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REVIEW

Thiol redox systems and protein kinases in hepatic stellate cell regulatory processes

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Abstract
Hepatic stellate cells (HSC) are the major producers of collagen in the liver and their conversion from resting cells to a proliferating, contractile and fibrogenic phenotype ('activation') is a critical step, leading to liver fibrosis characterized by deposition of excessive extracellular matrix. Cytokines, growth factors, reactive oxygen and nitrogen species (ROS/RNS), lipid peroxides and their products deriving from hepatocytes, Kupffer cells and other cells converge on HSC and influence their activation. This review focuses on glutathione and thioredoxin pathways, with particular emphasis on their role in HSC. These two systems have been shown to act in the metabolism of hydrogen peroxide, control of thiol redox balance and regulation of signalling pathways. Particular attention is paid to mitochondria and NADPH oxidase. Detailed knowledge of specific signalling, redox conditions and apoptotic processes will be of help in devising proper pharmacological treatments for liver fibrosis.

Keywords: *Glutathione system, hepatic stellate cells (HSC), liver fibrosis, mitochondria, NADPH oxidase, protein kinases, reactive oxygen species (ROS), reactive nitrogen species (RNS), redox regulation, thioredoxin system.*

Abbreviations: *2-AG, 2-arachidonoyl glycerol; ASK1, apoptosis signal-regulating kinase 1; CYP2E1, alcohol-inducible cytochrome P4502E1; DPI, diphenylene iodonium; ECM, extracellular matrix; EGCG, (-)-epigallocatechin-3-gallate; EGF, epidermal growth factor; Erk, extracellular signal-regulated kinase; ET-1, endothelin-1; FGF, fibroblast growth factor; γ-GCL, γ-glutamyl cysteine ligase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; Grx, glutaredoxin; GST, glutathione transferase; 4-HNE, 4-hydroxy-2-nonenal; HSC, hepatic stellate cells; MAP kinases, mitogen activated protein kinases; MMP, matrix metalloproteinases; MPTP, mitochondrial permeability transition pore; NASH, non-alcoholic steatohepatitis; NF-kB, nuclear factor-kB; NO, nitric oxide; PDGF, platelet-derived growth factor; PKA, protein kinase A; PKC, protein kinase C; Prx, peroxiredoxin; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSNO, nitrosothiols; SFK, Src family kinases; SOD, superoxide dismutase; TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of metalloproteinases; TNF-α, tumour necrosis factor-α; Trx, thioredoxin; TrxR, thioredoxin reductase; VEGF, vascular endothelial growth factor.*

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Hepatic stellate cells: Characteristics, functions and activation

Several chronic diseases affecting the liver, lung, nervous system and arteries are characterized by increased deposition of collagen, causing tissue fibrosis. A common mechanism appears to be involved in the fibrogenetic process and involves initial cell injury, leading to inflammation, phagocyte activation and production of a large network of cytokines, growth factors, chemoattractants and other active molecules eliciting the fibroproliferative response [1].

Hepatic stellate cells (HSC) are relatively undifferentiated pericytes of mesenchymal type located in

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the space of Disse between sinusoidal endothelial cells and hepatocytes [1–6]. HSC were formerly known as Ito cells, lipocytes, pericytes, perisynusoidal cells, fat-storing cells and vitamin A-storing cells. Their major specific functions include collagen production and remodelling of extracellular matrix (ECM), growth factor and cytokine production and retinoid storage [1–7]. It has been shown that early primary hepatocyte cultures may contain up to 10% HSC [8]. According to Giampieri et al. [9] there is an average of ~63 HSC per 1000 hepatocytes.

HSC have two main phenotypic states: 'quiescent' and 'activated'. Activation of HSC indicates the conversion of resting cells to a proliferating, fibrogenic and contractile phenotype and represents the major pathway leading to liver fibrosis [2] (Figure 1). Notably, formation of fibrogenic myofibroblasts occurs in various organs from a large spectrum of precursors and plays a crucial role in their respective tissues [10]. Activation of HSC comprises initiation and perpetuation phases [2]. Initiation results basically from paracrine stimulation elicited by all neighbouring cells including hepatocytes, sinusoidal endothelium, Kupffer cells and platelets [2]. Exposure to lipid peroxides and products deriving from damaged hepatocytes also contribute to the initiation phase of activation [2]. Hepatocytes are an important source of ROS, essentially produced by cytochrome P450, such as the alcohol-inducible cytochrome P450 2E1 (CYP2E1) [11]. HSC, cultured in the presence of CYP2E1-expressing HepG2 cell line (E47 cells), therefore undergo activation and increase their collagen formation [11]. However, this increase is prevented by inhibitors of CYP2E1, indicating that P450-mediated ROS production by hepatocytes is responsible for the stimulation of collagen formation by HSC [11]. Hepatocytes are also an effective source of fibrogenic lipid peroxides. HSC further stimulate collagen synthesis in the presence of E47 cells and arachidonic acid/iron, a combination that enhances lipid peroxidation [12]. Both necrosis and apoptosis of hepatocytes are involved in the fibrogenic response [2]. Necrosis depends mostly on lipid peroxidation and the associated inflammatory response [2]. Apoptosis

