Hepatic stellate cells and oxidative stress

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ABSTRACT

Hepatic fibrosis is a wound-healing response that takes place during chronic liver injury and is characterized by excessive production and deposition of extracellular matrix (ECM) components, mainly collagen type I. Hepatic stellate cells (HSC) are responsible for the excessive production of scar tissue during liver fibrosis. Activation of HSC, the main step in the development of hepatic fibrosis, is mediated by cytokines and reactive oxygen species (ROS) released by damaged hepatocytes and/or activated Kupffer cells and even HSC themselves. While HSC usually remain quiescent, in response to factors promoting liver injury they undergo activation and become highly proliferative and fibrogenic. Indeed a key feature of HSC activation is uncontrolled production of collagen type I. Collagen is a heterotrimeric protein composed of two α1 chains and one α2 chain forming a triple helix structure. Initiation of HSC activation is largely due to paracrine stimulation, whereas the perpetuation of such activated state involves autocrine as well as paracrine loops. This review focuses on the role of oxidant stress on the activation of stellate cells.

Key words: Hepatic stellate cells. Fibrosis. Extracellular matrix. Reactive oxygen species.

ABBREVIATIONS (in alphabetical order)

Alcoholic liver disease (ALD); arachidonic acid (AA); collagen α1 (I) promoter (COL1A1); collagen α2 (I) promoter (COL1A2); cyclooxygenase (COX); extracellular matrix (ECM); 4-hydroxyhexenal (4-HHE); 4-hydroxy-ynonenal (4-HNE); hepatic stellate cells (HSC); cytochrome P450 2E1 (CYP2E1); platelet-derived growth factor (PDGF); prostaglandin E2 (PGE2); polyunsaturated fatty acids (PUFA); reactive oxygen species (ROS); su-
peroxide anion (O$_2^-$); Transforming growth factor-β (TGF-β); and tumor necrosis factor-alpha (TNF-α).

**OXIDATIVE STRESS**

Generation of reactive oxygen species (ROS) during alcohol metabolism is suggested as one of the major mechanisms of alcohol-induced liver injury (1). ROS are considered hepatotoxic because of their potential to react with most cellular macromolecules, inactivating enzymes, causing DNA damage and posttranslational modifications of proteins, and inducing lipid peroxidation reactions, which result in disruption of biological membranes (2).

Among the different sources of ROS production in the cell are mitochondria, cytochrome P450s, NADPH oxidase, and xanthine oxidase, and the enzymes involved in the arachidonic acid pathways, such as lipoxygenase and cyclooxygenase (COX) (3-5). One of the major sources of ROS production in the cell is the mitochondrial respiratory chain, which utilizes approximately 80 to 90% of the O$_2$ consumed in humans (6). Even though only a small percentage of O$_2$ is then converted into ROS-producing oxidase (16) thereby enhancing oxidative stress. Other sources of ROS in the body.

Another major source of ROS, especially in the liver, is the cytochrome P450 mixed-function oxidases (7). Some cytochrome P450 enzymes are also important for metabolizing substrates naturally present in the body, such as fatty acids, cholesterol, steroids, or bile acids (8). The biochemical reactions catalyzed by the cytochrome P450 molecules use O$_2$, and during these reactions ROS are generated; however, the extent of ROS generation may vary considerably depending on the metabolite being degraded and the cytochrome P450 molecule involved (9).

The cytochrome P450 isofrom 2E1 (CYP2E1) is especially active in producing ROS, as it is an uncoupled protein which generates ROS even in the absence of any added substrate (10-12). This enzyme is of particular interest in the development of alcoholic liver injury as its activity increases after alcohol consumption (13), CYP2E1 protein is stabilized by alcohol itself (14), and alcohol is metabolized by CYP2E1 (10,15).

ROS are also produced by xanthine oxidase (12). Under physiological conditions, xanthine oxidase acts as a dehydrogenase removing hydrogen from xanthine or hypoxanthine transferring it to NAD to generate NADH. However, under certain conditions, such as the disruption of blood flow and alcohol consumption, xanthine dehydrogenase is converted to a ROS-producing oxidase (16) thereby enhancing oxidative stress. Other sources of ROS in the body are macrophages and neutrophils (17). Both of which express NADPH oxidase, which, when activated, generates O$_2^-$ which later on becomes decomposed to H$_2$O$_2$.

