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Oxidative and Nitrosative Stress and Fibrogenic Response

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Hepatic fibrosis represents a significant global health problem for which no adequate therapy exists.[1,2] Alcoholic fibrosis is the liver's wound-healing response to injury and it can lead to cirrhosis characterized by scar accumulation and nodule formation. Thus far, there is no proven therapy for hepatic fibrosis, which can be further complicated by hepatocellular carcinoma. Hence, prevention of liver fibrosis could help to ameliorate the complications of cirrhosis and improve the quality of life of many patients worldwide.[1,2]

Uncontrolled production of collagen I is the main feature of liver fibrosis.[1-13] Following a fibrogenic stimulus such as alcohol, hepatic stellate cells (HSC) transform into an activated collagen-producing cell. In alcoholic liver disease, numerous changes in gene expression are associated with HSC activation, including the induction of several intracellular signaling cascades, which help maintain the activated phenotype and control the fibrogenic and proliferative state of the cell.[1-16] Detailed analyses for understanding the molecular basis of the collagen I gene regulation have revealed a complex process involving reactive oxygen species (ROS) as key mediators.[1,2] Less is known, however, about the contribution of reactive nitrogen species (RNS). In addition, a series of cytokines, growth factors, and chemokines, which activate extracellular matrix (ECM)-producing cells through paracrine and autocrine loops, contribute to the fibrogenic response.[17]

RELEVANCE OF OXIDATIVE STRESS IN LIVER DISEASE

Following alcohol consumption, cholestasis, and iron overload, ROS and lipid peroxidation products are generated in large amounts, leading to Kupffer cell activation,[18-23] a key event in the liver inflammatory and profibrogenic response, and to the secretion of a myriad of growth factors, cytokines, and prostaglandins.[24,25] Among other stimuli, hepatocyte-derived lipid peroxidation induced by ethanol plays an important role in the pathogenesis of liver fibrosis by up-regulating collagen I synthesis.[26] Acetaldehyde is the first metabolite of ethanol and is a profibrogenic agent that stimulates intracellular accumulation of H_2O_2.[27] In addition, H_2O_2 has been also implicated in the onset of scleroderma, an event consistent with earlier clinical evidence for ROS participation in disease pathology.[28,29] Furthermore, ROS-sensitive cytokines contribute to HSC activation during inflammatory through paracrine signals released from immune cells.[30]

Alcohol metabolism by cytochrome P450 2E1 (CYP2E1) generates ROS.[31] Binge drinking triggers steatosis, a fibrogenic response, and apoptosis in rats fed a choline-deficient diet through increased oxidant stress, elevated phosphorylation of p38, and down-regulation of ECM proteolytic enzymes.[32] To better understand how HSC become activated in the presence of oxidative stress and to evaluate whether CYP2E1-derived ROS could play a role in HSC activation, our laboratory co-incubated primary HSC with HepG2 cells overexpressing or not

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CYP2E1.[8,9] There were enhanced proliferation rates and induction of a-smooth muscle actin, intracellular and secreted collagen I protein, and intra- and extracellular H_2O_2 and lipid peroxidation products in HSC co-incubated with HepG2 cells overexpressing CYP2E1 compared with HSC incubated with control HepG2 cells.[8,9] These effects were prevented by antioxidants and by CYP2E1 inhibitors, suggesting a role for CYP2E1-derived diffusible mediators on these effects.[8,9]

A causal relationship also exists between oxidative stress and chronic alcohol-induced liver injury, with sustained activation of Kupffer cells and HSC. A recent study[4] demonstrated that Kupffer cells induced a more activated phenotype, greater proliferation rates, and increased intra- and extracellular collagen I protein, H_2O_2, and IL-6 in HSC co-cultured with Kupffer cells when compared with HSC cultured alone. All these features were prevented by catalase, indicating a role for H_2O_2.[4] In addition, MMP-13, which degrades extracellular collagen I, decreased in the co-culture of HSC with Kupffer cells, while there was up-regulation of tissue inhibitor of metalloproteinase 1 (TIMP1), a MMP-13 inhibitor. A novel dual mechanism mediated by H_2O_2 and IL-6 was proposed for how Kupffer cells could modulate the fibrogenic response in HSC.[4]

Supporting the role of oxidative stress in fibrosis, a novel study showed a correlation between cellular activation and oxidative stress in the GRX cell line and how vitamin E and N-acetylcysteine prevented the activation.[33] Parola and colleagues[34] also have demonstrated that activated HSC are more vulnerable than quiescent HSC to aldehyde end products.

