

Oxidative Stress, Plant-Derived Antioxidants and Liver Fibrosis

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Abstract

There is accumulating evidence that oxidative stress plays a considerable role in the development of liver fibrosis by acting in different cell types and in different signaling pathways. Consequently, antioxidants, particularly those of plant origin, have emerged as potent antifibrotic agents. This review briefly sum-

marizes current views of the mechanisms of fibrogenesis and recent findings on the antifibrotic potential of plant-derived antioxidants.

Key words

Antioxidants · fibrogenesis · flavonoids · hepatic stellate cells · reactive oxygen species

Introduction

Liver fibrosis and cirrhosis developing in response to chronic hepatocellular injury (Table 1) show general features of a wound repair process [1], [2] characterized by specific cellular reactions that are orchestrated by a set of cytokines and other signaling molecules and finally lead to the excessive deposition of extracellular matrix proteins [2], [3], [4], [5]. As these processes continue, remodelling of the liver architecture is apparent resulting in severe pathophysiological consequences such as liver insufficiency, portal hypertension and hepatic encephalopathy. Although fibrosis and cirrhosis are of high incidence worldwide, therapeutic management of these diseases is still insufficiently based on therapeutic concepts that focus mainly on symptoms rather than on blocking central fibrogenic mechanisms [6], [7], [8]. Recent progress in the understanding of the pathological mechanisms, however, may open new strategies with which to interfere at early steps in the development of these diseases [3], [9], [10].

Oxidative stress has long been known to be involved in the pathogenesis of hepatic fibrosis [1], [11], [12], [13]. In the past, the main focus was placed on the damaging potential of oxygen radi-

Table 1 Natural and experimental causes of liver fibrosis

Viral hepatitis (chronic)	Chemicals (carbon tetrachloride, dimethylnitrosamine, stilbestrol, yellow phosphorus)
Alcohol abuse	
Radiation	Drugs (methyldopa)
Cholestasis (bile duct ligation)	Diet (choline deficient) Heterologous serum (pig in rats)
Oxidative stress	TGF- β transgenic mice

cals and other radicals for parenchymal cells (PC) [11], [14]. Thus, oxidative stress and radicals were considered as special primary causes of the disease. Consequently, antioxidants were recommended only in rare occasions [14] and were not recognized as therapeutic drugs with a potentially wide application. With time, it became increasingly evident that oxidative stress is associated with various cellular reactions during the development of fibrosis [15] (and of other pathologies [16]) and, thus, may not only be cause of but also mediator in this process (see below). Despite this fact, the therapeutic potential of antioxidants in fibrosis was not estimated adequately in reviews on traditional and more recent therapeutic approaches [8], [9].

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Current View of Fibrogenesis

As illustrated in Fig. 1 our present understanding of the key events in hepatic fibrosis reflects a complex interplay of cells and mediators. Roughly, the pathophysiological process can be divided into three phases [17], the preinflammatory, the inflammatory, and the postinflammatory phase. During the preinflammatory phase complete or minor damage of hepatic parenchymal cells (PC) initiates or facilitates the release of paracrine acting mitogen(s) and cytokines that have a dual effect. First, they induce proliferation of hepatic perisinusoidal stellate cells (PSC) [18]. Second, they activate resident macrophages, the Kupffer cells (KC), which, in turn, by releasing a whole spectrum of different cytokines initiate the inflammatory phase [17], [19]. This phase is associated with a number of cellular changes in liver parenchyma. Stimulated by TGF- β , the prototype of a fibrogenic cytokine, normal PSC transform into a myofibroblast-like phenotype [20], [21]. Other cytokines may participate in this process as well [4], [17], [19], [22], [23], [24]. This transformation is considered a central event, since the resulting myofibroblasts have been shown to contribute in many ways to the excessive deposition of extracellular matrix [22], [25], [26] and to produce several other mediators that further promote the cellular changes in a paracrine and autocrine fashion [19], [27], [28]. Production of such factors may lead to the recruitment of new cells (neutrophils, mast cells, etc.) in liver parenchyma [28], [29], [30]. In addition, the activated KC attract other cell types such as polymorphonuclear leukocytes (PML) and may even potentiate damage of PC via release of proteases, reactive oxygen species (ROS) and toxic cytokines (e.g., TNF- α) [17], [31]. In general, the inflammatory phase may be characterized as the most aggressive period, maintained in a vicious cycle by various (chronic) exogenous and endogenous signals. Despite the fulminant dynamic of this phase, fibrogenesis is still reversible as obvious from spontaneous regeneration, when primary stimuli are withdrawn [32] or after interference with anti-inflammatory cytokines [4], [19], [33]. In contrast of the detailed view of the pathogenesis, information on the mechanisms leading to (spontaneous) regeneration is relatively sparse as yet. Apoptosis of stellate cells seems to be important [9], [32], [34], [35], but little is known about the regulation of this process [36]. From recent studies, however, it is emergent that signals transmitted through neuronal factors like nerve growth factor [37] or through the peripheral-type benzodiazepine receptor [38] may be involved in this regulation as may be fibronectin-derived antiadhesive peptides [39].