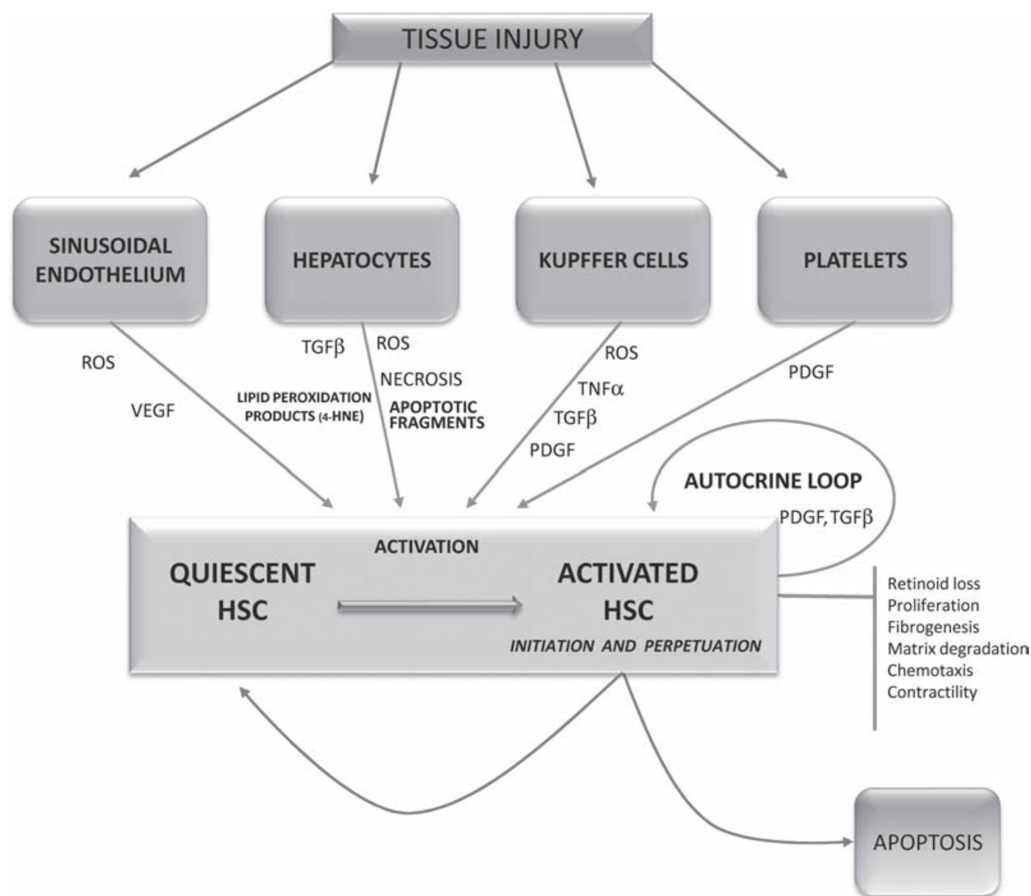

Figure 1. Pathways of activation of hepatic stellate cells and cellular events leading to liver fibrosis. Tissue injury involving hepatocytes, endothelial cells, Kupffer cells and also neutrophils and lymphocytes gives rise to production of a large array of cytokines, growth factors, peroxides and lipid peroxidation products such as 4-hydroxynonenal (4-HNE), all able to stimulate quiescent stellate cells to convert to an activated condition. Activation comprises initiation and perpetuation phases. Once activated, HSC set up an autocrine loop, but paracrine effects persist. Perpetuation phase of activation is characterized by retinoid loss, proliferation, chemotaxis, contractility, fibrogenesis and increased production of extracellular matrix. A resolution phase may take place in activated cells, which can either revert to quiescent state or be cleared in an apoptotic process, making this phase critical for regression of fibrosis.

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of hepatocytes stimulates initiation of HSC in a Fas-mediated process which is fibrogenic *in vivo* [13–15]. Reactive aldehydes produced from lipid peroxidation, such as 4-hydroxynonenal (4-HNE), are also important contributors to activation. Platelets are a quite important source of paracrine stimuli including PDGF, TGF-β and EGF [16]. Activated Kupffer cells play a role in activating HSC, mediated by the production of various cytokines, especially TGF-β and TNF-α, and also by reactive oxygen and nitrogen species [17]. However, nitric oxide can antagonize the stimulatory effect of ROS and thus decrease HSC proliferation and contractility [18,19]. Lastly, lymphocytes, neutrophils and endothelial cells are also involved in HSC activation [18]. Leucocytes act through cytokine production and neutrophils mostly produce ROS/RNS [20]. Injured endothelial cells are able to activate TGF-β from latent to profibrogenic forms [21].

The perpetuation phase of HSC activation is characterized by several phenotypic and functional changes. Notably, during perpetuation, in addition to paracrine stimuli, autocrine stimuli, including PDGF, TGF-β1, FGF and endothelin-1 (ET-1) also markedly contribute to the promotion of HSC activation [18] (Figure 1). An early event of activation is the loss of the perinuclear droplets of retinoids, stored as retinylesters and released as retinol after hydrolysis before export [7,22]. HSC also undergo proliferation, mostly stimulated by PDGF [23], although other mitogenic factors such as FGF, ET-1, thrombin, vascular endothelial growth factor and insulin-like growth factor are also operative [2,24]. Activated HSC generate fibrosis by increasing production of extracellular matrix protein. In particular, production of collagen (essentially collagen type I) is stimulated by TGF-β of paracrine or autocrine origin [25]. Matrix metalloproteinases (MMP) are a family of calcium-dependent enzymes playing a crucial role in matrix remodelling. They can be regulated at different levels, including inactivation by specific tissue inhibitors of metalloproteinases (TIMP) produced by activated HSC [26]. As a consequence of the inhibition of MMP, reduced degradation of the matrix takes place, resulting in excessive matrix accumulation. Other features of the perpetuation phase of HSC are contractility and chemotaxis [2].

After activation, the resolution phase ensues and deserves particular attention in view of its potential interest in medical treatment of fibrosis and devising new anti-fibrotic therapies [27]. In this phase, activated HSCs can either revert to the quiescent phenotype or be removed by an apoptotic process.

HSC play a central role in liver physiology, as they convert from resting cells to a proliferating, fibrogenic and contractile phenotype, in a process stimulated by neighbouring cells and regulated by several signalling cascades.

Cell signalling by oxidant species

HSC are sensitive to a large array of stimuli coming from both surrounding cells and an autocrine production, including cytokines, growth factors and lipid peroxidation end-products. Most of these signalling events depend on or are mediated by the action of reactive oxygen and nitrogen species (ROS and RNS). Although ROS and RNS are potentially responsible for the damage inflicted on almost all biological molecules, it was recently observed that relatively low concentrations of oxidants can behave as second messengers in cell signalling. After interaction with their receptors, growth factors and cytokines stimulate an intracellular transient increase in ROS [28–34], mostly represented by hydrogen peroxide, which activates specific signalling pathways. Important examples are tumour necrosis factor-α [35], platelet-derived growth factor [36], epidermal growth factor [37] and insulin [38]. Cellular thiols are critical in sensing and coupling redox changes to biochemical pathways [30,32,39–41], as they can react with oxidants faster than other aminoacids; in addition, most of their oxidized states, such as disulphide, can be reversed back to thiols [39,42].