SYNERGISM BETWEEN REACTIVE OXYGEN SPECIES AND GROWTH FACTORS

The role of pro-fibrogenic cytokines and growth factors is central for the development of liver fibrosis because they allow cross-talk between ECM-producing cells.[3-6] ROS play a decisive role in the initial phase of fibrosis by integrating different profibrotic stimuli independently of TGFβ. By contrast, progression to the subsequent stage depends on TGFβ production and the canonical Smad pathway.[35] Liver fibrosis involves elevated expression of TGFβ in rodents[36,37] and in humans.[38] In normal liver and in CCl_4-induced fibrosis, TGFβ1 is mainly produced by Kupffer cells and HSC although a small amount is generated by endothelial cells.[36,39] TGFβ is secreted as a latent complex that is trapped among matrix fibbers, from which it is released and activated during tissue remodeling.[40,41] Activated TGFβ signals through transmembrane serine/threonine kinases and intracellular Smad proteins, which translocate into the nucleus, activate gene transcription by binding the “CAGA” consensus sequence, and interact with promoter-specific transcription factors and general co-activators[42] to initiate and perpetuate the fibrogenic response.[43] TGFβ1 is a redox sensitive gene;[44] indeed, ROS (eg, H_2O_2) up-regulate TGFβ1 expression in rat HSC, which is blocked by catalase.[45] In addition, TGFβ per se increases $O_2^{\cdot -}$ production in fibroblasts.[46] Other studies have described how TGFβ increases ROS production by activating the membrane-bound enzyme NADPH oxidase[47,48] and by impairing complex IV in the mitochondrial respiratory chain.[49] The canonical signal transduction pathway of TGFβ plays a central role in fibrosis[42,43,50,51] even though phosphatidylinositol 3-kinase (PI3K)/Akt is a well-documented example of an alternative pathway that is induced by TGFβ in different cell lines and profibrogenic conditions.[35,52]

PDGF is the most potent mitogen for transdifferentiated HSC during liver fibrosis, and the expression of PDGF receptors is important for mitogenesis and chronic inflammation of the liver,[53-55] and particularly chemo-attraction of mononuclear cells.[56] The expression of PDGF is also influenced by intracellular redox changes.[57] TGFβ1 regulates the PDGFRb in human HSC.[58] Moreover, the PDGF-dependent activation of HSC is followed by phosphorylation of

PI3K.[59,60] PI3K activation is essential for both mitogenesis and chemotaxis induced by PDGF during liver injury in vivo.[61] The 70-kDa ribosomal S6 kinase is activated in a PI3K-dependent manner and plays an important role in HSC proliferation, collagen expression, and cell cycle control, representing a potential therapeutic target for liver fibrosis.[62] FoxO1 inhibits PDGF-induced HSC proliferation via G1 cell cycle arrest suggesting that FoxO1 is a crucial downstream target of the PI3K/Akt pathway in regulating HSC proliferation.[60] PI3K is induced by oxidative stress in rat HSC following exposure to CCl_4.[63] HSC isolated from CCl_4-treated rats or from acute liver damage in vivo show increased ERK activity, which modulates HSC proliferation and chemotaxis and regulates nuclear signaling.[64]

The proliferative effect of PDGF requires the activation of PDGFRb.[65,66] Furthermore, the activation of cultured rat HSC by Kupffer cell-conditioned medium directly enhances matrix synthesis and stimulates HSC proliferation via induction of PDGF receptors.[65] The myristoylated alanine-rich protein kinase C substrate is a downstream effector in PDGF-induced motility of activated human HSC.[67] Different compounds that activate the AMPK pathway inhibit PDGF-stimulated proliferation and migration of human HSC and reduce the secretion of monocyte chemoattractant protein-1, suggesting that AMPK negatively modulates the activated phenotype of HSC.[68] Nitric oxide (NO·) donors also exert a direct antifibrogenic action by inhibiting PDGF-induced proliferation, motility, and contractility in HSC, in addition to lowering ECM proteins.[69] A regulatory mechanism of reactive aldehydes on PDGFRb signaling and biologic actions is relevant to liver fibrosis.[70]

TNFα secreted by macrophages, Kupffer cells, and HSC, as well as IFNγ secreted by T cells, are well-known antifibrogenic signals.[71,72] TNFα reduces ECM deposition by inhibiting the synthesis of structural components, including elastin, osteocalcin, and collagen I.[73-76] TNFα counteracts TGFβ-stimulation of collagen I gene in different cell types.[77,78] Both TNFα and IFNγ blunt the TGFβ-mediated up-regulation of the *COL1A2* promoter by interfering with the formation of the TGFβ1-responsive element complex.[79-81] TGFβ antagonism by TNFα involves c-Jun N-terminal kinase-1 phosphorylation of c-Jun, which leads to off-DNA interference of Smad3 binding to the cognate DNA site and/or interaction with the p300/CBP co-activators.[82] Moreover, a previous report has shown that p38 MAPK is a key mediator of the antifibrogenic effect of TNFα in regulating the expression of *col1A1* in HSC in response to cytokines.[83]