Oxidative stress usually reflects the balance between the rate of production, the rate of removal of ROS, and the subsequent repair of damaged cellular macromolecules and membranes (18). Enzymatic and non-enzymatic antioxidant mechanisms are available to protect cells against ROS. Among them are: a) superoxide dismutases (SOD1 and SOD2) which dismutate O$_2^-$ and convert it into H$_2$O$_2$ and O$_2^-$; b) catalase and the glutathione peroxidase system which decompose H$_2$O$_2$; c) glutathione S-transferases which can remove reactive intermediates and lipid aldehydes; d) metallothioneins, heme oxygenases, thioredoxins; ceruloplasmin and ferritin which help to remove metals such as iron, which promotes oxidative reactions; and e) non-enzymatic, low molecular weight antioxidants such as glutathione, vitamins A, C and E, ubiquinone, uric acid, bilirubin (19,20).

**STELLATE CELL ACTIVATION AND LIVER FIBROSIS**

HSC have been the central focus in efforts to identifying the source of ECM since they undergo activation during liver injury of many different etiologies (21). Their activation is characterized by the development of a proliferative, contractile, migratory, fibrogenic, and inflammatory phenotype (22) (Fig. 1).

Indeed, the model of sequential stellate cell activation to myofibroblasts has provided a robust conceptual framework, allowing new advances to be placed in a clear biological context. Recent studies have underscored the heterogeneity of mesenchymal populations in liver ranging from classic HSC to portal fibroblasts, with the variable expression of neural, angiogenic, contractile and even bone-marrow-derived markers (23). Moreover, experimental genetic marking of HSC, by expressing fluorescent proteins downstream of either fibrogenic or contractile gene promoters, illustrates the plasticity of the fibrogenic cell populations in vivo (24). In view of this capacity for transdifferentiation between different mesenchymal cell lineages and possibly even epithelial cells, the issue is not necessarily where fibrogenic cells arise from, but rather whether they express target molecules such as receptors or cytokines in sufficient concentrations in vivo to merit their targeting by diagnostic agents or antifibrotic compounds (25).

Following liver injury of many different etiologies HSC become activated, undergoing a transition from quiescent cells to proliferative, fibrogenic, and contractile myofibroblasts (26). The initial events involve rapid changes in gene expression and phenotype rendering the cells responsive to cytokines and ROS (26). They are a results from paracrine stimulation due to rapid, disruptive effects of liver injury on the homeostasis of neighbouring cells and from early changes in ECM (27,28). Stimuli initiating HSC activation derive
mostly from injured hepatocytes, Kupffer cells, and endothelial cells in addition to rapid, subtle changes in ECM composition (27,28).

Hepatocytes and Kupffer cells are a potent source of ROS and reactive nitrogen species, which exert paracrine stimulation on HSC (27-31). Moreover, their activity is amplified in vivo by depletion of antioxidants as typically occurs in liver disease (32). Kupffer cells produce cytokines and growth factors, key molecules participating in HSC activation (33). Endothelial cells play a dual role in early HSC activation: injury to sinusoidal endothelial cells stimulates production of a splice variant of cellular fibronectin, which has an activating effect on HSC (34). Endothelial cells also convert latent TGF\(\beta\) to the active fibrogenic form through the activation of plasmin (35).

The perpetuation of such activation involves cellular events that amplify the activated phenotype through enhanced cytokine expression and responsiveness; this component of activation results from autocrine and paracrine stimulation, and from accelerated ECM remodelling (26,36,37). Enhanced cytokine responses occur through multiple mechanisms (35), among these, increased expression of cell membrane receptors and enhanced signalling are especially important (38). Receptor tyrosine kinases, which mediate many of the stellate cell responses to cytokines, are broadly up-regulated during liver injury (39). Continued ECM remodelling during this phase underlies virtually all cellular responses characterizing increasing liver injury. The low density subendothelial matrix is progressively replaced by one rich in fibril-forming collagen. This fundamental shift in ECM composition affects the behaviour of hepatocytes, sinusoidal endothelium, Kupffer cells, and HSC (26,29,40).