In cells, ROS are generated from several enzymatic sources, but the most significant producers of oxidants are NADPH oxidases and mitochondria. The latter, when the scavenging capacity of ROS is exceeded, give rise to a continuous flow of hydrogen peroxide, potentially involved in the pathogenesis of several diseases. Mitochondrial production of ROS stimulated by agents such as inhibitors of the respiratory chain, leads to cell growth arrest or cell death by apoptosis or necrosis [43–46]. For instance, in activated primary human HSC, the endocannabinoid 2-arachidonoyl glycerol (2-AG) is a potent inducer of mitochondrial ROS production [47]. This may lead to the resolution of hepatic fibrosis due to induction of cell death [47]. Consequently, continuous production of ROS by mitochondria appears to be more involved in apoptosis and/or cell cycle arrest than in cell proliferation. Conversely, short-lived production of ROS by NADPH oxidases is preferentially associated with physiological signalling processes. Therefore, mitochondria and NADPH oxidases must be regarded as playing potentially distinct roles in their production of hydrogen peroxide [44]. However, in view of the complexity of the cellular environment, signalling of mitochondrial ROS cannot be excluded [28,46,48–54], although mitochondrial ROS formation is not subjected to the strict regulation that occurs with NADPH oxidases [54,55].

Nitric oxide (NO) acts as a second messenger by activating soluble guanylate cyclase which, in turn, catalyses the formation of cyclic GMP, controlling multiple signalling events leading to smooth muscle relaxation, inhibition of platelet aggregation and cell

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proliferation [42,56]. However, nitric oxide and its derivatives also exert a signalling action through other pathways, such as formation of nitrosothiols (RSNO) with protein cysteines and nitration of tyrosine residues. Although nitrosothiols are not formed by direct reaction of thiols with NO, there are many indirect pathways. NO can react with oxygen, forming nitrogen oxides capable of nitrosylating thiols directly. It can also react with superoxide anion in a near-diffusion controlled reaction to form peroxynitrite ($ONOO^-$), a potent oxidizing agent that is a major cause of nitrosative stress and may favour apoptosis [19]. Peroxynitrite can oxidize thiols and induce tyrosine nitration, thereby interfering with cell signalling processes in tyrosine kinase/phosphatase systems by preventing phosphorylation of tyrosine kinase substrates [19,56]. In the liver, all major cell types such as hepatocytes, Kupffer cells, endothelial sinusoidal cells and HSC can produce nitric oxide [19,57] and upregulation of inducible nitric oxide synthase (NOS2) has been observed in pathologic conditions [19]. In HSC, nitric oxide contrasts the effects of ROS by acting as a regulator of contractility, collagen induction and cell proliferation, but, in certain conditions, can also reinforce the action of ROS [19].

ROS, RNS and lipid peroxidation end-products are critical regulators of HSC activity and, through specific signalling pathways, play a crucial role in all the events to which these cells are subjected, particularly proliferation and collagen production.

Involvement of glutathione and thioredoxin systems in redox signalling

The low molecular weight thiol compound glutathione and the small protein thioredoxin play pivotal roles in signalling mediated by oxidant species. Glutathione and thioredoxin systems both maintain a reducing environment in the cell and use NADPH as the same upstream source of reducing equivalents (Figure 2). Thus, the 2GSH/GSSG and $Trx(SH)_2$/TrxSS ratios do not simply reflect the thiol redox balance of the cell but mainly determine the specific involvement of these species in well-defined signal

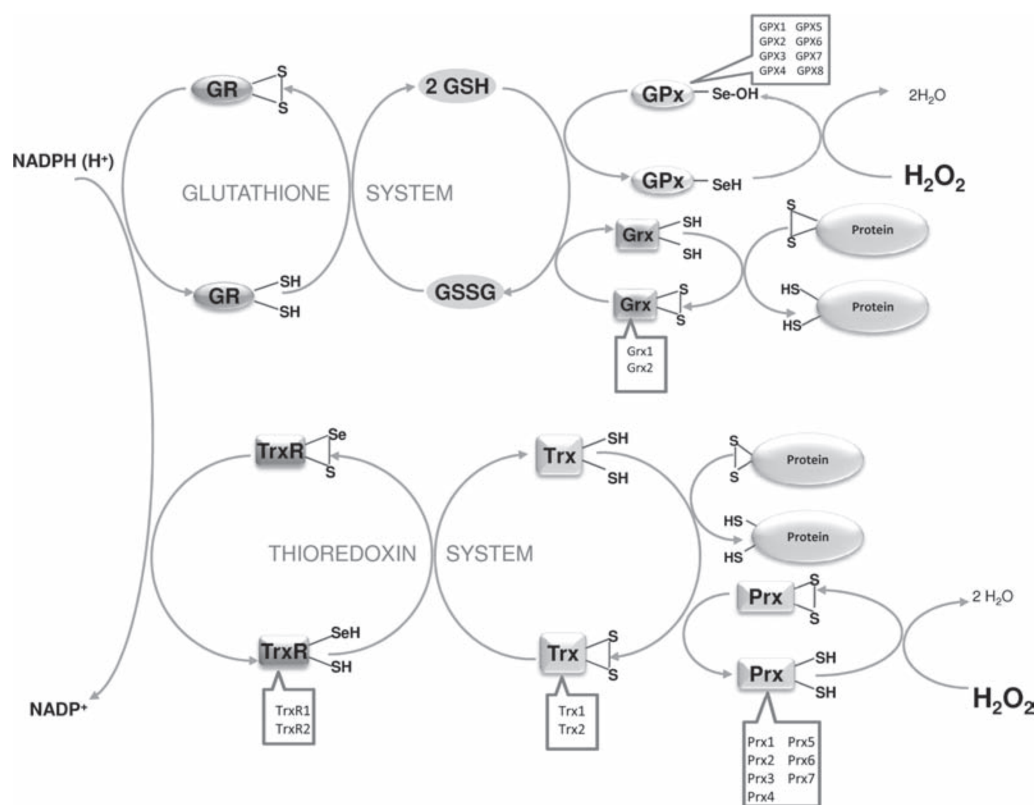

Figure 2. Control of thiol redox state by glutathione and thioredoxin systems. In cells, maintenance of thiol balance is mostly controlled by thioredoxin and glutathione redox systems. In glutathione system, the small protein glutaredoxin (Grx) also plays an important role. Hydrogen peroxide oxidizes glutathione (GSH) and thioredoxin ($Trx(SH)_2$) in reactions mediated by glutathione peroxidase (GPx) and peroxiredoxin (Prx), respectively. All thiol intermediates receive electrons from NADPH which, in turn, is maintained reduced by the pentose phosphate pathway in the cytosol. By transferring reducing equivalents from NADH to $NADP^+$, membrane-bound enzyme transhydrogenase forms NADPH in mitochondria. Further mitochondrial sources of NADPH are glutamate and isocitrate dehydrogenases which directly reduce $NADP^+$. NADPH, through glutathione reductase (GR) and thioredoxin reductase (TrxR), reduces glutathione (GSSG) and thioredoxin ($Trx(S)_2$), respectively. Thioredoxin and glutaredoxin, in their reduced forms, are able to reduce several protein substrates and then play a major role in signalling processes. For most of these enzymes, several isoforms exist, corresponding to diverse locations and functions.