In addition, interferon regulatory factor-binding site IF3 is a novel target of the pathways elicited by IFNγ to blunt *COL1A2* promoter transcription.[84] IFNγ promotes occupancy of the *COL1A2* transcription start site by the RFX5/CIITA complex, which interacts with CBF/NFY and/or YB-1.[79] These antifibrogenic cytokines down-regulate *COL1A2* transcription and antagonize TGFβ through multiple pathways, which converge on the same promoter elements.[85,86] TNFα–mediated down-regulation of the mouse *col1a1* gene is associated with the activation and binding of C/EBPδ-and C/EBPβ-containing complexes to the -370 to -344 region of the mouse *col1a1* promoter (Table 1).[87]

EFFECTS OF REACTIVE OXYGEN SPECIES ON THE *COL1A1* AND *COL1A2* PROMOTERS

Collagen I, the most abundant collagen type found in liver fibrosis, is a heterotrimeric protein composed of two α1 chains and one α2 chain forming a triple helical structure. The human α-chains genes are located as single copies on different chromosomes. The α1(I) chain gene is on chromosome 17q21-22 whereas the α2(I) chain gene is located on 7q21-22.[88,89] In normal tissue, both genes are co-ordinately expressed,[90] whereas a homotrimer of three α1(I) chains occasionally occurs in tumors[91] and in cultured cells.[92] Structural and metabolic deficiencies

of the collagen I chains lead to several heritable and acquired disorders of connective tissue,[93] in general, and impair liver function, in particular.[94]

The IL-4-induced transcriptional activator STAT6 binds to various sequences within the *COL1A1* and *COL1A2* promoters.[95] An AP-2 site adjacent to the reverse-oriented STAT6 consensus motif TTCN3/4 GCT is located within 205 bp from the transcription start site and seems to support the moderate IL-4-induced *COL1A1* gene activation. Furthermore, IL-6 up-regulates the expression of type I collagen in vivo[96] and in cultured HSC.[97-99] HSC respond to IL-6 with a transient increase in *col1a1* mRNA expression.[97,100] A TGFβ1-responsive element is found in the -370 to -344 bp region of the mouse *col1a1* gene.[45] TGFβ1 induces the activation and binding to the TGFβ1-responsive element of a protein complex that contains C/EBPβ, and H_2O_2 acts as a second messenger for the TGFβ1-mediated *col1a1* gene up-regulation.[45] In fact, acetaldehyde induces *col1a1* up-regulation via H_2O_2.[101] However, IFNγ and TNFα down-regulate transcription of the *COL1A1* promoter.[95] The TNFα-mediated down-regulation of the *col1a1* gene is associated with activation and binding of C/EBPδ- and C/EBPβ-containing complexes to the -370 to -344 bp region of the mouse *col1a1* promoter. Indeed, over-expression of C/EBPδ or p20C/EBPβ down-regulates the expression of a reporter construct driven by the -412 to +110 bp sequence of the *col1a1* promoter, validating the relevance of these two transcription factors in the TNFα-mediated *col1a1* down-regulation.[87]

The functional properties of the proximal promoter of the *COL1A2* gene have been studied in transfection experiments,[102] which have described the minimal upstream sequence directing high and cell type-specific expression of the CAT-reporter gene. The proximal promoter of the *COL1A2* spans from −380 to +54 bp relative to the transcription start site and contains several overlapping DNA elements that are bound by ubiquitous transcription factors. Constitutive transcription of the proximal promoter of the *COL1A2* is under the control of four clusters of *cis*-acting elements, which are involved in mediating the transcriptional response to cytokines implicated in tissue remodeling and fibrosis.[103] Recent studies have underscored the importance of the proximal promoter in proper *COL1A2* expression, describing, in addition, that a far-upstream enhancer and a downstream silencer are also part of the regulatory network of the *COL1A2* gene.[104,105] A model has been proposed whereby *COL1A2* expression is the result of combinatorial interactions amongst *trans*-acting factors bound within the promoter, enhancer, and repressor sequences.[85] The downstream repressor resides within the first intron of *COL1A2* and contains a DNase I hypersensitive site located around a cluster of three *cis*-acting elements, which contain binding sites for 'GATA' (FIi1 an FIi2) and IRF (FIi3) nuclear proteins.[105]

Many nuclear factors that have been implicated in regulating the *COL1A2* proximal promoter in fibroblasts, including Sp1/Sp3, NFkB, C/EBPδ and β, AP1, Fli-1/Ets-1, CBF/NFY, YB-1 and the Smads 3/4 and RFX5/CIITA complexes.[76,79,81,106] Specifically, either single or multiple "CAGA" boxes are present in the TGFβ -responsive element on the *COL1A2* gene; hence, the Smad3/4 complex represents a common mediator of the TGFβ signaling for ECM accumulation.[107] The ubiquitous transcription factor Sp1, the Smad3/4 complex, and the co-activators p300/CBP mediate the response of the *COL1A2* promoter to TGFβ stimulation.[85, 108,109] A Smad complex may represent the alleged Sp1 co-factor involved in *COL1A2* trans-activation.[110] Transient over-expression of Smad3 and Smad4 can transactivate the *COL1A2* promoter.[111] Sp1 and Smad3/Smad4 cooperate synergistically in transactivating the *COL1A2* promoter after binding to the TGFβ1-responsive element. Furthermore, there is additional evidence for a critical role of Sp1 in constitutive *COL1A2* expression, and in integrating the transcriptional responses of the gene to antagonistic cytokines.[112] Sp1/Sp3 proteins bound to the *COL1A2* promoter interact with CBF/NFY to strengthen promoter activity and patterned transgene expression.[113,114] Fli-1, a member of Ets transcriptional factors, is a negative regulator of the *COL1A2* gene expression in dermal fibroblasts.[115]

