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Review

# Role of hydrogen peroxide and oxidative stress in healing responses

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**Abstract.** Oxidative stress is a host defense mechanism whose involvement in maintaining homeostasis and/or inducing disease has been widely investigated over the past decade. Various reactive oxygen species (ROS) have been defined and the enzymes involved in generating and/or eliminating them have been widely studied. In this review we briefly discuss general mechanisms of oxidative stress and the oxidative stress response of the host. We focus primarily on hydrogen peroxide and summarize the systems involved in its formation and elimination. We describe mechanisms whereby hydrogen peroxide and other ROS can modify protein conformation and, thus, alter protein function, and describe a group of transcription factors whose biological activity is modulated by the redox state of cells. These basic aspects of oxidative stress are followed by a discussion of mechanisms whereby hydrogen peroxide and other ROS can modulate some physiological and pathological processes, with special emphasis on wound healing and scarring of the liver.

**Key words.** Hepatic stellate cells; extracellular matrix; fibrosis; wound healing; oxidative stress; organ scarring.

## Introduction

In aerobic organisms, $O_2$ is the final electron acceptor for mitochondrial cytochrome oxidase in a complex chain of events leading to the formation of high-energy phosphates required for multiple cellular functions. During this process, $O_2$ undergoes a four-electron reduction to form $H_2O$. A side-product of mitochondrial oxidative phosphorylation is the accumulation of reactive oxygen species (ROS), some of which, such as hydroxyl radicals ($^{\bullet}OH$), are very unstable, highly reactive, induce membrane lipid peroxidation, generate multiple reactive aldehydes and cause cell death. Others, such as $H_2O_2$, are more stable, less reactive and may act as second intracellular messengers (fig. 1). However, they can alter protein conformation after oxidation of cysteine and methionine residues. Moreover, in the presence of $Fe^{2+}$ or $Cu^{+}$, via the so-called Fenton reaction, $H_2O_2$ is converted to $^{\bullet}OH$. However, although the iron-dependent formation of $^{\bullet}OH$ occurs in vivo, the physiological significance of the copper-dependent formation of $^{\bullet}OH$ is still debated. The amount of ROS formed is not negligible because of the high amount of $O_2$ consumed by aerobic organisms. Indeed, approximately 2–4% of oxygen consumed in mitochondria is converted to the superoxide ion by iron-sulfur proteins. Thus, to maintain homeostasis, accumulation of excess ROS is prevented by multiple enzymatic and non-enzymatic systems that receive the generic name of 'host antioxidant defense systems.' When they fail, either because of excess production of ROS and/or because of a relative or absolute de-

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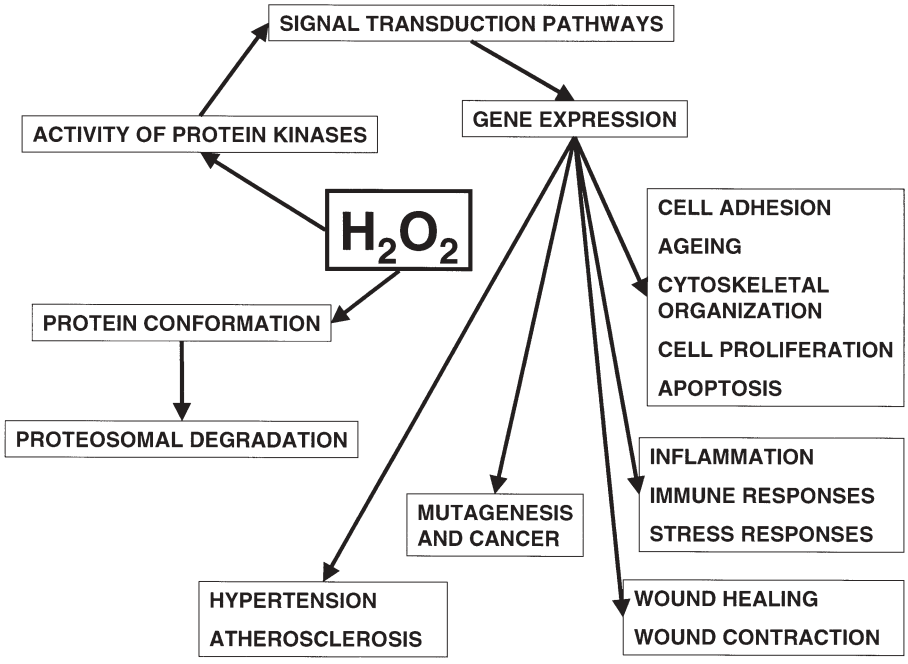


Figure 1. Schematic representation of the multiple actions of $H_2O_2$. $H_2O_2$ is involved in multiple physiological and pathological processes via its capacity to regulate the expression of several protein kinases and phosphatases. Through these mechanisms and also via the oxidation of sensitive amino acid residues in proteins, it regulates protein gene expression and affects protein half-life. This latter effect results from the induced changes in protein conformation that trigger proteosomal degradation. These are by no means the only mechanisms whereby $H_2O_2$ modifies gene expression, nor does the figure contain an exhaustive list of pathological conditions affected and/or induced by this ROS. Nonetheless, it provides an overview of an interesting and important host defense mechanism that can result in cell damage, cell death and/or scarring of tissues.

crease in the capacity of the cells to eliminate them, the alteration in homeostasis results in oxidative stress. In many instances, as occurs during wound healing and/or organ scar formation, ROS are key elements of the healing response leading to excess collagen accumulation. However, while this is a desirable event during skin wound healing, excess collagen deposition that occurs in parenchymatous organs results in organ fibrosis, which in turn may lead to organ failure. Thus, a better understanding of the molecular events whereby oxidative stress induces cell death, organ scarring and/or accumulation of undegraded proteins in cells could result in a more rational therapeutic approach to disease.