**OXIDATIVE STRESS IN THE PROGRESSION OF SCAR FORMATION AND ACCUMULATION**

Hepatic ECM synthesis is typically associated with activated HSC. In addition, with the progression of the fibrogenic response, qualitative and quantitative changes in ECM may also occur, as HSC not only synthesize new ECM proteins but also produce metalloproteinases leading to the disruption of the normal physiological matrix (41-45).

As far as HSC-dependent ECM synthesis, detailed analysis of the different mediators and their sources has depicted a main role of mesenchymal cells in the expression of TGF\(\beta\)1 and the other fibrogenic cytokines (46). In the early steps of excessive fibrogenesis, mediators like PDGF and TGF\(\beta\)1 are only produced by Kupffer cells, sinusoidal endothelial cells, and hepatocytes, so that a paracrine loop with HSC as a potential target is likely to occur (37). If the fibrotic process is sustained, HSC may synthesize such mediators in rather large amounts maintaining and amplifying their own fibrogenic activity through an autocrine loop (26).

An increasing volume of literature suggests the involvement of H\(_2\)O\(_2\) in the induction of the COL1A1 promoter under TGF\(\beta\) treatment (47,48). Thus, the idea that both the expression and the activity of selected profibrogenic cytokines is significantly modulated by ROS-dependent reactions, is gaining relevance (47,48). Lipid peroxidation-derived reactions are also involved in the profibrogenic response (28-30). Acetaldehyde has also been demonstrated to have profibrogenic actions (49,50). ROS (H\(_2\)O\(_2\)) and lipid peroxidation products [hydroxy-2,3-nonenal (4-HNE), 4-hydroxyhexenal (4-HHE), malondialdehyde (MDA)] can easily diffuse through the plasma membrane (51,52); therefore it is likely that dif-

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**Fig. 1.** Primary HSC isolated from control rats. Light micrographs were taken at 0 days (A) and 6 days (B) (200x).
...and LPO-derived products and not only cytokines and growth factors may also impact on the HSC in vivo to induce a fibrogenic response (28,30,32,53). Moreover, supporting evidence is provided by work from Jezequel's et al. (32) suggesting increased proliferation and elevated collagen type I synthesis in rat HSC cultured in conditioned medium derived from hepatocytes undergoing stimulated lipid peroxidation, and the work of Nieto et al. showing that HepG2 cells overexpressing CYP2E1 release ROS-derived from CYP2E1 metabolism which impact on the HSC triggering HSC activation, proliferation, and a profibrogenic response as assessed by α-smooth muscle actin and collagen I expression as well as the rate of incorporation of methyl[3H]-thymidine into the DNA of HSC (28,54).

The signaling from CYP2E1-derived ROS triggering activation of HSC may play a role in alcohol-induced liver injury and fibrosis (27,28). Similarly recent studies using primary Kupffer cells in co-culture with HSC have demonstrated a profibrogenic mechanism whereby Kupffer cell-derived H2O2 may up-regulate COL1A1 and COL1A2 transactivation and simultaneously prevent collagen I protein degradation via α IL6-dependent mechanism (53).

PARACRINE EFFECTS ON STELLATE CELLS

Paracrine effect of hepatocytes

There is considerable interest in the role of oxidative stress and the generation of ROS in the mechanism by which ethanol is hepatotoxic (55). A major advance has been the development of the intragastric infusion model of ethanol feeding in which induction of CYP2E1 occurs along with significant alcoholic liver injury (56,57). In this model, the ethanol-induced liver pathology correlates with CYP2E1 levels and elevated lipid peroxidation (58). Understanding how CYP2E1-derived oxidative stress stimulates the fibrogenic response in liver could be of value in ameliorating some of the toxic effects of alcohol.