The autocrine stimulation of the myofibroblasts initiated already in the inflammatory phase may be the most important feature of the third, the postinflammatory phase which is characterized by an increasing self-perpetuation of the fibrogenic process [27]. Apparently, cellular reprogramming has reached a degree that is independent of the primary stimuli [40]. One reason for this may be the continuous deposition of extracellular matrix which modulates the environment of the cells and is known to considerable influence gene expression in transformed PSC [17], [41], [42], [43]. Another reason may be changes in the response to cytokines characteristic for the myofibroblast phenotype as found for TGF- β signaling [44]. To what extent the pathogenesis is reversible at this point, is still a matter of debate and has to await further elucidation of regenerating mechanisms. When the process continues to develop into cirrhosis, however, the irreversible state is reached undoubtedly.

Fibrogenesis in Alcoholic Fibrosis and Chronic Hepatitis

Although the principal sequence of events is likely to be similar in fibrosis of different etiology, inflammation may not always be overt as, for instance, in alcoholic fibrosis [45]. There, hypoxia and products of oxidative stress may be more important [46], [47], since enhanced ROS production occurs in response to CYP11E1-induction by ethanol [48]. However, the pathogenesis of alcoholic liver disease involves additional mechanisms specific to excessive alcohol consumption [49], [50]. In chronic hepatitis C, PSC activation and fibrosis seem to be associated with necroinflammation and a Th1-like response, but independent on viral load [51]. While elimination of hepatitis C virus in responders to interferon therapy led to a deactivation of stellate cells [52], in non-responders *d*-alpha-tocopherol was able to prevent fibrogenesis [53], again favoring oxidative stress as mediator. Apparently, various aspects of the general scheme of fibrogenesis may be emphasized differently depending on the type of primary stimulus, its duration and other circumstances.

Specific Involvement of Oxidative Stress in Fibrogenesis

ROS may be created by a variety of different mechanisms and usually are under close control by sophisticated cellular detoxification systems (Fig. 2). Within the cascade of events outlined above for fibrogenesis, oxidative stress is enhanced and seems to be involved in many ways. This will be summarized for each of several main liver cell types individually.

Hepatocytes

On the level of the parenchymal cells, production of ROS may be cause and consequence of cellular damage. For instance, many hepatotoxins (often through metabolism by the cytochrome P450 system [48], [54] lead to increased concentrations of ROS that cannot be handled in a normal way by the protective machinery of the cells [55]. Excessive production of ROS results in lipid peroxidation leading to an increase in highly reactive aldehydic end products, altered signal transduction, modulation of gene expression, alteration of the redox state including decrease of glutathione levels, and induction of apoptosis and necrosis [56], [57], [58]. With respect to fibrogenesis, enhanced rates of lipid peroxidation that surmount those associated with physiological events (e.g., hepatocyte regeneration) seem to be of considerable importance [59]. Thus, additional factors such as the availability of heavy metals, particularly of free iron, may be cofibrogenic by enhancing oxidative stress [13]. However, this may vary for hepatocytes and KC in different types of fibrosis [13]. Under the conditions of high lipid peroxidation, the aldehydes formed (e.g., malondialdehyde and 4-hydroxynonenal) attack various cellular and extracellular proteins in the hepatocytes but also in adjacent cell types [60]. They seem to affect gene expression in adjacent PSC as demonstrated by the induction of matrix components (collagen type I, fibronectin), matrix metalloproteinases and other factors [56], [61], [62], [63], [64], [65]. Fibroblasts could also be a potential target [59], [66].

