is a pro-oxidant agent [25]. Moreover, in isolated rat HSC, prostaglandin F2-like compounds, mediators in lipid peroxidation, stimulated HSC proliferation and ECM production, supporting the role of oxidative stress in HSC activation and fibrogenesis [26]. In addition to the effect of ROS on HSC, it was proposed that HSC may be responsible for the generation of an excess of ROS as both the phagocytic NOX (NOX2) and the non-phagocytic NOX (NOX1 and NOX4) isoforms are expressed in HSC [16, 27].

Since it is clear that ROS-derived oxidative stress plays a negative role in the physiological functions of liver-specific cells (hepatocytes, KC and HSC), which ultimately leads to fibrogenesis of the liver; therefore, mitigating oxidative stress could be a promising therapy for the treatment of liver fibrosis.

Oxidative stress as a therapeutic target

In order to cease oxidative stress, scavenging of ROS has been a mainstay therapeutic strategy for a very long time [28]. Nevertheless, multiple potential intracellular sources that generate ROS by specific enzymatic reactions have been currently identified. These ROS generators are promising targets for the treatment of liver fibrosis. In this section, the molecular and subcellular generators of ROS that play a role in liver injury and may become a therapeutic target are introduced.

Several organelles, *i.e.* mitochondria, peroxisomes and endoplasmic reticulum (ER), produce ROS during normal physiological processes as illustrated in Figure 2. Mitochondria, particularly complex I, II and III of the respiratory electron transport chain, are an essential intracellular source of ROS [29]. ROS generated by mitochondria mediate the release of cytochrome C and pro-apoptotic proteins to initiate cellular inflammation and apoptosis [29]. In addition, mitochondrial β-oxidation of long-chain fatty acids requires oxidative enzymes, such as acyl-CoA oxidase, that possess ROS-generating capacities, and therefore may play a role in cellular injury under certain conditions [30]. Peroxisomes, which play a major role in metabolism of fatty acids, contain similar pro-oxidant enzymes as mitochondria; however, the contribution of peroxisome-derived ROS in liver injury remains unclear [30, 31]. The ER contains two key enzymes responsible for oxidative protein maturation: endoplasmic reticulum oxidoreductin 1 alpha (Ero1α) and protein disulfide isomerase (PDI), and disruption of these enzymes leads to protein misfolding, which contributes to ER stress-associated oxidative stress [32, 33]. ROS-derived oxidative stress in the ER is a consequence of the excessive utilization of reduced glutathione (GSH), the most abundant anti-oxidative molecule in the ER lumen for reducing oxidized misfolded proteins [33]. In addition, improper configured proteins also trigger the release of calcium (Ca^{2+}) from the sarcoplasmic reticulum of the ER to induce the generation of ROS in the mitochondria, thereby causing apoptosis and injury [32, 33].

Next to ROS production in organelles, ROS-generating enzymes can also be found in the cytosol and in the plasma membrane. Enzymes such as NOX, cytochrome P450 (CYP), xanthine oxidase, lipoxygenases, cyclooxygenases, myeloperoxidase and nitric oxide synthases can be a cause of oxidative stress, which also contributes to injury [1, 2]. For example, it was shown that ROS-derived oxidative stress generated by various isoforms of NOX, a membrane-bound enzyme, are deleterious to the liver [16]. Additionally, ROS-generating cytosolic and transmembrane enzymes may work in concert with other fibrosis-related pathways to induce oxidative stress and enhance fibrogenesis. This is well demonstrated in the crosstalk between NOX and TLR, a group of transmembrane receptors responsible for the recognition of microbial components in the innate immune response [34, 35]. This crosstalk was further clarified in a molecular level that the cytoplasmic tail of TLR interacted with the carboxyl terminal region of NOX to mediate ROS production [36]. Interestingly, both NOX and TLR are found in the liver, and ROS generation as a result of this crosstalk may negatively affect liver cell function and contribute to fibrosis [34, 35].