Competition between Fli-1 and Ets-1 for binding to the same promoter sequence is associated both with modulating constitutive *COL1A2* activity and inhibiting TGFβ signaling.[116] In addition, a recent study has shown that TGFβ -dependent acetylation and inhibition of Fli-1 may represent the principal mechanisms responsible for the TGFβ -induced dissociation of Fli-1 from the *COL1A2* promoter.[117]

Several studies indicate that adiponectin has antifibrotic properties because hepatic fibrosis produced by chronic CCl_4 was enhanced in adiponectin-knockout mice as compared with wild-type mice,[118,119] while leptin has an opposite effect of enhancing fibrogenesis.[120-122]

Acetaldehyde up-regulates type I collagen in HSC[123-125] and the *COL1A1* and *COL1A2* induction occurs through a TGFβ-dependent mechanism.[126] In addition, acetaldehyde stimulates intracellular accumulation of H_2O_2 and *COL1A2* promoter activity in HSC,[29] strongly suggesting that the early stage of ethanol-induced liver fibrosis induces a H_2O_2-dependent loop, which triggers and amplifies autocrine TGFβ production, via activation of the Sp1-Smad3/4 complex, conceivably through the PI3K pathway.[29,127] Another report has implicated H_2O_2 in the pathology of scleroderma, demonstrating that PDGF treatment of primary human fibroblasts triggers an intracellular loop involving Ha-Ras, ERK1/2, and ROS, ultimately leading to *COL1A2* up-regulation.[128] The TGFβRI-dependent program and the up-regulation of collagen I does not involve Smad2/3 activation but is mediated by ALK1/Smad1 and ERK1/2 pathways in scleroderma fibroblasts.[128] The involvement of H_2O_2 in the induction of the *COL1A1* promoter under TGFβ treatment has been defined.[45,76,101] Primary Kupffer cells in co-culture with HSC induce a profibrogenic response mediated by H_2O_2 that leads to up-regulation of *COL1A1* and *COL1A2* trans-activation and simultaneously prevents collagen I protein degradation via an IL-6-dependent mechanism.[4] Work from our group has described a role for H_2O_2 in the up-regulation of *COL1A2* expression by ethanol and arachidonic acid whereby COX-2 appears to mediate the arachidonic acid-mediated induction of *COL1A2* expression (see Table 1).[11]

STABILITY AND DEGRADATION OF COLLAGEN I: ROLE OF METALLOPROTEINASES

Liver fibrosis is characterized by activation of HSC and subsequent ECM synthesis, along with insufficient degradation. Matrix remodeling occurs mainly due to the action of MMPs, a multidomain family of zinc-dependent endopeptidases. These enzymes are secreted into the extracellular space as zymogens that require activation by a variety of stimuli. The active enzymes can, in turn, be inhibited by the family of tissue inhibitors of metalloproteinases (TIMPs). ECM remodeling is, therefore, highly regulated under physiologic conditions. Alteration of the balance between MMPs plays a role in scarring.[129,130]

The three most relevant MMPs are gelatinase A (MMP-2), gelatinase B (MMP-9), and stromelysin (MMP-3). In liver fibrosis, the expression of the MMPs involved in fibrillar collagen degradation (eg, MMP-1 in humans and rodents and MMP-13 also in rodents) is limited, whereas the expression of MMP-2 is markedly increased.[131] TGFβ1 modulates MMP-13 expression in HSC by complex mechanisms involving p38 MAPK, PI3K/AKT and p70-ribosomal S6 kinase.[132] MMP-2 can degrade several components of the subendothelial matrix, including collagen IV, laminin, and fibronectin, and it may be important in the remodeling of matrix during tissue repair processes.[133] A recent study has shown that oxidative stress induces MMP-2 expression, proliferation, and invasiveness of HSC; these effects could be prevented by specific MMP inhibitors and antioxidants.[62] Increased expression of pro-gelatinase and formation of active enzyme occurs in human liver disease and in animal models of liver fibrosis.[134,135] Sustained over-expression of MMPs like gelatinase A, with the consequent degradation of basement-membrane collagen IV, represents a basic

mechanism in the remodeling of the space of Disse with capillarization of the sinusoids.[136] In progressive liver fibrosis, the overall MMP activity decreases,[137] due to increased expression of TIMPs and other anti-proteases expressed by HSC and hepatocytes.[62,138] In liver fibrosis, hepatic TIMP-1 expression is markedly up-regulated both in humans and in murine fibrosis models.[130,139,140] Both TIMP-1 and TIMP-2 are released by fully activated HSC.[141] The increased expression of TIMPs is important in advanced liver fibrosis both in rodents and in humans.[142,143] In fact, increased TIMP-1 and TIMP-2 mRNA levels have been demonstrated by in situ hybridization in CCl_4-induced rat fibrosis,[144] as well as in primary biliary cirrhosis and biliary atresia.[140]