Kupffer cells

Similar events from increased lipid peroxidation to induction of apoptosis may act on the level of KC. However, in the case of

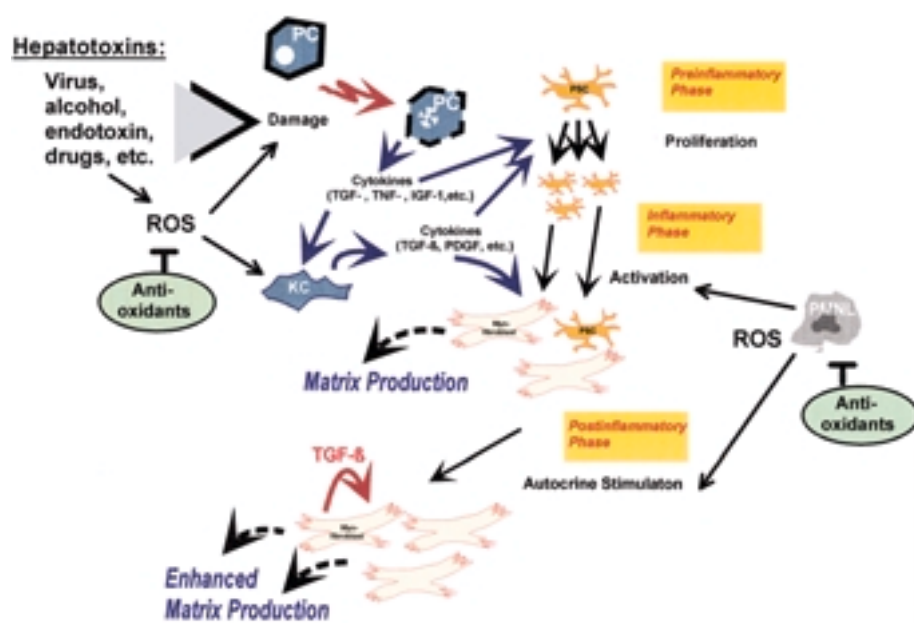


Fig. 1 Key events in hepatic fibrosis. Schematic illustration of key events in fibrogenesis emphasizing the role of reactive oxygen species (ROS) and some cytokines. Possible major sites for intervention by antioxidants are also indicated. KC, Kupffer cells; PC, parenchymal cells; PMNL, polymorphonuclear leukocytes; PSC, perisinusoidal stellate cells.