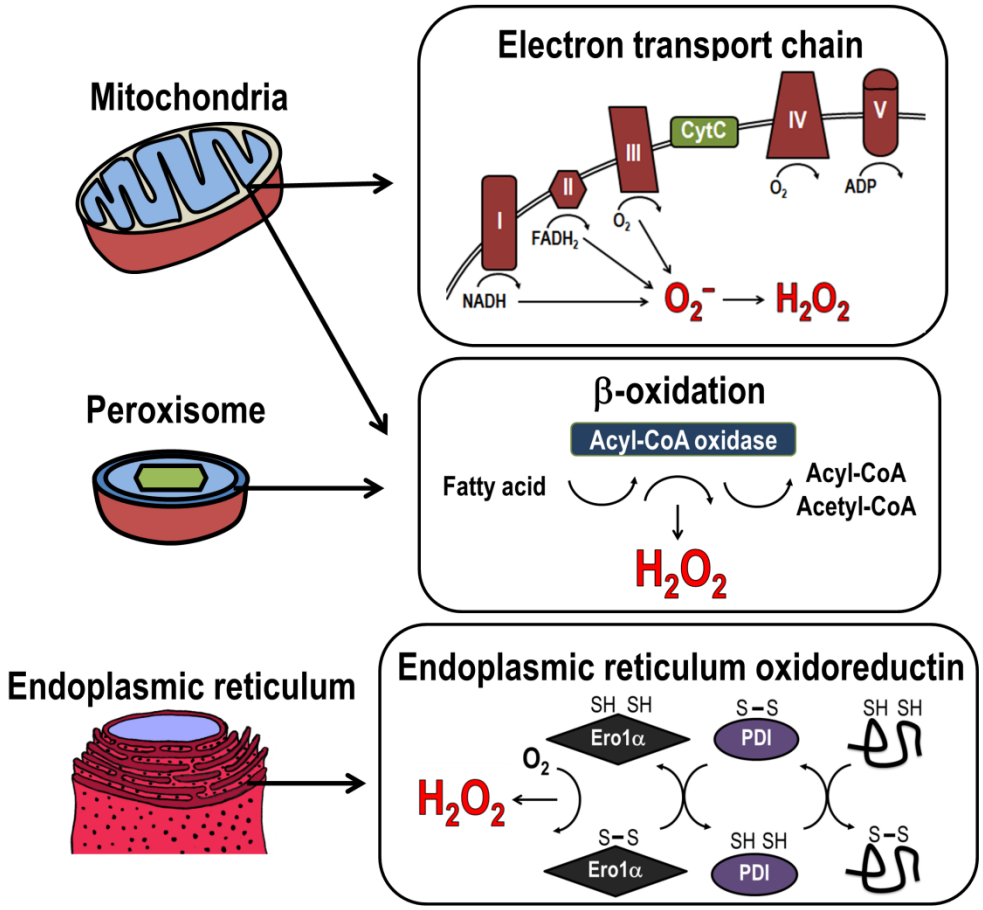

Figure 2: Organelles involved in the generation of ROS (I, II, III, IV, V, mitochondrial respiratory complexes I-V; ADP, adenosine diphosphate; CoA, coenzyme A; CytC, cytochrome C; Ero1α, endoplasmic reticulum oxidoreductin 1 alpha; $FADH_2$, flavin adenine dinucleotide; H_2O_2, hydrogen peroxide; NADH, nicotinamide adenine dinucleotide; O_2, oxygen; O_2^-, superoxide anion; PDI; protein disulfide isomerase).

Presently, the therapeutic potency of drugs inhibiting ROS generated from mitochondria, the ER, NOX and TLR have recently been shown. Therefore, the current status of these drugs for the management of liver fibrosis is described in the next section.

Drugs targeting ROS generators as possibility for the treatment of liver fibrosis

Although the pathogenesis of liver fibrosis has been extensively studied, and new promising therapeutic targets were discovered, to date, none of the potential drugs that target signaling pathways in fibrosis have been clinically approved due to the lack of efficacy [9]. Therefore, characterization of novel targets for the treatment of liver fibrosis is crucial. As oxidative stress plays a significant role in fibrosis progression, inhibiting oxidative stress should be further explored as a therapeutic target. Conventionally, unspecific alleviation of ROS accumulation can be achieved by using anti-oxidant therapy, such as administration of anti-oxidant vitamins; however, their therapeutic efficacy for several pathologies including fibrosis remains debatable [28]. Recently, specific oxidative molecular components, subcellular processes and oxidative-related pathways, such as mitochondrial dysfunction, ER stress, and NOX/TLR activation, have been shown to be an important generator of oxidative stress. Therefore, mitigation of these contributors would be beneficial in the treatment of liver fibrosis. As illustrated in Figure 3, inhibition of these targets may directly or indirectly alleviate oxidative stress and thereby halt fibrosis progression. In this section, the possibility and current status of drugs affecting these targets for the treatment of liver fibrosis are discussed.