Plasmin is a broad-spectrum protease capable of directly degrading matrix components, including fibronectin, laminin, and proteoglycans[145] and also participates in matrix degradation indirectly by activating MMP-13, MMP-1, MMP-3, interstitial collagenase, and stromelysin.[146,147] PAI-1, a physiologic inhibitor of plasminogen activator, inhibits protease-dependent fibrinolytic activity and subsequent ECM degradation. PAI-1 expression is up-regulated in a variety of fibrotic diseases as well as in experimental animal models such as CCl_4-induced liver fibrosis,[148] and PAI-1-deficient mice develop less severe fibrosis in lung, [149] suggesting a role for PAI-1 in the progression of fibrosis. Furthermore, GSH inhibits TGFβ -induced collagen I accumulation by blocking TGFβ-induced PAI-1 expression, and thus stimulating collagen degradation.[150] Many other studies have also described TGFβ as an inducer of ROS production and ROS mediate PAI-1 induction by different stimuli.[151-154]

SOURCES OF REACTIVE NITROGEN SPECIES IN THE LIVER

Nearly all cell types in the liver, including hepatocytes, Kupffer cells, HSC, and endothelial cells, have the capacity to generate NO·.[155] The reactivity of NO· per se has been greatly overestimated in vitro because no drain is provided to remove NO·.[156] NO· remains in solution for several minutes in micromolar concentrations before it reacts with O_2 to form much stronger oxidants like nitrogen dioxide and others.[157] Most biological actions of NO· appear to be mediated by interactions with paramagnetic centers in effector proteins, such as heme- or iron-sulfur centers, but NO· is also known to react rapidly, via radical termination reactions, with other targets that carry unpaired electrons.[158] These reactions include interactions with ROS such as $O_2^{\cdot -}$, or with radical intermediates in proteins or lipids.[156] Furthermore, NO· reacts with O_2 to form higher oxides of nitrogen, in a relatively slow reaction (Fig. 1).[159] The eventual biologic fate of NO· is oxidation to nitrite and nitrate, end products of NO· metabolism that are rapidly distributed throughout the body and excreted in urine.[158]

There are a number of RNS derived from NO·.[160] Of these, peroxynitrite ($ONOO^-$) is the best characterized and appears to have the highest biological activity.[160] $ONOO^-$ is formed by the bi-radical reaction of NO· and $O_2^{\cdot -}$. The reaction is extremely fast and will occur at a near diffusion-limited rate.[161] NO· is the only biological molecule produced in concentrations large enough to compete with superoxide dismutase for $O_2^{\cdot -}$.[156] $ONOO^-$ reacts relatively slowly with most biological molecules, which defines it as a selective oxidant. Effects of $ONOO^-$ may also be beneficial or detrimental depending on the concentration and local environment, the level of cellular activation, and the endogenous GSH pool, which acts as a natural $ONOO^-$ scavenger.[162] On the other hand, direct in vivo and in vitro evidence that, at low concentrations, $ONOO^-$ is actively involved in triggering cellular survival signals has been reported in studies demonstrating protection against myocardial ischemia-reperfusion injury and neuronal apoptosis.[163,164]

REACTIVE NITROGEN SPECIES AND HEPATOTOXICITY

NO· is a short-life gaseous free radical known to exert many actions in the liver as well as in other tissues and organs.[165] This review summarizes only the major notions, with special

reference to interactions with ROS at the molecular level leading to collagen I regulation, and effects at the cellular level (ie, HSC activation). In normal liver, low fluxes of NO· are produced by constitutive endothelial nitric oxide synthase (eNOS, mainly in endothelial cells) and are considered sufficient to maintain perfusion of liver sinusoids by acting on vascular tone (ie, vasodilatation) and on vascular permeability.[166] NO· regulates leukocyte adhesion to sinusoidal endothelium and inhibits platelet adhesion and aggregation.[167] In pathologic conditions, including endotoxemia and chronic inflammation, nitric oxide synthase 2 (NOS2) is up-regulated in almost all liver cells, including HSC,[168] by several mediators and, consequently, NO· generation increases.[166] Under these conditions, NO· acts either as cytoprotective or as cytotoxic depending on the cellular microenvironment.[169]