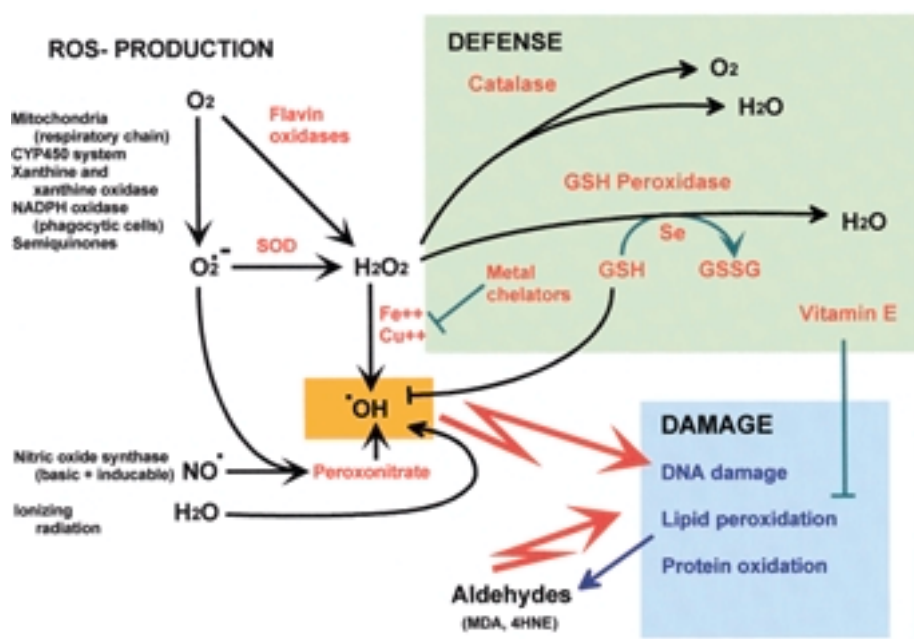


Fig. 2 Major reaction of reactive oxygen species (ROS). Schematic illustration of basic mechanisms of the formation, detoxification, and damaging reactions of reactive oxygen species. Cu^{++} , copper; Fe^{++} , iron; HNE, 4-hydroxynonenal; MDA, malon dialdehyde; SOD, superoxide dismutase.

these cells excessive production of, rather than ultimate injury by, ROS seems to prevail in their activated state. Thus, phagocytic activity is accompanied by increased formation of toxic oxygen metabolites and other radical species such as NO [24], [67]. Similar products may be generated by PML [24]. These products are released into the extracellular space and may attack other cells in the vicinity. Interestingly, however, NO has been found to act as a scavenger of ROS *in vitro* inhibiting PSC proliferation [68]. Thus, the precise role of peroxonitrate formed from NO (see Fig. 2) remains to be clarified.

Perisinusoidal stellate cells

Besides the parenchymal cells and macrophages, PSC may be attacked by liberated ROS. Due to their central role in fibrogenesis, these cells may represent the most important cellular target. However, although ROS may promote transformation of PSC [69], such molecules may also be mediators of transformation by other stimuli, e.g., TGF- α and collagen type I [17]. Apparently,

there is considerable cross-talk between ROS and various signaling pathways [70], [71] (see below). Moreover, ROS seem to be involved in further regulating and influencing many cellular events once the myofibroblast-like phenotype has been established. Thus, ROS have been shown to directly affect the synthesis of monocyte chemoattractant protein 1 [72] and connective tissue growth factor (CTGF) [73]. It is likely that other proteins produced by PSC (myofibroblasts that are related to fibrogenesis are directly induced as well. On the other hand, the fibrogenic effect of oxidative stress induced by ferric nitrotriacetate in cultured PSC seems to be mediated through induction of the Na^+/H^+ exchanger [74]. The importance of the Na^+/H^+ exchange which can also be induced by other factors like PDGF and IGF-1 [75] for the fibrogenic process has recently been stressed *in vitro* and *in vivo* [76]. In other models, NF- κ B and c myb seem to be essential mediators [3], [77], indicating again that ROS and other radicals have to interfere with certain signaling pathways, in order to exert their effects. Indeed, activation of NF- κ B might play a potent

role in hepatic injury and fibrosis [78] through activation of PSC [79]. Although NF- κ B is believed to mediate a general antioxidant cellular response [80], its activation is subject to complex control opening the possibility for alternative functional consequences. Thus, it was reported that NF- κ B may be involved in apoptosis rather than proliferation and activation of HSC in culture [81], [82]. Moreover, the distal effects of this transcription factor seem to depend on the cellular background [83], and, in particular, on the timing of activation in relation to other signals [84]. Therefore, it is hard to predict whether activation of NF- κ B will result in cell survival, proliferation, apoptosis or another fate.

As indicated above, aldehydes produced in response to oxidative stress through lipid peroxidation such as 4-hydroxy-2,3-alkenals (e.g., HNE) may play an additional role as mediators [85] both in PSC as well as other cells (for review see [3], [60]. Apparently, transformed PSC are less sensitive to toxic effects of these aldehydes than normal PSC [86]. This seems to be due to a higher rate of metabolism [86] although the level of GSTs which detoxify HNE is decreased [87].

Correspondence of *in vitro* and *in vivo* Results

There is still some uncertainty whether the sequence of events outlined above or their extent is comparable *in vivo* and *in vitro*. For instance, this has been questioned with respect to cellular glutathione [88] and the site or temporal pattern of the production of superoxide radicals [89]. Furthermore, modulation of glutathione content may not have easily predictable effects on the function of stellate cells [90]. The context in which these parameters are tested, however, seems to be of considerable influence. It is also possible that the general scheme may vary to some extent depending on the specific cause of fibrosis. Thus, it was doubted that PSC are subject to oxidative stress during iron-induced fibrogenesis in rodents [13]. On the other hand, the level of oxidative stress rather than cytokines seems to be responsible for the progression of the activation of PSC during CCl₄-induced fibrogenesis [91]. In this respect it is of interest that the glutathione level can discriminate between oxidative stress and TGF- β signaling in activated PSC [92].