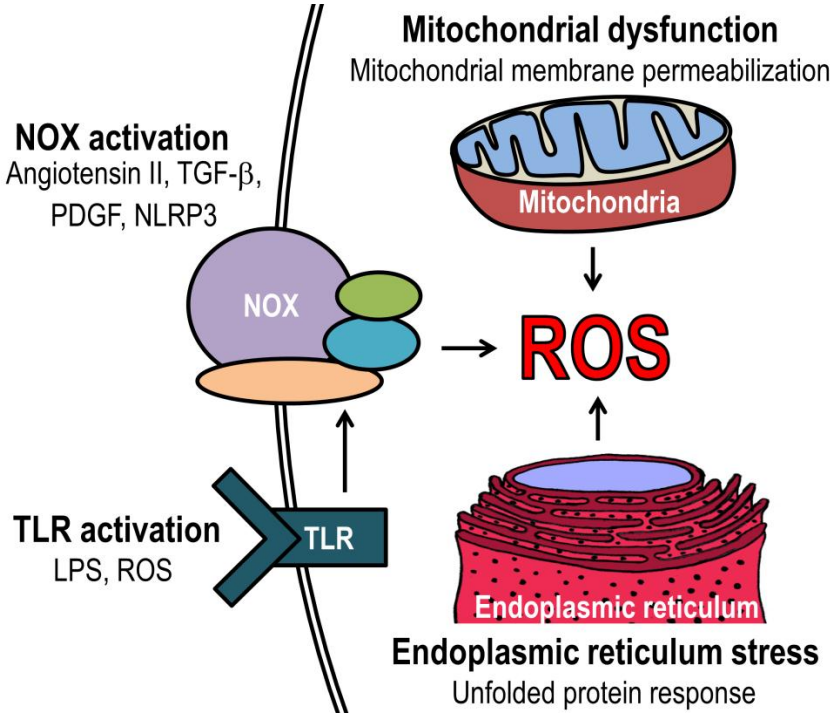

Figure 3: Promising targets for inhibiting oxidative stress in the treatment of liver fibrosis (LPS, lipopolysaccharide; NOX, NADPH oxidase; NLRP3, nucleotide-binding domain, leucine-rich repeat family, pyrin domain containing 3; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; TGF-β, transforming growth factor beta).

Mitochondrial dysfunction inhibitors

Besides their well-known function in cellular energy supply, mitochondria are also involved in signaling, differentiation and homeostasis [29]. These functions of mitochondria are dependent on mitochondrial membrane permeabilization, which is modulated by the concentration of Ca^{2+} [29]. The loss of function of mitochondria, usually characterized as mitochondrial dysfunction, leads to chronic diseases including liver fibrosis [37].

Because of the high number and density of mitochondria in the liver, oxidative stress derived from mitochondrial dysfunction can lead to the pathogenesis of various chronic liver diseases, particularly metabolic disorders, which ultimately cause non-alcoholic fatty liver disease (NAFLD) and NASH [37, 38]. In genetically modified obese-diabetic mice and choline-deficient diet fed rats, the generation of mitochondrial ROS was significantly increased in the liver [39, 40]. In addition, CYP2E1 was up-regulated in mitochondria of various tissues including livers of streptozotocin-induced diabetic rats indicating a role of CYP2E1 in the generation of mitochondria-derived ROS in the liver also [41]. As CYP2E1 is also one of the enzymes responsible for ethanol metabolism, not surprisingly, an association between mitochondrial dysfunction and ROS generation was found in alcoholic liver disease (ALD) [42]. In addition, mitochondrial ROS production was additionally activated due to the increased availability of cytosolic NADH, which is the metabolic product of alcohol metabolism [42]. Furthermore, generation of ROS as a result of mitochondrial dysfunction also plays an essential role in fibrogenesis after HBV and HCV infection [43, 44].