The molecular regulation of NOS2 expression is complex and occurs at multiple levels. NOS2 expression requires the transcription factor NFκB and is down-regulated by steroids, TGFβ, the heat shock response, p53, and NO· itself.[169] NO· also presents a protective effect both in vivo and in vitro by blocking TNFα-induced apoptosis and hepatotoxicity, in part by a thiol-dependent inhibition of caspase-3-like protease activity.[170] These studies demonstrate the cytoprotective effects of NO· in the liver and suggest that hepatic NOS2 expression may function as an adaptive response to minimize inflammatory injury.[170] Thus, numerous mechanisms have evolved to regulate NOS2 expression during hepatocellular injury.[171] The activation of NO· synthesis can be considered as an early adaptive response, which may become a mediator of tissue damage in excess. Whether or not NO· or secondary oxidants generated from NO· act as mediators of tissue injury or protect against toxicity will likely depend on the precise targets of these RNS, the levels of $O_2^{\cdot -}$, and the extent to which tissue injury is mediated by ROS.[155]

SYNERGISM BETWEEN REACTIVE NITROGEN SPECIES AND REACTIVE OXYGEN SPECIES

ROS may synergize with or antagonize RNS during liver injury and inflammation. ROS and RNS are important in the process of energy generation, lipid peroxidation, protein and DNA oxidation, nitration, nitrosation, or nitrosylation and catecholamine response.[172] ROS and RNS also strongly interact with reactive sulfur species, ie, derivatives of reduced thiols (RSH) including the thiolate anion (RS^-), thiyl radical (RS·) , sulfenic acid (R-SOH), sulfinic acid (R-SOO), and sulfonic acid (R-SOOH) derivatives.

Among the many types of oxidative modifications induced by $ONOO^-$ and other RNS are the characteristic addition or substitution products in which $NO^{\cdot}$ is essentially incorporated into the target molecule (ie, nitrosation and nitration reactions).[158] For instance, reactions with thiol residues to form *S*-nitrosothiols have been proposed as a mechanism of either enzyme regulation or $NO^{\cdot}$ transport, and may provide a unique signaling mechanism induced by nitrosative stress. *S*-Nitrosothiols in proteins (eg, albumin) or in low-molecular-weight thiols, such as GSH, have been detected in the circulation, bile, as well as in respiratory tract lining fluids.[158] There is a mechanism for pro-MMPs activation caused by *S*-glutathiolation whereby the GSH adduct of pro-MMP may be produced through disulfide *S*-oxide formation involving generation of *S*-nitrosoglutathione (GSNO2) by $ONOO^-$.[173]

The amino acid tyrosine appears to be a particularly susceptible target for nitration, and the formation of free or protein-associated 3-nitrotyrosine has received much recent interest as a potential biomarker for the generation of RNS in vivo.[158] Furthermore, there is considerable evidence in the protein chemistry literature that nitration of essential tyrosine residues can inactivate many enzymes or prevent phosphorylation of tyrosine kinase substrates,[174] and these findings have supported the hypothesis that tyrosine nitration might result not only in the formation of inactive “footprints” of RNS but might also be functionally related to the

pathobiology of inflammatory diseases.[175] For example, $ONOO^-$ promoted nitration and/or phosphorylation of regulatory sites at tyrosine kinase receptors coupled to the well-known anti-apoptotic pathways involving PI3K/Akt or MAPK.[176,177] Moreover, one of the most interesting protective effects of NO· is represented by the NO-dependent blocking of hepatocyte apoptosis induced either by removal of growth factors or by exposure to TNFα or anti-Fas antibody. This anti-apoptotic effect has been ascribed to *S*-nitrosylation of caspase-3 and -8, with the subsequent inhibition of their activity.[178]

REACTIVE NITROGEN SPECIES AND HEPATIC STELLATE CELL ACTIVATION

Paracrine signaling is also important for nitrosative stress as it is for oxidative stress. Kupffer cells also produce NO·, which can counterbalance the stimulatory effects of ROS by reducing HSC proliferation, contractility, and collagen I production.[179]

Neutrophils are an important source of ROS, which have a direct stimulatory effect on HSC collagen I synthesis. Activated neutrophils increased HSC collagen synthesis 3-fold over control levels. $O_2^{\cdot -}$ was identified as the principal mediator of the neutrophils' effect. Activated neutrophils also produce NO·, which dampened the effect of $O_2^{\cdot -}$ on collagen I expression but did not abrogate it completely.[180]

Activated HSC have contractile features[181] that may contribute to increased intrahepatic portal hypertension via constriction of the sinusoid or by contraction of fibrous ECM rich in collagen I with concomitant disruption of lobular architecture.[182] Endothelin and NO· play a major role in the modulation of HSC contractility, and are therefore important in the pathogenesis of intrahepatic portal hypertension.[182]

Therefore, NO· and NO· donors are capable of preventing or reducing proliferative responses of activated HSC. NO· donors can efficiently inhibit PDGF-dependent proliferation and chemotaxis in activated human HSC by activating an ibuprofen-sensitive, prostaglandin E_2 and cAMP-dependent pathway which interferes negatively with PDGF signaling.[69] Similar results (ie, inhibition of stimulated proliferation of HSC by NO· donors) have been found with angiotensin II as proliferative stimulus.[183]

There is recent work showing lipopolysaccharide-induced synthesis of IL-6, TNFα, and NO· via NOS2 in HSC.[184] This group presented evidence that activation of p38 by lipopolysaccharide initiates signaling via NFκB and ROS (eg, H_2O_2) leading to the induction of NOS2 and expression of IL-6 and TNFα, major players in hepatic hemodynamic regulation, inflammation, and immune responses.