Therapeutic Potential of Plant-Derived Antioxidants in Liver Fibrosis

For a long time, the therapeutic potential of antioxidants with respect to fibrosis was realized only marginally [3], [6], [7], [8]. In the last few years, however, a number of studies has dramatically changed this situation [3], [93] placing oxidative stress and the antifibrotic efficacy of antioxidants into focus. Even traditional drugs such as pentoxifylline [94], as well-known phosphodiesterase inhibitor, were unexpectedly found to block PSC activation by interfering with the oxidative stress cascade suggesting new mechanisms for their antifibrotic activity.

Antifibrotic efficiency – *in vivo* studies

Accumulative evidence for the effectiveness against fibrosis is now available for several plant-derived antioxidants. Silymarin, the active principle from *Carduus marianus* L., proved to be antifibrogenic in humans [95], [96] and in a rat fibrosis model [97], [98] where it led to a reduction of hepatic collagen accumulation

by more than 35%. It is well known that silymarin and its component silibinin have potent antioxidant activity [99], [100]. Likewise, in a bile duct ligation model, the antifibrotic influence of extracts from *Stephania tetrandra* and *Polygonum aviculare* [101], [102] was described, although the active compounds have not yet been identified. In the same experimental model quercetin was found to ameliorate liver damage [103]. Furthermore, antioxidants from the herbal medicine Sho-saiko-to (e.g., baicalin, baicalin and wogonin) were recently found to act antifibrogenic in different animal models, namely choline-deficient L-amino acid-defined diet [104] and fibrosis induced by pig serum [105] or dimethylnitrosamine [105], [106]. Sho-saiko-to had already been known for beneficial effects in patients with chronic active hepatitis [107] and may have an even broader range of potential applications [108]. Also, antioxidants in red wine have long been suspected of exerting antifibrotic effects [50]. Likewise, diosmin was found *in vivo* to reduce fibrosis associated with edema [139]. These promising reports on plant-derived antioxidants are complemented by other recent findings that polyenylphosphatidylcholine and vitamin E were also effective as antifibrogenic drugs in alcohol-induced fibrosis in the baboon [109] or in carbon tetrachloride-induced liver cirrhosis [14]. Thus, there is no doubt that plant-derived antioxidants represent valuable antifibrotic drugs.

However, what may render these compounds (particularly the flavonoids) so efficient may not be their antioxidative potential alone. This is apparent from reports comparing different antioxidant vitamins (C and E), selenium and antioxidants from *Nigella sativa* in CCl₄-induced liver fibrosis in rabbits [110]. In this model, *Nigella sativa* proved to be effective, while vitamin C was not. Concerning vitamin E and selenium results were less promising [110] than in a similar rat fibrosis model [14]. Using this latter model, hepatoprotective effects of other plant extracts from *Emblica officinalis* (syn. *Phyllanthus embilca* L.) and *Artemisia iwayomogi* were found [111], [112] that may only partially involve antioxidant functions. Olive oil in contrast to sunflower, corn or fish oil was also protective against CCl₄-induced fibrosis [113].

Possible mechanisms – *in vitro* studies

Although the efficacy of antifibrotic agents can best be demonstrated by *in vivo* studies, mechanistic details of this activity that can be derived from these studies, however, are sparse. Therefore, many *in vitro* studies with plant extracts, less complex fractions or isolated compounds were performed. Obviously, the antioxidative activity was considered first. In most of the cases cited above, the antioxidants were polyphenols, flavonoids or structurally related compounds [95], [105], [114] that are known as strong antioxidants [115], [116], [117]. As pointed out above, many points of interference of antioxidants with single steps in the fibrogenic process seem possible. Central points seem to be NF- κ B, bcl-x_L and c-myc which are upregulated in activated PSC [82]. Indeed, silymarin and silibinin inhibit NF- κ B activation [83] and seem to retard PSC activation [118] (R. Gebhardt, G. Buniatian, unpublished results). Likewise, *trans*-resveratrol (from grapes), a strong antioxidant, was found to deactivate the myofibroblast phenotype [119]. Another target may be the induction of CTGF by ROS such as hydrogen peroxide [73]. In general, antioxidant such as baicalin, baicalin, quercetin, apigenin and *trans*-resveratrol were shown to interfere with fibrogenic functions of PSC and KC *in vitro* [105], [114], [118], [119], [120], [121].