After establishing a role for mitochondrial dysfunction in the pathogenesis of liver diseases, treatment strategies were recently developed to directly supplement the endogenous components required for proper mitochondrial functioning to prevent ROS formation. **Coenzyme Q10** (CoQ10), or ubiquinone, is a major component of the mitochondrial electron transport chain and is widely used as an anti-oxidant supplement. Oral administration of CoQ10 reduced oxidative stress and liver fibrosis in a rat model of poor maternal nutrition [45], and in a mice model of dimethylnitrosamine-induced liver fibrosis [46]. Since CoQ10 does not directly target the mitochondria, **mitoquinone mesylate** was developed as a mitochondria-targeted anti-oxidant. Preclinical experiments showed that mitoquinone mesylate attenuated oxidative stress and liver fibrosis in CCl_4-treated mice and rats, and also in human precision-cut liver slices, an *ex vivo* model of liver fibrosis [47, 48]. Additionally, mitoquinone mesylate alleviated mitochondrial oxidative damage and improved liver function in a phase II study in HCV patients [49].

Mitochondrial function can also be controlled by modulating mitochondrial membrane permeabilization and mitochondria-cytosol Ca^{2+} homeostasis. **Minocycline**, a member of the tetracycline-class of anti-microbial drugs, exhibited beneficial effects in rodent hepatic ischemia/reperfusion injury model through reducing oxidative stress [50, 51], and the protective effect appeared to be related with the modulation of mitochondrial membrane potential due to the Ca^{2+} chelating property of minocycline [52, 53]. However, the observed autoimmune hepatotoxicity of minocycline is a concern [54]. Furthermore, the anti-fibrotic efficacy of **NIM811**, a cyclosporin analogue, which is an inhibitor of the mitochondrial permeability transition pore, was evaluated. It was demonstrated that NIM811 inhibited TGF-β signaling and the expression of fibrosis markers in HSC-T6 cells and in CCl_4-induced liver fibrosis in rats [55]. In addition, treatment of NIM811 in massive hepatectomy mice prevented mitochondrial dysfunction, attenuated liver injury and stimulated liver regeneration [56].

Thus, mitochondrial dysfunction plays a significant role in various liver diseases, and modulating electron transport chain and/or mitochondrial membrane potential could be promising strategy for the treatment of liver fibrosis. Nevertheless, mitoquinone mesylate appears to be the only drug which was evaluated in patients with chronic liver diseases.

ER stress inhibitors

The ER acts as a cellular machinery to facilitate and regulate protein folding, but accumulation of unfolded/misfolded proteins in the ER causes stress and activates the unfolded protein response (UPR) pathway [57]. UPR activation mainly reduces ER stress by halting protein translation, degrading deformed proteins and increasing the repair of unfolded proteins; however, sustained UPR activation leads to ROS formation and apoptosis [57]. Ero1α and PDI are essential enzymes responsible for the generation of disulfide bonds of proteins in the ER [32, 33]. Together with Ero1α and PDI, reduced level of GSH, disruption of the mitochondrial electron transport chain proteins, which subsequently affect cytoplasmic/mitochondrial/ER Ca^{2+} homeostasis, and also NOX4 play a crucial role in ER stress-induced production of ROS [32, 33]. Disruption of these enzymes and corresponding network will contribute to ER stress-associated ROS, which is a mediator of signal transduction in various oxidative stress-related diseases including liver fibrosis, particularly ALD and NAFLD [58]. In ALD, it was found that the oxidative stress markers: GSH utilization and protein glutathionylation, were significantly increased in hyperhomocysteinemia, a common manifestation in patients with alcoholic steatohepatitis [59]. In NAFLD, the contribution of free fatty acids, a harmful factor in the pathogenesis of NAFLD and NASH, to ER stress was recognized. It was demonstrated in primary rat hepatocytes and H4IIEC3 cells, a rat hepatoma cell line, that palmitic acid, the most abundant saturated fatty acid in the human body, triggered oxidative stress and Ca^{2+} release from the sarcoplasmic reticulum, thereby depleting Ca^{2+} storage in the ER. This alteration of Ca^{2+} homeostasis impaired the protein folding function of enzymes including Ero1α [60, 61]. Additionally, it was found that ER stress and the UPR were activated in obese-patients who were diagnosed with NAFLD [62].