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transduction pathways. However, one major difference is immediately apparent, as the intracellular concentrations of glutathione and thioredoxin are in the millimolar and micromolar ranges, respectively [58–60]. The two systems appear to operate independently in cell-reducing processes and signalling pathways [61,62].

Glutathione system

Glutathione is the main non-protein thiol in cells, playing an essential role in protecting against xenobiotics and oxidants [60]. Glutathione, kept reduced by glutathione reductase, acts as a substrate for glutathione peroxidase (GPx) [63], glutathione transferase (GST) and glutaredoxin. The latter is a 12-kDa protein, present in both cytosol and mitochondria, and interacts with ribonucleotide reductase and a range of proteins involved in cell signalling and transcription control [64]. Glutaredoxin accepts reducing equivalents from glutathione (Figure 2) and complements the action of thioredoxin in modulating cell responses to alterations in cell redox state [65]. For instance, suppression of proliferation was observed in myocardial H9c2 cells over-expressing Grx and treated with PDGF-BB [66]. This effect is due to enhanced dephosphorylation of PDGFR-β, depending on the retention of activity of low molecular weight protein tyrosine phosphatases by increased expression of Grx [66].

Glutathione can detoxify many electrophilic compounds in a reaction mediated by the glutathione S-transferase enzyme superfamily. These enzymes are involved in the metabolism of xenobiotics and some also show glutathione peroxidase-like activity with organic hydroperoxides [58]. They are present in various sub-classes with different substrate specificity [58]. Several isoforms of glutathione S-transferase, such as alpha, mu and pi, have been identified in HSC associated with significant levels of glutathione [67]. Glutathione S-transferase, coupled with GSH, plays an important role in detoxification of toxic aldehydes deriving from lipid peroxidation. In experimental animals, membrane phospholipids undergo lipid peroxidation after chronic administration of carbon tetrachloride or ethanol or bile duct ligation, generating several aldehyde end-products responsible for liver fibrosis [1,68]. These derivatives, e.g. 4-hydroxy-2-nonenal (4-HNE), play quite an important role in the adaptive response to oxidative stress and are included among the signalling species [56]. Glutathione S-transferase can modulate this type of signalling, catalysing GSH addition to electrophilic compounds such as 4-HNE [56]. However, as HSC, cultured for a few days, undergo depletion of most forms of GST [69], in the absence of GST, GSH is unable to conjugate the 4-HNE which can act on its cell targets and signalling pathways, independently of GSH concentration [69]. Altering GSH concentration therefore has limited effects on collagen synthesis after treatment of activated HSC with 4-HNE [69,70]. 4-hydroxynonenal and other 4-hydroxyalkenals can markedly induce procollagen type I synthesis [68]. Induction of the pro-fibrogenic stimulus by 4-HNE occurs at low, non-cytotoxic doses like those found after acute liver injury induced by CCl_4 (0.5–10 μM) [71]; at higher, experimental concentrations, it induces apoptosis in cultured rat HSC [72]. Whatever their source, the various aldehyde end-products of lipid peroxidation are all able to diffuse freely from their generation site and interact with targets inside and outside the cell, suggesting a close *in vivo* link between lipid peroxidation and liver fibrosis. Although the expression of most isoforms of GST falls during activation of HSC [69], stellate cell lines isolated from the liver of cirrhotic rats can still degrade HNE through aldehyde dehydrogenase activity [73]. Like aldehydic lipid peroxidation products, also F_2-isoprostanes, deriving from peroxidation of arachidonic acid, have been shown to mediate proliferation and collagen hyperproduction in HSC [74].

In HSC, reduced glutathione is recognized as a powerful anti-fibrogenic metabolite [75]. The glutathione content of HSC is present in an amount comparable to that of other cells of the myofibroblastic phenotype [76]. Notably, when plated in primary culture, HSC progressively increase their glutathione content, due to upregulation of γ-glutamyl cysteine ligase (γ-GCL) [76], the rate-limiting enzyme in glutathione biosynthesis. Although, during primary culture, HSC show all the phenotypic changes observed during activation [76], culture-induced accumulation of glutathione does not occur *in vivo*, i.e. in HSC isolated from fibrotic organs [76]. This discrepancy constitutes an uncommon case in which the pathways of HSC activation *in vivo* are different from those in culture-induced activation [2,76]. In HSC treated with the polyphenol compound curcumin, upregulation of γ-GCL has been observed, concomitant with increased GSH concentration, inhibition of cell activation and αI procollagen synthesis [77]. Similar effects were found for epigallocatechin gallate [78], which increases the level of both cytoplasmic and mitochondrial glutathione by stimulating the expression of gene coding for the catalytic sub-unit of γ-GCL. The increased activity of the latter leads to *de novo* synthesis of glutathione [78]. According to the same authors [78], EGCG interrupts TGF-β signalling and hence increases γ-GCL. TGF-β signalling is mediated by Smad proteins, which can activate or repress the expression of specific genes [79]. In particular, phase 2 gene expression is a target for TGF-β repression, leading to suppression of the synthesis of several enzymes, including superoxide dismutase 1, catalase, glutathione transferases (pi2, mu1, alpha4), the γ-GCL catalytic sub-unit [79] and glutathione

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peroxidase [80,81]. Therefore, interruption of this inhibitory pathway by EGCG leads to increased expression of γ-GCL and thus to enhanced capacity of the cell to synthesize glutathione.

Glutathione is maintained reduced by glutathione reductase in the presence of NADPH (Figure 2), as the GSH/GSSG ratio is critical for preserving the redox homeostasis of cells. According to De Bleser et al. [82], in activated HSC, glutathione levels play a role in discriminating whether hydrogen peroxide acts as a second messenger in TGF-β1 signalling or is the result of oxidative stress. The same authors also found an over-expression of catalase, modulating hydrogen peroxide internally generated in the TGF-β signalling pathway [82]. In contrast, in these cells, glutathione peroxidase is specifically involved in the removal of hydrogen peroxide deriving from extracellular sources [82]. According to Jameel et al. [83] the enzymatic activities of superoxide dismutase, catalase and glutathione peroxidase are lower in activated than in quiescent HSC. In addition, when HSC were treated with a controlled flux of superoxide generated by hypoxanthine/xanthine oxidase, they exhibited increased activities of SOD, catalase and GPx only in quiescent but not in activated HSC [83].