Alcohol metabolism via CYP2E1 leads to ROS production and lipid peroxidation-end products (58,59). Acetaldehyde, ROS, and long-chain polyunsaturated fatty acids can activate HSC in a paracrine mode (27,28). Acetaldehyde induces COL1A1 and COL1A2 through a TGFβ-dependent mechanism (49,60). TGF-β is considered to be the most potent pro-fibrogenic cytokine (35,61). It suppresses hepatocyte proliferation, stimulates HSC activation, promotes ECM production, and mediates hepatocyte apoptosis (62). ROS and lipid peroxidation products MDA, 4-HNE, and 4-HHE can increase collagen type I production in HSC (29,63). ROS also modulate the binding of transcription factors (e.g. c-Jun/AP1, NFκB, Sp1, and Smads) which modulate COL1A1 and COL1A2 transactivation in HSC (63).

Direct stimulation of HSC proliferation and collagen type I protein synthesis by products generated from hepatocytes is a leading hypothesis to account for iron- and alcohol-related fibrosis (64,65). Several studies have evaluated the role of conditioned medium from hepatocytes in stimulating HSC. Chen et al. (66) found an inhibitory effect of medium from murine hepatocytes on HSC proliferation; Gressner et al. (37) showed a strong stimulation of HSC proliferation in 0.2% fetal bovine serum (FBS) during a 48-hour coculture with parenchymal, rat hepatoma, and human hepatoma cells. Faouzi et al. (67) found that tumoral rat hepatocyte–conditioned medium induces the activation of rat HSC in culture, and Hu et al. (68,69) showed that conditioned medium from carbon tetrachloride-treated hepatocytes induces HSC activation. In cocultures of freshly isolated hepatocytes and a liver HSC line, ethanol induced COL1A1 mRNA in a dose- and time-dependent manner via its metabolism by alcohol dehydrogenase (70).

Paracrine effect of Kupffer cells

Kupffer cells are resident macrophages in the liver that account for 70-80% of total body macrophages. They provide the first line of defense in the organ that receives nutrient-rich splanchnic and arterial blood. They offer defensive mechanisms against invading microorganisms and function as the major site for endotoxin clearance (71). They release a wide array of soluble mediators, including reactive species such as O2•−, H2O2, and nitric oxide, cytokines, chemokines, growth factors, cyclooxygenase and lipoxigenase metabolites, all of which provide physiologically diverse and pivotal paracrine effects on all other liver cell types (72,73).

Kupffer cells are also central to the liver homeostatic response to injury. Upon degenerative changes in hepatocytes, Kupffer cells immediately respond to the insult and release mediators to orchestrate inflammatory and reparative responses (74). Kupffer cell-derived TNFα along with other cytokines and unknown mediators stimulate HSC expression of matrix metalloproteinases MMPs which can degrade the perisinusoidal matrix, allowing migration and proliferation of HSC to set a stage for scarring (75). Thus, the homeostatic responses are initiated by Kupffer cell-derived mediators at the cellular level and underlie the liver’s defense and reparative mechanisms against injury (76).

Influx of Kupffer cells coincides with the appearance of HSC activation markers (e.g. PDGFRβ and α-smooth muscle actin) (77). Kupffer cells may stimulate matrix synthesis, cell proliferation, and release of retinooids by HSC through the actions of cytokines and reactive species (78). They can also influence HSC through secretion of MMP-9 because it can activate latent TGFβ1, stimulating HSC collagen I synthesis (79). Lastly, Kupffer cells generate ROS either via NADPH oxidase, xan-
The effect of endotoxin on Kupffer cell biology is a key event in the pathogenesis of alcoholic liver fibrogenesis (84). In Kupffer cells, O$_2^•$ is also produced during the oxidative burst of phagocytic activity (31). A membrane respiratory burst oxidase catalyzes one-electron reduction of O$_2$ to O$_2^•$ at the expense of reduced form of NADPH (85). Another radical species, nitric oxide, is produced in Kupffer cells from L-arginine by catalysis of nitric oxide synthase after a different set of stimuli, including endotoxin, and the combined addition of PGE$_2$ and TNF-α (86). Both O$_2^•$ and nitric oxide possess a significant cytotoxic potential. Under normal conditions, Kupffer cells have a limited producing ability of reactive species. However, after a “priming” event (e.g., stimulation by cytokines), they increase reactive species, which become maximal during phagocytic activity (87).