ER stress-associated production of ROS can be mitigated by drugs affecting enzymes responsible for ROS production in the ER. A specific small molecule inhibitor against Ero1α, **EN460**, was found in a high throughput activity assay to evaluate potential inhibitors of mammalian Ero1α. EN460 inhibited Ero1α, promoted the UPR and exhibited protective effect during chemically induced ER stress [63]; however, to the best of our knowledge, no further studies on EN460 were published in the international literature. Interestingly, inhibiting PDI appeared to be deleterious as ER stress was increased contributing to apoptosis possibly due to the disruption of the oxidative networks in the electron transmission of the ER [64, 65]. Nevertheless, the negative impact on ER stress may also derive from unidentified off-target effect of the tested PDI inhibitors used, namely oxidized low-density lipoproteins and small molecule CCF642 [64, 65].

In addition, modulating non-oxidative enzymes via ER stress sensors which mediate the UPR can be effective targets to reduce ER stress [66]. **GSK2606414** and **GSK2656157**, inhibitors of protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), which is an ER stress sensor, were found to inhibit the UPR-mediated pathway and reduce oxidative stress [66]. GSK2606414 showed neuroprotective effects against prions in mice; however, weight loss and elevated blood glucose levels indicated an adverse effect on pancreatic function [67]. **Salubrinol**, a direct inhibitor of the PERK-eIF2α signaling pathway, significantly reduced apoptosis in hepatic cells of brain-death rats [68]. Salubrinol was experimentally used to protect against various xenotoxicant-induced cellular damages [69]; however, this strategy was questioned, as it aggravated cisplatin-induced oxidative stress and nephrotoxicity in mice [70]. **4-phenylbutyrate**, an orphan drug, was approved for the treatment of urea cycle disorders due to its activity to modulate protein maturation, and it is also used for the treatment of cystic fibrosis, a protein misfolding disease [71]. 4-phenylbutyrate was effective in the prevention of steatohepatitis in mice-fed with dietary trans-fatty acid plus fructose through amelioration of ER stress [72]. Moreover, 4-phenylbutyrate exhibited protective effect in liver ischemia/reperfusion injury models via an ER stress-dependent mechanism [73, 74].

Nevertheless, at this time, none of the above-mentioned ER stress inhibitors have been clinically evaluated for the treatment of liver fibrosis.

NOX-inhibitors

NADPH oxidases, abbreviated as NOX, are membrane-bound enzymes that generate ROS by transferring electrons from NADPH to molecular oxygen. Beyond a role in phagocytic cells, physiologic and pathophysiologic roles of NOX have been demonstrated [75].

The associations between NOX and liver fibrosis have been studied extensively. NOX1$^{-/-}$ and NOX4$^{-/-}$ mice exhibited less oxidative stress, inflammation, injury and fibrosis in the liver after CCl_4-treatment when compared to wild-type mice [27]. In addition, the expression of NOX2 subunits (P22phox, P40phox and P67phox) was increased in western diet-induced fatty livers in mice and correlated with the degree of liver steatosis [76]. After BDL in rats, NOX4 mRNA was up-regulated, and furthermore in CCl_4-treated or BDL mice, elevated hepatic NOX1 and NOX2 mRNA and protein levels were found [77]. Moreover, phagocytosis of apoptotic bodies in LX-1 cells, a human HSC cell line, activated NOX, which in turn is associated with liver fibrosis [78]. This association was supported by immunohistochemical analysis showing that NOX4 protein expression was dramatically increased in the livers of NASH patients suggesting a role of NOX in the pathogenesis of this disease [79]. Thus, HSC appear to play a major role in NOX-mediated ROS generation, and NOX1, NOX2 and NOX4 are most likely the main isoforms associated with liver fibrosis [16, 80].

Crosstalk between NOX and other fibrogenesis-related proteins such as TLR, angiotensin II, TGF-β, platelet-derived growth factor (PDGF) and nucleotide-binding domain, leucine-rich repeat family, pyrin domain containing 3 (NLRP3) has been demonstrated. Angiotensin II activation increased NOX4 protein expression and H_2O_2 production in mouse primary hepatocytes and in a human hepatocyte cell line [81]. In primary rat HSC, angiotensin II-induced TGF-β and ROS production appeared to be dependent on the activity of NOX [82]. Treatment of rat HSC-T6 cells with PDGF increased the expression of NOX2 and ROS [83]. In macrophages, NOX4-mediated fatty acid oxidation promoted the formation of a NLRP3 inflammasome, a component of the innate immune system that plays a role in fibrosis [84]. Moreover, in human cirrhotic livers, NLRP3 and NOX4 were found to co-localize implicating an associated role in the progression of liver fibrosis [77].