A cytoplasmic haemoprotein specific for HSC has been found by means of a proteomic approach. This protein, named Cygb/STAP (cytoglobin/stellate cell activation-associated protein), is significantly increased in HSC and shown to possess peroxidase activity [84] and oxygen binding properties [84,85]. In HSC, its expression is stimulated by sera, PDGF-BB and TGFβ-1 [85].

Thioredoxin system

The thioredoxin system is a redox transfer pathway which plays a crucial role in modulating cell viability and proliferation via thiol redox state [65,86,87]. This system is composed of NADPH, thioredoxin reductase (TrxR) and thioredoxin (Trx). Thioredoxin reductase is found in cytosol, nucleus (TrxR1) [88,89] and mitochondria (TrxR2) [90,91]. Thioredoxin, the major 12-kDa protein substrate of thioredoxin reductase, is also present in both cytosol (Trx1) and mitochondria (Trx2) and the cytosolic isoform can also enter the nucleus. Reduced thioredoxin donates electrons to a number of enzymes such as ribonucleotide reductase [92], methionine sulphoxide reductase [93] and peroxiredoxins [94,95]. Peroxiredoxins play a critical role in cell signalling, as they can act either as antioxidants by rapidly removing hydrogen peroxide [95] or in redox regulation of various signalling molecules by modulating the redox state of thioredoxin. Reduced thioredoxin-1, by binding to ASK1, inhibits apoptosis, which, however, takes place upon oxidation of thioredoxin and its dissociation from ASK1 [96]. In alveolar macrophages, it was recently shown that production of hydrogen peroxide by stimulated Nox2 activates ASK1 depending on thioredoxin-1 oxidation [97]. The function of several transcription factors depends on the redox state of thioredoxin, which interacts by means of its redox-sensitive cysteines [98–101].

Cells with dendritic and stellate morphology of various origins have all been shown to be positive for thioredoxin [102], which can be either constitutive or inducible. Therefore, thioredoxin appears to be critical in the general stress response of these cells. Transgenic mice over-expressing thioredoxin showed attenuated hepatic fibrosis induced by thioacetamide [103]. In addition, in HSC isolated from the same transgenic mice, proliferative capacity was lower than in the corresponding wild type and supplementation of thioredoxin to activated HSC significantly inhibited DNA synthesis upon stimulation with serum or PDGF [103]. These results partly explain the mechanism of thioredoxin-dependent decrease in hepatic fibrosis, which depends on inhibition of HSC proliferation [103].

Collagen expression was not inhibited in HSC isolated from thioredoxin transgenic mice compared with the wild type [103]. However, thioredoxin may act by controlling the maturation or degradation of collagen fibres at the post-transcriptional level [103]. It has been observed that Trx can exert redox control on collagen biosynthesis [104]. The C-propeptide region of human pro α1 type I collagen can bind wild-type Trx but not mutant Trx, in which the cysteine of the redox active site is replaced by a serine [104]. Pancreatic fibrosis was also attenuated in mice over-expressing Trx-1 and observed to be concomitant with suppression of pancreatic stellate cell activation [105]. Strong enhancement of thioredoxin expression was observed in hepatocellular carcinoma, whereas it was moderate in chronic hepatitis or cirrhosis [106]. Serum Trx levels were also significantly increased in patients with hepatocellular carcinoma, liver cirrhosis and, especially, non-alcoholic steatohepatitis (NASH) [106]. Thioredoxin binding protein 2 (TBP2) is a negative regulator of the expression and functions of thioredoxin. It binds the reduced form of Trx [106] and its over-expression results in growth suppression [106]. TBP2 is effective not only in cell growth regulation, but also in glucose and lipid metabolism and, together with thioredoxin, has been suggested as a potential marker of liver diseases like NASH [106].

In HSC, thioredoxin is maintained reduced by thioredoxin reductases, which are active in both cytosolic and mitochondrial compartments of HSC (unpublished results). Thioredoxin reductase is a selenium enzyme which is easily inhibited by a large number of compounds, including natural and synthetic organic compounds, metals and metal complexes [107,108]. As reported above, aldehyde products,

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essentially deriving from hepatocyte lipid peroxidation, play an important role in liver fibrogenesis. Aldehydes such as 4-HNE readily react with and inactivate both thioredoxin and thioredoxin reductase, provided the latter are in their reduced forms [109]. However, HSC contain both alcohol and acetaldehyde dehydrogenase [110,111], which cooperate in removing aldehyde products. In addition, gliotoxin, an anti-fibrogenic and pro-apoptotic agent, interacts with the thioredoxin system, displaying thioredoxin-dependent hydrogen peroxide-reducing activity [112].

Mitochondrial peroxiredoxin (Prx3) is an acceptor of electrons from thioredoxin and contributes toward controlling the intracellular balance of hydrogen peroxide. Prx3 is completely oxidized after inhibition of mitochondrial thioredoxin reductase [113] and may be responsible for the permeabilization of the outer mitochondrial membranes and the consequent release of pro-apoptotic factors. Peroxiredoxin 2 of HSC cells was found by proteomic analysis and shown to be under-expressed in specific conditions, e.g. high glucose concentrations [114]. In conclusion, in HSC, both glutathione and thioredoxin redox systems are active and play important roles in controlling activation and fibrogenesis.

Apoptosis of hepatic stellate cells: The role of mitochondria

As resolution of fibrosis largely depends on the increased apoptotic death of HSC [115–117], medical treatment of hepatic fibrosis can be potentially based on selective induction of apoptosis in them [118]. Activated HSC can undergo either spontaneous or receptor-mediated cell death [119]. In response to several stimuli, they can increase expression of Fas or TNF-α receptors and their ligands, leading to a caspase-8/caspase-3-linked apoptosis. Over-expression of pro-apoptotic proteins, such as Bax and p53, can also activate the mitochondrial pathways of programmed cell death mediated by caspase-9 [119].