Concerning flavonoids, it is known that they exert a variety of other effects besides acting as antioxidants. In particular, they are inhibitors of different protein kinases [122], [123], [124] and other kinases involved in signal transduction [125], which they inhibit with different potency. As a result, they may interact with intracellular signaling as has been demonstrated recently for luteolin in cultured hepatocytes [126]. Furthermore, flavonoids are known to interrupt the cell cycle at different points [127], [128]. Accordingly, proliferation of PSC usually associated with the fibrogenic process *in vivo* was effectively inhibited *in vitro* by baicalein, the major flavonoid in Sho-saiko-to [129]. A similar function was described for *trans*-resveratrol which shows some structural similarity to flavonoids [119]. Since protein kinases, in particular stress-activated protein kinases, also play a role in the activation of PSC [130] as well as other cell types [131], such inhibitory effects of flavonoids may be of significance for interrupting the pathogenic process in various different ways rendering these natural compounds particularly interesting for therapeutic use. It remains a challenge for the future to unravel the definitive antifibrotic potential of flavonoids and to elucidate whether there is any synergistic influence between the antioxidative functions and the inhibitory effects on kinases, transporters and other proteins in the prevention of hepatic fibrosis.

Bioavailability, biotransformation and responsiveness

An issue of particular concern linking *in vivo* and *in vitro* studies is the question of bioavailability of genuine compounds on administration of plant extracts or complex fractions. Concerning silymarin and silipide (lipophilic silybin-phosphatidylcholine complex), detailed comparative studies on solubility and pharmacokinetics in animals and patients with extrahepatic biliary obstruction or cholecystectomy have been performed [132], [133], [134], [135]. These studies revealed the rapid availability in serum and bile of silybin (particularly from silipide) in free and conjugated form, but also of silydianin, silycristin and isosilybin. Studies on the bioavailability of baicalin in rats revealed that it is mainly absorbed as the aglycone after hydrolysis by the intestinal microflora and is re-conjugated in intestine and liver [136]. Similar results were reported by Li and coworkers for baicalin and other flavonoids from Sho-saiko-to [137]. Interestingly, these authors demonstrated a delayed excretion of the flavonoids after administration of Sho-saiko-to compared to isolated components [137] indicating an enhanced efficacy of the flavonoids as part of a complex herbal medicine. Although these studies consistently demonstrate the bioavailability of the flavonoids, two aspects deserve further investigation. First, it remains uncertain which molecules (aglycones, conjugates or degradation products) are responsible for the biological effects. Regarding quercetin, for instance, it was shown that some metabolic products (e.g., 3,4-dihydroxytoluene) were almost as efficient as the flavone itself with respect to the antioxidative function as well as the inhibition of metabolic pathways [138]. Thus, biotransformation and breakdown do not necessarily lead to inactivation. Second, patients might be divided into responders (showing high serum levels) and non-responders [137]. The basis for these interindividual differences is not yet known, but may be due to differences in metabolism by the intestinal microflora, in the extent of absorption, and in biotransformation and excretion. Since such question are of considerable importance, if herbal medicines shall be used effectively, these aspects should be investigated in more detail.

Conclusions

As demonstrated by recent studies *in vivo* and *in vitro*, plant antioxidants, particularly flavonoids, show a remarkable potency to block liver fibrogenesis of different etiology. Since the few examples studied so far have just opened a wide horizon, it is worth to screen other plant extracts and natural compounds in appropriate model systems and to look for further compounds combining antioxidative properties with other effector functions. Such studies may lead to new drugs particularly suited and specifically tailored to block liver fibrosis at early steps of pathogenesis. On the basis of preliminary experimental results, it may be expected that such drugs are suitable also for preventive care and for supporting the endogenous regenerating capacity of the liver, once fibrosis has already developed.

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