Due to the clear association between NOX and fibrosis, inhibiting NOX has become an interesting target to alleviate oxidative stress, and drugs affecting NOX activity have been shown to attenuate fibrosis. **GKT137831**, a dual NOX1/NOX4 inhibitor, suppressed the production of chemokines, inhibited HSC activation and attenuated fibrosis after lipopolysaccharide (LPS) induced injury in primary mouse HSC [27]. Another study confirmed the effect of GKT137831 using multiple *in vitro* and *in vivo* models of liver fibrosis demonstrating a decrease in ROS production, inflammation and fibrogenesis [85]. Also several natural-derived agents were shown to exhibit promising anti-fibrotic potency via the interruption of NOX activity. In Ldlr$^{-/-}$ mice, a genetically modified NAFLD model, dietary supplementation of microalgae-derived **docosahexaenoic acid** reversed the western diet-induced up-regulation of NOX2 and attenuated hepatic fibrosis [76]. **Decursin**, isolated from roots of *Angelica gigas* Nakai., decreased HSC activation, attenuated liver fibrosis and ameliorated liver injury in CCl_4-treated mice via TGF-β- and NOX-dependent inhibition [86]. **Chlorogenic acid**, a phenolic compound found in coffee, fruit and vegetables, inhibited PDGF-induced NOX expression and ROS production in HSC-T6 cells. Moreover, in CCl_4-treated rats, chlorogenic acid attenuated liver fibrosis via up-regulation of NFE2L2 which is a transcription factor that regulates the expression of

anti-oxidant enzymes [83]. In chronic HCV patients treated with **losartan**, an angiotensin II receptor blocker commonly used for management of hypertension and congestive heart failure, expression of pro-fibrogenic and NOX genes were decreased [87]. Thus, inhibition of the crosstalk between NOX and other pathways might be beneficial in the alleviation of oxidative stress and thereby the treatment of fibrosis.

Among known drugs designed to specifically inhibit NOX, GKT137831, named GKT831 currently, should be considered the most advanced drug in development. Although the clinical efficacy of GKT831 was not impressive in patients with diabetic kidney disease, this study showed that GKT831 significant reduced liver enzyme and inflammatory marker levels, with attractive safety profile (clinicaltrials.gov: NCT02010242). Therefore, a phase 2 study is now started to evaluate the therapeutic efficacy of GKT831 in the treatment of primary biliary cholangitis (clinicaltrials.gov: NCT03226067).

Drug affecting TLR

Toll-like receptors (TLR) are a class of transmembrane proteins recognizing structurally conserved molecules derived from microorganisms. TLR are usually expressed in phagocytic cells including KC to act as a sensor in physical barriers such as the skin and intestinal mucosa. Essential roles of TLR in the innate immune system have been clearly recognized [34]. There are many subtypes of TLR found in humans, and their association with oxidative stress has been demonstrated [35]. As mentioned earlier, crosstalk between TLR and NOX is associated with fibrogenesis.

ROS may directly activate TLR and induce liver injury. For example, it was shown that superoxide acted through TLR4, which subsequently activates NOX, leading to inflammation and injury after liver ischemia/reperfusion in mice [88]. Beyond direct activation by ROS, lipopolysaccharide (LPS), a membrane component of gram-negative bacteria that is an agonist of TLR4, also induced inflammation and oxidative stress thereby contributing to liver steatosis as observed in liver samples obtained from NASH patient after pancreaticoduodenectomy [89]. The mechanism of LPS-induced ROS generation is still unknown; however, it is suggested that NOX4 is essential for LPS-triggered ROS production and inflammatory response through a direct interaction with TLR4 [90]. Moreover, alcohol induced oxidative stress in mouse livers by up-regulating TLRs [91]. In addition, high mobility group box 1 (HMGB1) released from hepatocytes in response to hypoxia was dependent on TLR4-induced ROS production and downstream Ca^{2+}/calmodulin-mediated signaling [92, 93].