There are therefore many conditions, factors, drugs and chemically unrelated compounds that can direct HSC toward apoptosis (Table I). There is evidence that ROS and RNS can stimulate apoptosis in HSC. When treated with systems producing superoxide, HSC undergo apoptosis with cytochrome *c* release, caspase-3 activation, increased Bax expression and hydrolysis of PARP (polyADP-ribose polymerase) [120]. However, increased expression of the anti-apoptotic protein Bcl-xL and translocation to the nucleus of NF-kB were also observed [120]. Novo et al. [121] showed the dose-dependent effects of superoxide anion on cell death, as low levels of superoxide were able to up-regulate procollagen type I expression, whereas a high rate of superoxide generation caused both apoptotic and necrotic cell death. Activated HSC, lacking retinoids, appear particularly subject to superoxide-induced apoptosis [83]. Dunning et al. [122] recently showed that ROS inhibit proliferation of HSC and, depending on concentration, promote different modes of cell death. Superoxide production was obtained by addition of menadione to the cells, which caused a flux of endogenous superoxide anion by a redox cycling process. RNS are also able to promote HSC apoptosis. Nitric oxide (NO) can induce apoptosis in HSC by a mechanism involving mitochondria and ROS [123]. The effects of RNS on the fibrogenic response of HSC are reported and discussed in a recent review [19].

The role of mitochondria in apoptosis of HSC is clearly evident when considering the effects of endocannabinoids [47,124–127]. Endocannabinoids such as 2-AG promote the resolution of hepatic fibrosis efficiently by inducing apoptosis in HSC [47]. The mechanism involved in 2-AG-induced apoptotic cell death depends essentially on enhancement of mitochondrial ROS production. Mitochondria are therefore a major source of oxidant formation after 2-AG treatment and mitochondrial components of the membrane permeability transition pore complex (MPTP), such as adenine nucleotide translocase, VDAC and cyclophilin D, very probably act as

Table I. Conditions, compounds and drugs stimulating or preventing fibrogenesis.

Activation/fibrogenesis	Refs	Resolution/apoptosis	Refs
Ethanol, carbon tetrachloride	[1–5]	Serum deprivation, senescence	[119]
Viral hepatitis, biliary obstruction	[119]	TNF-α, FasL	[132,133]
Increase of TIMP-1	[26]	TIMP-1 inhibition	[26]
ROS	[1–5]	ROS	[47]
TGF-β, PDGF	[1–5]	Gliotoxin	[135]
4-hydroxynonenal (4-HNE)	[1–5]	Endocannabinoids	[47,124–127]
F_2-isoprostanes	[74]	Epigallocatechingallate (EGCG)	[78]
		Resveratrol, taurine	[136,138]
		Tanshinone I, leflunomide	[139,140]
		Sulphasalazine, celecoxib, curcumin	[116,141,142]

Apoptosis of activated HSC is important in resolution of hepatic fibrosis and constitutes an attractive target for pharmacological therapy. In addition to oxidative stress and well-known protein factors and drugs, several natural secondary plant metabolites, such as terpenes, flavonoids and other polyphenols, can direct HSC to apoptosis.

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redox-sensitive targets involved in apoptosis [128,129]. Alteration of mitochondrial membrane permeability leads to cytochrome *c* release and caspase activation, well-known hallmarks of apoptosis. In the same experimental conditions, hepatocytes were also observed to be more resistant to 2-AG-induced apoptosis than HSC [47] and this behaviour was due to the high glutathione content of hepatocytes [127]. Unlike 2-AG, the endocannabinoid anandamide mostly induces necrosis in HSC [124].

Several studies, e.g. [130,131], report that ROS stimulate HSC activation and proliferation instead of inducing apoptosis. This apparent contradiction, discussed by Siegmund et al. [47] was solved on the basis that low levels of ROS, mostly produced by NADPH oxidase, have a proliferative and activating effect whereas high levels of ROS, essentially of mitochondrial origin, direct cells to the death pathways. 2-AG may act as an endogenous anti-fibrogenic agent, being more specific [47] than other endogenous mediators of cell death such as TNF-α [132] and FasL [133].

A specific apoptotic effect on HSC was obtained with the fungal metabolite gliotoxin, conjugated with mannose-6-phosphate-modified serum albumin, which can selectively accumulate in liver fibrogenic cells and reduce their number [134]. Previous works have shown that gliotoxin has anti-fibrogenic properties *in vivo* and acts as a potent pro-apoptotic agent in HSC [117,135].

Flavonoids such as epigallocatechin gallate (EGCG) can direct HSC to apoptosis by increasing levels of the pro-apoptotic protein Bax and decreasing anti-apoptotic Bcl-2 [78]. EGCG also reduces the level of cyclin D1 and enhances the quantities of two proteins ($p21^{(WAF1/Cip1)}$ and $p27^{(Kip1)}$) which act as inhibitors in regulating cell cycle progression [78].

Resveratrol (3,5,4′-trihydroxy-trans-stilbene), found in a variety of plants, is well-known to induce apoptosis in several types of cells [136]. At concentrations similar to those estimated in biological fluids of subjects with diets rich in food containing polyphenols, resveratrol causes cell cycle arrest and apoptosis of HSC, observed as an increase in the cell fraction with sub-diploid DNA content and from the direct inspection of nuclear morphology [136]. However, resveratrol has also been shown to amplify the pro-fibrogenic effect of fatty acids by up-regulating several genes, including the anti-apoptotic protein Bcl-2 [137]. Other compounds inducing apoptosis in HSC are taurine [138], tanshinone I, a diterpene quinone [139], leflunomide [140], sulphasalazine [116], celecoxib [141] and curcumin [142]. Lastly, inhibition of heat shock protein 90 [143], activated NK cells [144] and interferon α [145] can also induce the death of HSC. In addition, the number of drugs and chemicals reported to be able to stimulate apopotosis of HSC is rapidly increasing (for a selection see Table I). Many of these compounds may represent novel therapeutic approaches in treating hepatic fibrosis.

NADPH oxidase of hepatic stellate cells and related signalling pathways involving protein kinases and redox regulation

NADPH oxidase represents a well-controlled source of ROS, playing a critical role in signalling processes involved in the modulation of fibrogenesis (Figure 3). NADPH oxidase is a multi-enzyme complex producing superoxide via the one electron-reduction of oxygen by NADPH. In phagocytic cells, this complex contributes to host defence, whereas, in non-phagocytic ones, it acts as a regulator in intracellular signalling [146–148]. In neutrophil granulocytes, NADPH oxidase consists of two trans-membrane proteins, the catalytic sub-unit gp91phox (Nox2) and the regulatory sub-unit p22phox, together forming cytochrome b558 [149]. In addition, four regulatory proteins (p47phox, p67phox, p40phox and the Rac1/2 GTPase) are located in the cytosol of resting cells [150]. Following stimulation, the cytosolic components are rapidly recruited to the plasma membrane, where they assemble with flavocytochrome b558 to form the active enzyme in a highly regulated process consisting of a sequence of events involving phosphorylation, GTPase activation and protein–protein interactions [146,151]. In particular, the mechanism of recruitment involves multiple SH3-domain interactions, coupled with the interaction of phox homology domains with phosphoinositides [147,152]. However, the signalling pathways leading to activation of the enzyme complex in phagocytic and non-phagocytic cells are still not completely elucidated and several lines of evidence suggest a role played by protein kinases/phosphatases and phospholipases in the post-translational modifications of the various components.