While hepatocytes have been the central focus of most studies investigating the effects of ethanol on liver function and on the activation of HSC (76,88), several reports have demonstrated that Kupffer cells produce mediators that stimulate ethanol metabolism and initiate early ethanol-induced liver injury (89). The cascade of events leading to hepatocyte-mediated hepatotoxicity is initiated by an increase in the delivery of endotoxin from the gut, which activates Kupffer cells to release oxygen radicals, growth factors, cytokines, nitric oxide, and prostaglandins, which are either hepatotoxic or serve as chemoattractants for cytotoxic neutrophils that invade the liver (90). Subsequently, hypoxia develops in pericentral regions of the liver lobule, where toxic free radicals are formed upon reintroduction of oxygen, resulting in cell death (91,92).

**POLY UNSATURATED FATTY ACIDS AND HSC ACTIVATION**

In addition to effects of alcohol metabolism, broad evidence implicates dietary fat as contributing to the severity of alcoholic liver disease. In animal models, diets containing polyunsaturated fatty acids (PUFA) enhance the toxic potential of ethanol (93). Arachidonic acid (AA) (a n-6 series PUFA), as a component of cell membranes, is a target for autoxidation, and it is prone to lipid peroxidation (94,95), and lipid peroxidation-derived products such as MDA and 4-HHE can increase collagen I expression (27-29). Alternatively, the AA metabolic pathways lead to generation of prostaglandins, thromboxanes, and leukotrienes, which may also cause a fibrogenic response due to their potential to induce oxidative stress-dependent reactions (29).

Cell culture models have been developed to explore the relationships between ethanol, AA, and CYP2E1 in mediating liver cell injury by oxidant stress. By overexpressing CYP2E1 in HepG2 cells, for example, AA can lead to oxidant stress-dependent toxicity (59,96). AA stimulates HSC proliferation and collagen I expression in HSC co-cultured with HepG2 transfected with CYP2E1-expressing HepG2 cells or hepatocytes (29).

HSC proliferation, morphologic changes, loss of lipid droplets, increased α-smooth muscle actin, elevated intracellular and secreted collagen I and laminin I proteins, higher intra- and extracellular H$_2$O$_2$ and lipid peroxidation products were more apparent in HSC grown on plastic or on matrigel™ and co-cultured with the CYP2E1-enriched cells than in control empty insert co-cultured HSC (27,30). Mechanistic studies reveal a critical role for H$_2$O$_2$ in the up-regulation of *COL1A2* expression by ethanol and AA; moreover, COX-2 mediates the AA-mediated induction of *COL1A2* expression. These effects were prevented by antioxidants and CYP2E1 inhibitors, suggesting a role for hepatocyte-derived ROS in stellate cell activation (28,30).

Whereas many studies have been carried out using n-6 series PUFA, less is known on the role of n-3 series PUFA in the development of alcoholic liver disease. Administration of n-3 PUFA may have the potential down side of inducing LPO reactions and the subsequent collagen I up-regulation. Multiple studies have suggested an important role for LPO in the pathogenesis of alcoholic liver disease (97,98). 4-HHE and 4-HNE are the major aldehydes generated by microsomal peroxidation of n-3 and n-6 PUFA, respectively, both present in fish oil (99). They are highly toxic and have been shown by *in vivo* and *in vitro* experiments to inhibit biological functions of rat liver microsomes and mitochondria and to alter rat liver membrane structure (100). A recent study focused on analyzing potential mechanisms by which co-administration of a diet enriched in FO (n-3 series PUFA), which generated abundant LPO products (i.e. 4-HHN and 4-HNE), plus ethanol could increase collagen I deposition (101). These studies suggested that fish oil can synergize with ethanol to induced collagen I, transactivating the *COL1A2* promoter through a lipid peroxidation-PKC-Pi3K-Akt-NFκB-driven mechanism in the absence of overt steatosis and inflammation.
REFERENCES