Inhibition of TLR-mediated ROS in the mitigation of liver fibrosis was demonstrated using various natural-derived agents. Interestingly, the activity of natural-derived agents was predominantly on TLR4 inhibition. **Curcumin**, the principal phenolic compound of turmeric, reduced high-fat diet-induced NASH and oxidative stress in mice possibly via inhibition of HMGB1-induced TLR4 signaling [94]. **Pomegranate** extract inhibited sepsis-induced oxidative stress and liver injury in rats by inhibiting TLR4 signaling and inflammation [95]. **Quercetin**, a natural flavonoid, ameliorated hepatic oxidative stress in CCl_4-treated mice possibly due to inhibition of the TLR2/TLR4 pathway [96]. In another

study, it was found that the anti-inflammatory, anti-oxidative and hepatoprotective properties of quercetin in methionine-choline deficient diet-induced NASH mice model was due to interference with TLR signaling [97]. ***Lactobacillus plantarum*** **NDC 75017**, a potential probiotic, showed hepatoprotective effects against LPS-induced oxidative stress and inflammation in mice via inhibition of the TLR4/NF-κB pathway [98]. Similarly, **bicyclol**, an anti-hepatitis drug available in China, also exhibited hepatoprotective effects and attenuated oxidative stress during liver injury in mice via inhibiting the TLR4/NF-κB pathway [99]. **Asiatic acid** from *Potentilla chinensis* attenuated alcohol-induced liver injury in rats by reducing oxidative stress and inhibiting KC activation by down-regulating TLR4 signaling [100]. **(-)-Epigallocatechin-3-gallate**, a polyphenolic compound in green tea, rescued concanavalin-A-induced liver injury in mice by inhibiting TLR2, TLR4 and TLR9 signaling resulting in reduced ROS production and increased anti-oxidant capacity [101]. In Otsuka Long-Evans Tokushima Fatty (OLETF) rats, alpha-lipoic acid inhibited TLR4/HMGB1 signaling and downstream inflammation, reduced ROS production and increased the anti-oxidant capacity of the liver [102]. Although TLR4 signaling was closely related to oxidative stress, this association was likely mediated via interleukin-1 receptor associated kinase-1 (IRAK-1) [103].

Besides specific interactions with TLR4, other natural-derived agents also reduced oxidative stress via unidentified TLR-subtypes or various TLR-related pathways. ***Agrimonia eupatoria*** water extract ameliorated chronic alcohol-induced liver injury in rats probably via down-regulating TLR-signaling and suppressing oxidative stress [104]. ***Lonicera caerulea*** berry extract suppressed inflammation via TLR and oxidative stress-associated mitogen-activated protein kinase signaling in the livers of LPS-treated rats [105]. **Limonin**, from citrus fruits, exhibited hepatoprotective, anti-oxidative and anti-inflammatory effects by down-regulating TLR-signaling after liver ischemia/reperfusion in rats [106]. **Aloin**, a major anthraquinone extracted from *Aloe ferox* Mill. and *Aloe vera* L., ameliorated alcoholic liver injury in mice by reducing lipid accumulation, oxidative stress and LPS-induced inflammatory responses [107]. **Polyenephosphatidylcholine** showed anti-inflammatory and anti-fibrotic effects in ethanol-fed mice possibly due to inhibition of NOX4-mediated ROS production, amplification of LPS-mediated signaling and down-regulation of TGF-β signaling [108].

Even though the earlier described natural-derived compounds can inhibit TLR4 or associated signaling pathways and attenuate ROS formation, liver inflammation and/or fibrosis, unfortunately, these compounds have not been systematically tested in human yet.

Challenges for targeting oxidative stress in the management of liver fibrosis

Despite the potential of inhibition of oxidative stress for the treatment of liver fibrosis, clinical development of these promising drugs faces several challenges. In this section, two major challenges; maintaining physiological needed ROS and monitoring individual's redox state, are demonstrated.