A functionally active form of NADPH is expressed in HSC and has been reported to be a critical mediator of liver fibrogenesis [130,153]. In this type of cells, fibrogenic factors, such as angiotensin II [130], PDGF [154], leptin [155,156] and apoptotic bodies [157] activate NADPH oxidase to produce ROS which stimulate several intracellular signalling pathways including MAP kinases and phopshatidylinositide 3-kinase. For instance, PDGF potently induces proliferation of HSC mediated by NADPH oxidase-dependent production of ROS which, in turn, stimulate the phosphorylation of p38 MAP kinase [154]. HSC prepared from p47phox-deficient mice and incubated with leptin do not induce ROS production, in contrast to wild-type HSC which, in the same conditions, give rise to a marked formation of ROS [156]. Similarly, leptin stimulates proliferation of wild-type HSC, while the proliferation of $p47phox^{-/-}$ HSC is considerably lower [156]. Also, treatment with specific inhibitors of NADPH oxidase such as apocynin or diphenylene iodonium (DPI) decreases HSC proliferation mediated by fibrogenic factors [156]. In addition, in HSC stimulated with leptin, both pharmacological treatment with specific inhibitors or genetic inhibition of NADPH

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oxidase are able to reduce the production of markers of fibrogenesis, such as collagen α1(I) and α-smooth muscle actin [156]. Furthermore, mice lacking p47 phox oxidase do not develop liver fibrosis after bile duct ligation [130].

Some specific characteristics differentiate the role played by NADPH oxidase in non-phagocytic and phagocytic cells. For instance, non-phagocytic NADPH oxidase is constitutively active and consequently produces relatively low levels of ROS; moreover, in response to various stimuli, it generates high levels of oxidants. However, in non-phagocytic cells, the exact features of the components of this complex and their interactions are not completely known [158].

Several human homologues of gp91phox (Nox2), encoded by distinct genes, have been identified in non-phagocytic cells. These include Nox1 and Nox3–5 as well as Dual oxidase (Duox) 1 and 2 [146,158]. The expression of these proteins depends on the cell and tissue types, functions and on sub-cellular distribution [159]. In HSC, the quiescent state is associated with a strong expression of Duox, whereas other Nox need induction to become activated [160].

Homologues of the cytoplasmatic components p47phox and p67phox, named NoxO1and NoxA1, respectively, were also found [153]. A relevant difference between p47phox and NoxO1 is the lack of the C-terminal domain in the latter. Therefore, NoxO1 and p47phox affect oxidase activity in a different manner [161]. In p47phox, the C-terminal domain is regulated by phosphorylation in response to different stimuli. Serine phosphorylation of p47phox [162] seems to be an important post-translational modification regulating the assembly, translocation and activation of NADPH oxidase. Phosphorylation of p47phox makes the phox homology domain capable of binding phosphatidylinositol 3,4-bisphosphate and phosphatidic acid [163]. This binding contributes to the aggregation of the NADPH oxidase complex and is critical for its localization to the membrane. Notably, p47phox, poorly expressed in quiescent HSC, is highly expressed in activated HSC in culture or in cells obtained from patients with liver fibrosis [153,164]. Besides, angiotensin II was shown to stimulate the phosphorylation of p47phox in activated HSC [164].

In non-phagocytic cells, the activation of NADPH oxidase has recently been shown to be regulated by tyrosine phosphorylation of p47phox mediated by c-Src, a member of the Src family kinases (SFKs) [165,166]. These findings are also confirmed by the observation that specific SFK inhibitors such as PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine), after stimulation of HSC by PDGF, prevent the translocation of p47phox to the plasma membrane, with a significant decrease of ROS production (unpublished data). c-Src can also indirectly support the function of the NADPH oxidase complex by association to cortactin which acts as a scaffold protein to link the components of NADPH oxidase with the actin cytoskeleton [167].

There is increasing evidence that c-Src activity is controlled not only by phosphorylation but also by a redox regulatory process [168–172]. c-Src tyrosine kinase has been shown to be activated after oxidation,

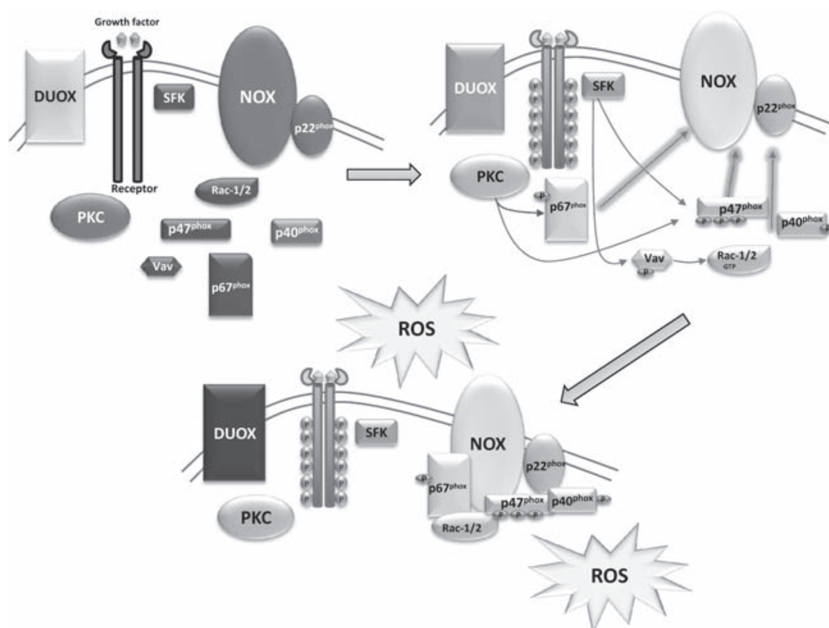

Figure 3. Proposed model depicting effects of growth factor interactions with cells and consequences on signalling pathways, depending on kinases and redox-regulated processes. A variety of growth factors interact with membrane tyrosine kinases receptors which, in turn, phosphorylate and activate several signalling molecules including PKC and SFK. Activated kinases phosphorylate cytosolic components of NADPH oxidase such as p47phox, which assemble with membrane components of enzyme. Active NADPH oxidase generates superoxide, which dismutates to hydrogen peroxide. H_2O_2 modulates a large number of signalling pathways.