REACTIVE NITROGEN SPECIES AND COLLAGEN I

Oxidative stress may represent a direct or indirect relevant profibrogenic stimulus for HSC, as suggested by in vivo experimental studies in which administration of antioxidants prevents oxidative stress, lipid peroxidation, and liver fibrosis.[185] Furthermore, oxidative stress and lipid peroxidation are concomitant or precede HSC activation and collagen I deposition.[4] Exposure of cultured human or rat HSC to pro-oxidants or to medium containing products released from hepatocytes undergoing oxidative stress (ie, to mimic a possible paracrine effect by damaged parenchymal cells) is followed by increased pro-collagen I gene expression.[10]

In contrast to ROS, which have been typically considered pro-fibrogenic agents,[4,11] NO· may be anti-fibrogenic.[69,186] In the wound-healing response that restores tissue integrity, NO· is synthesized in the early phase by inflammatory cells, mainly macrophages.[187,188] However, many cells participate in NO· synthesis during the proliferative phase after wounding. NO·

released via NOS2 regulates collagen formation, cell proliferation, and wound contraction in distinct ways in animal models of wound healing. Although NOS2 gene deletion delays, and arginine and NO· administration, improve healing, the exact mechanisms of action of NO· on wound-healing parameters are still unknown.[187,188]

$ONOO^-$ can down-regulate type I collagen in dermal and cardiac fibroblasts,[189] smooth muscle cells,[190] and other cell types.[191] In addition, $ONOO^-$ can act as a potent antifibrotic effector in animal models of experimental fibrosis,[192] and in the long-term inhibition of NOS2 in rats.[186] However, in early traumatic wound-healing conditions, $ONOO^-$ favors collagen synthesis and the formation of granulation tissue.[193] There are different mechanisms to explain the inhibition of collagen by $ONOO^-$, ie, $ONOO^-$ may act through direct inhibition of collagen synthesis by proline hydroxylation,[191] stimulation of MMPs,[194,195] reduced production of TGFβ,[189,195] initiation of fibroblast apoptosis,[196] and/or neutralization of profibrogenic ROS.[156,164]

Recent data from our group indicate that $ONOO^-$ and its secondary products may have potential beneficial effects in the early fibrogenic response of HSC, which are exposed to reactive species (ie, $O_2^{\cdot -}$ and NO·) per se and also able to generate them (Fig. 2). The authors found a time- and dose-dependent down-regulation of intra- and extracellular collagen I protein along with an up-regulation of MMP-1 and TNFα in HSC treated with either pure $ONOO^-$ or a $ONOO^-$ donor, which were blocked by $ONOO^-$ scavengers. The addition of $ONOO^-$ increased nitration of MMP-1 and MMP-13 leading to increased activity as the cleaved active isoforms 22/25 kDa for MMP-1 and 44/48 kDa for MMP-13 were undetected in the absence of $ONOO^-$. However, the protective role of $ONOO^-$ occurs only in the early fibrogenic response, and it is lost at more advanced stages of the disease when other factors may hit in a synergistic way.[197]

The temporal expression profiles of profibrogenic genes in HSC and their coordination concerning cell proliferation in alcoholic liver disease still needs further clarification. Stress-derived mediators may activate seemingly contradictory signaling pathways and the ultimate outcome may be dependent on the balance between these stress-activated pathways because they could determine whether the cell proliferates or undergoes a fibrogenic response.