Maintaining ROS needed for physiological processes

Even though oxidative stress is involved in the pathogenesis of numerous diseases, ROS can also be beneficial. In the innate immune system, neutrophils utilize NOX-derived ROS for attacking and killing pathogens [109]; therefore, the effect of NOX inhibitors on neutrophil function may be of concern and needs to be further elucidated. Additionally, low levels of ROS appear to positively affect aging processes by modulating mitochondrial hormesis, also known as mitohormesis. In mitohormesis, ROS were found to function as signaling molecules to prevent and delay a number of chronic diseases, thereby extending the lifespan of various species including mice [110]. The opposing functional roles of ROS complicate the choice of agents to efficiently target oxidative stress for the treatment of various diseases including fibrogenesis of the liver. By using systemic anti-oxidant therapy for the treatment of liver fibrosis, alleviation of oxidative stress in the liver may be accompanied by the inhibition of beneficial ROS in other tissues [1-3]. Furthermore, determining the cellular specificity of anti-oxidative agents is a challenge. As discussed earlier, availability of mitoquinone mesylate tends to be higher in the liver due to the high number and density of mitochondria when compared to other tissues where mitochondria are less abundant [47-49]. Thus, liver specific anti-oxidants would be a valuable therapy when liver-specific molecular or subcellular targets are characterized [111]. Additionally, modern drug delivery technologies such as using protein carrier or polymeric nanoparticles containing the active drug inhibiting oxidative stress that directly target liver cells might be a successful approach [112, 113].

Monitoring individual's redox state

Due to the duality of ROS function, an accurate determination of an individual's redox state, ideally in the liver, is required to correctly administer drugs that target oxidative stress. In order to clinically assess the redox status, easy applicable and non-invasive biomarkers are necessary [114]. It seemed that using biomarkers of lipid peroxidation such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) were frequently accepted for measurements of oxidative stress in clinical practice [115, 116]. Nevertheless, none of these promising candidates are acknowledged as a surrogate biomarker [114]. Sensitivity, specificity and reproducibility of oxidative stress biomarkers are important issues. For instance, in the colorimetric measurements of plasma MDA levels, invalid results may be obtained due to the interference with other plasma components [117, 118]. Recently, unreliable results of MDA measurements were observed in healthy people and psychiatric disorder patients illustrating the shortcoming of this popular biomarker of oxidative stress [119]. Ideally, identification of biomarkers that exclusively reflect the liver redox status would be practical; however, this desire seems difficult to achieve since a liver-specific biomarker is unavailable [120]. Therefore, clinical manifestations of liver injury should always be simultaneously assessed with biomarkers of oxidative stress. This principle was used in the evaluation of the oxidative stress in patients with a chronic fascioliasis infection for optimization of individual’s therapeutic strategy [121].

Finally, although numerous studies have been conducted to explore the role of ROS, oxidative stress and anti-oxidants in health and disease, proper management for the complicated regulation of ROS is still under debate [1-3]. Therefore, more studies must be performed to elucidate the optimal redox status of the liver. Also new strategies to directly target oxidative stress in the liver need to be further explored. Such studies will accelerate the development of therapeutic modalities to reduce oxidative stress and consequently liver fibrosis.

Conclusion

ROS are generated in various molecular processes and organelles. Oxidative stress affects several cellular functions of the liver and plays a significant role in the progression of liver fibrosis. Today, novel drugs that directly target oxidative pathways, particularly ROS generators, that is, inhibitors of mitochondrial dysfunction, endoplasmic reticulum stress and NOX, and drugs affecting TLR, are promising new therapies to modulate oxidative stress. Nonetheless, the effectiveness of these promising drugs is mostly acknowledged in preclinical stage, and systematically evaluation in patients are awaiting in the future. In addition, it should be noted that numerous challenges need to be overcome before targeting the complicated redox status for the treatment of liver fibrosis.

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Part B

Development of novel disease models

Supplementary box IV

Metabolic syndrome

- Metabolic syndrome is a cluster of risk factors that can lead to diabetes, heart disease, stroke and other serious health problems. Noticeably, prevalence of metabolic syndrome is increasing globally [1, 2].
- Western diet, increased age, obesity, sedentary lifestyle, insulin resistance, genetics, stress and excessive alcohol use can contribute to the development and progression of metabolic syndrome [3].
- Metabolic syndrome is diagnosed when any three of the following five risk factors are present: high blood glucose, low levels of high-density lipoprotein (HDL), high levels of triglycerides, large waist circumference and high blood pressure [3].
- The first line treatment of metabolic syndrome is lifestyle modification, in particular, eating a healthy diet, losing weight (especially reducing central obesity), increasing physical activity and treating individual risk factors [3].

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