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leading to disulphide bond formation between Cys245 and Cys487, as apparent from the insensitivity of mutants to oxidation [169]. Interestingly, heavy metals such as mercury ions, that are well-known to react preferentially with thiol residues in proteins, markedly enhance the activity of c-Src [172], again indicating that thiol alterations influence functioning of these proteins. However, the cysteines involved in mercury ion activation are different from those previously reported to be involved in disulphide bond formation [169]. As a result, in addition to the known phosphorylation/dephosphorylation cycle regulating the activation state of c-Src [173–175], several evidences underline the role of redox regulation [169,170]. A redox circuit has been proposed and involves c-Src kinase-dependent activation of NADPH oxidase. In turn, the latter, through hydrogen peroxide production, promotes the complete activation of c-Src. Fully active c-Src acts as a mediator in transmitting the downstream signalling dependent on ROS [165].

A further component needed for activation of NADPH oxidase is Rac GTPase, in particular Rac1 in HSC [153]. Rac belongs to the Rho family of small GTPase proteins, involved in the regulation of several cell functions and acting as molecular switches able to cycle between GDP-bound inactive and GTP-bound active forms which stimulate downstream effectors [176]. The Rac active form is adopted after interaction with GDP/GTP exchange factors (GEFs). The latter includes a variety of molecules such as Vav1/2 [176] which, in order to be activated, needs to bind inositides such as phosphatidylinositol 3,4 and 3,4,5-trisphosphate. In this process, a critical role is exerted by phosphatidylinositol 3-kinase which, by producing phosphoinositides, acts as an important participant upstream of NADPH oxidase activation. The role of phosphatidylinositol 3-kinase has also been studied in HSC upon PDGF stimulation [154]. Transgenic mice over-expressing Rac1 are a good model to show the role played by this component of NADPH oxidase in mediating liver injury [177]. In fact, Rac-transgenic mice treated with carbon tetrachloride (CCl_4) were characterized by a large number of activated HSC. In addition, they showed a more severe liver damage and a more sustained hepatocyte apoptosis, liver fibrosis and mortality in comparison to CCl_4-treated wild-type animals [177].

c-Src-mediated generation of ROS by NADPH oxidase is also regulated by tyrosine phosphorylation of GEFs. In HT29 human colonic adenocarcinoma cells, tyrosine phosphorylation of nucleotide exchange factor Vav2 is abolished in cells treated with PP2 [168], a selective inhibitor of c-Src, already described. Furthermore, Vav2-specific siRNA also blocks Src-mediated ROS generation [168]. The data reported highlight the prominent role of SFKs in connecting the signalling pathways, triggered by specific agonists, to the activation of NADPH oxidase.

On the whole, it is clearly apparent that the finely regulated NADPH oxidase complex plays an essential role in controlling the fibrogenic signalling pathways of HSCs. In addition, NADPH oxidase may be an attractive target of selective drugs to be used in the anti-fibrotic therapy for liver diseases.

Conclusions

HSC are multiform 'protean' cells [2], in which the pathophysiology of oxidative stress, redox signalling

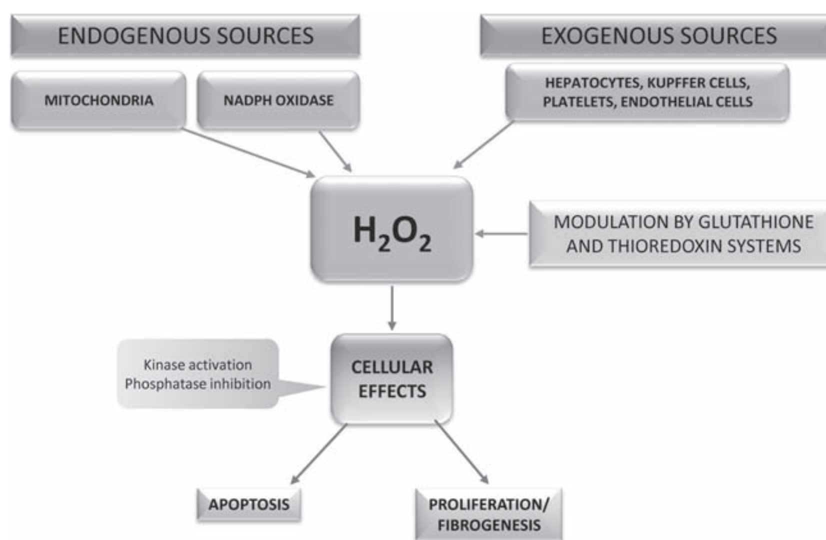

Figure 4. Redox biology of hepatic stellate cells. HSC are challenged by oxidants from exogenous and endogenous sources. Hydrogen peroxide is the most important signalling compound, with concentration controlled by glutathione and thioredoxin systems through their respective peroxidases. H_2O_2 acts on several targets in cells, eliciting, according to conditions, apoptosis or proliferation/fibrogenesis.

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and the various pathways depending on the kinase/phosphatase system are closely intertwined. Oxidative stress is intimately involved in the stimulation of HSC, as shown by the effects of ROS/RNS and lipid peroxidation products which, in addition to cytokines and growth factors, alter the intracellular redox conditions of HSC resulting in excessive ECM deposition. A decisive role in the control of redox signalling pathways is played by the glutathione and thioredoxin systems which, in many instances, can link redox processes to the kinase pathways. Intracellular signalling pathways, involving kinases such as MAP kinases, PI 3-K and PKC, are also activated by ROS and cytokines in a process in which NADPH oxidase plays a critical role.

The most significant features of the redox biology of HSC are shown in Figure 4. It is apparent that several exogenous or endogenous sources give rise to an increase in ROS, mostly hydrogen peroxide, which is strictly controlled by the thiol-dependent redox systems linked to glutathione or thioredoxin. Hydrogen peroxide can act on several intracellular targets eliciting effects which, depending on conditions, give rise either to apoptosis or cell proliferation and fibrogenesis.

As hepatic fibrosis is the result of several chronic liver diseases and essentially depends on HSC activation, one research challenge is the development of pharmacological therapies able to reverse this process. The induction of apoptosis, in which mitochondria play a fundamental role, is being actively studied. Several natural substances, such as terpenes, flavonoids and other polyphenols with recognized anti-fibrotic properties may constitute a potential nutritional approach which, in addition to well-established pharmacological therapies, may contrast the progression of fibrosis in the liver.

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