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KEYWORDS

CYP2E1
Cytochrome P450 2E1

ECM
Extracellular matrix

ERK 1/2
Extracellular signal-regulated kinase 1/2

GSH
Glutathione

HSC
Hepatic stellate cells

H_2O_2
Hydrogen peroxide

IFNγ
Interferon γ

IL
Interleukin

MMPs
Matrix metalloproteinases

MAPK
Mitogen-activated protein kinase

NO·
Nitric oxide

NOS2
Nitric oxide synthase 2

$ONOO^-$
Peroxynitrite

PI3K
Phosphatidylinositol 3-kinase

PDGF
Platelet-derived growth factor

RNS
: Reactive nitrogen species

ROS
: Reactive oxygen species

$O_2^{\cdot -}$
: Superoxide anion

TIMPs
: Tissue inhibitor of metalloproteinases

TGFβ
: Transforming growth factor-beta

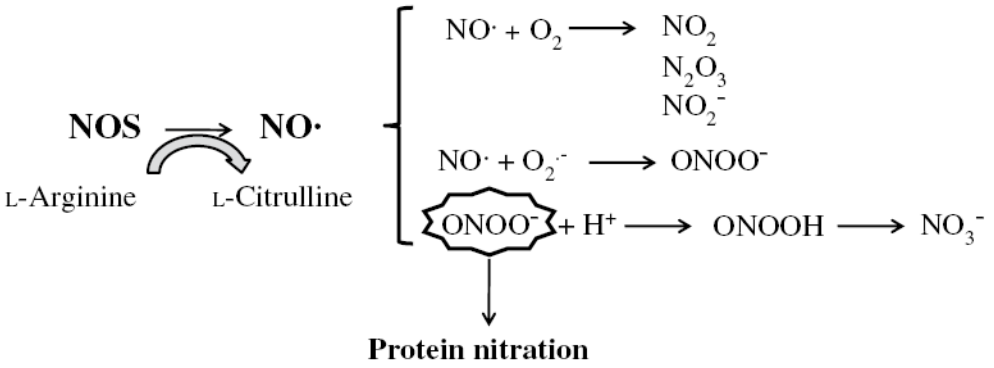

Fig. 1.

Generation of NO· by NOS in liver cells. NO· is relatively unstable in the presence of O_2 and will rapidly and spontaneoulsy auto-oxidize to yield a variety of nitrogen oxides.NO· also reacts with $O_2^{\cdot -}$ to generate $ONOO^-$. Although $ONOO^-$ is relatively stable, it has a pKa of 6.8, which implies that substantial amounts of $ONOO^-$ will be protonated at physiologic pH to yield peroxynitrous acid. This conjugate acid rapidly decomposes to yield NO_3^-. Nitration of tyrosine residues by $ONOO^-$ forms the stable product, 3-nitrotyrosine (3-NT, the footprint for $ONOO^-$) by addition of a nitro group to the 3-position adjacent to the hydroxyl group of tyrosine.

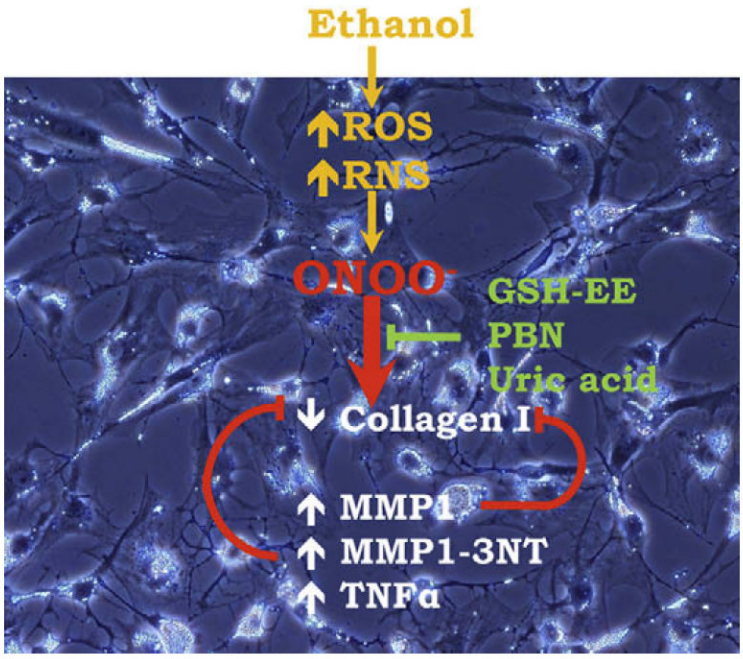

Fig. 2.

In liver injury, ROS and RNS are generated in all liver cells. $O_2^{\cdot -}$ can react with NO· to generate $ONOO^-$ and other metabolites that may impact the HSC fibrogenic response in the early stages of cellular activation. $ONOO^-$ and its metabolites lower collagen I protein by increasing TNFα and inducing nitration of MMP1 with the subsequent cleavage of collagen I. These effects can be reverted by $ONOO^-$ chelating agents.

Table 1

Summary of different factors that modulate collagen I expression

	Collagen I
ROS($O_2^{\cdot -}$ and H_2O_2) [11,29,47,64,91,133,157-160]	↑
IL-4[100]	↑
IL-6[101,102]	↑
Acetaldehyde[29,106,128-131]	↑
Arachidonic acid[11]	↑
Malondialdehyde[134]	↑
PDGF[61,62,67]	↑
Nitric oxide[71]	↓
TGFβ[47,112,113]	↑
TNFα[78,85,89]	↓
INFγ[81,83,86]	↓
Fli-1[120-122]	↓
Sp1/Sp3[118,119]	↑
Adiponectin[123,124]	↓
Leptin[125-127]	↑