The role of oxidative stress in general and of $H_2O_2$ in particular in physiological and pathological conditions has been established using different animal models and a large variety of cultured cells. Thus, although we have taken the simplistic approach of unifying molecular events described in the literature, these may not be identical in all cell types and/or animal species. Strong evidence supports the notion that distinct cell types within a single animal species, as well as similar cell types derived from different animal species, may express significant differences in their response to oxidative stress. Accordingly, to establish the physiological relevance of an oxidative stress event, a causal relationship needs to be established. During the past few years, the field of oxidative stress has expanded considerably and multiple reviews implicating ROS in organ pathology have been published [1–21]. To avoid an unnecessarily long list of older references, when possible, the above-mentioned review articles will be cited.

## Cellular sources and general properties of $H_2O_2$

$H_2O_2$ is a bacteriostatic agent widely used in dental and medical practices and is a key element in the defense mechanisms of inflammatory cells. Many cells produce it and its presence can be detected in urine, blood and exhaled air. Consequently, measurements of $H_2O_2$ in body fluids and/or exhaled air could be used to measure oxidative stress status during physiological and pathological conditions and/or to monitor the response to treatment [22–26]. $H_2O_2$ is a small and relatively stable molecule that diffuses readily and thus, like other small molecules, such as $Ca^{2+}$, diacylglycerol or cAMP, has the potential to act as a second messenger and modulate gene expression. $H_2O_2$ is generated by two general mechanisms: (i) by enzymatic or chemical dismutation of superoxide ions and (ii) by the action of certain oxidases via a two-electron reduction of oxygen. The superoxide ion is produced in many subcellular compartments by multiple enzymatic mechanisms, most of them belonging to the following

categories: oxidases, peroxidases, mono- and dioxygenases as well as cytochrome P450s [4]. These include, among others, xanthine oxidase [27], nitrous acid synthase [28], NAD(P)H-dependent oxidoreductases [29], cyclooxygenases and lipooxygenases [30] and aldehyde oxidase [31]. The enzymes that generate $H_2O_2$ directly include the following peroxisomal oxidases: glycolate, D-amino, ureate, L-$\alpha$-hydroxyacid and fatty-acyl-CoA oxidases. In addition, $H_2O_2$ is generated by monoamino oxidase [32–35] and lysyl oxidase [36], enzymes localized to the mitochondria and the extracellular space, respectively.

Because of the different mechanisms involved in $H_2O_2$ formation as well as the implications in disease states, the formation of ROS could be classified into two general categories: those derived from mitochondrial oxygen consumption and those that are mitochondrial independent. The efficiency of oxygen consumption by the mitochondrial respiratory chain is highly dependent on its coupling state and, thus, changes in $O_2$ consumption can result in alterations in $H_2O_2$ formation. Moreover, cellular injury leading to $Ca^{2+}$ accumulation will accelerate the electron transfer process and, thus, increase ROS formation. Similarly, chemicals that alter coupling of the respiratory chain, such as antimycin, or those that change the redox state of the cell, such as alcohol or its metabolite acetaldehyde, could result in excess formation of ROS [11, 37–39].

**NADPH oxidase**

Of all the systems involved in $H_2O_2$ formation, the best characterized is that of the NADPH oxidase localized to the plasma membranes of phagocytic and non-phagocytic cells [40–46]. It is a multimeric complex made of several subunits localized to the plasma membrane and cytosol. The plasma membrane component of this enzymatic complex is cytochrome $b_{(559)}$, a heterodimer made of $gp91_{phox}$ and $p22_{phox}$. The redox activity of the NADPH oxidase complex is located in the $gp91_{phox}$ subunit. Activation of NADPH oxidase is associated with a rapid depolarization of membrane potential due to electron passage sing through the enzyme complex from intracellular NADPH to extracellular molecular oxygen. Interestingly, data from several laboratories suggest that this enzyme may play a role in oxygen-sensing processes underlying many biological processes including hypoxic pulmonary vasoconstriction and smooth muscle cell proliferation [9]. However, this complex is not functional unless there is recruitment of the cytosolic proteins $p40_{phox}$, $p47_{phox}$ and $p67_{phox}$, the extrinsic factor Rac2 and members of the Ras superfamily of small GTP-binding proteins. Although the existence of the complete NADPH oxidase complex has not been demonstrated in non-phagocytic cells, some components of the system are present [40–46].

**Superoxide dismutases (EC 1.15.1.1)**

Superoxide dismutase (SOD) catalyzes the conversion of $O_2^-$ to $H_2O_2$ and $O_2$. Of the three SODs described in eukaryotic cells, two are Cu/Zn and one Mn dependent. Of the two Cu/Zn SODs, one is localized to the cytosol and nucleus (SOD-1) and the other is an extracellular enzyme (ECSOD or SOD-3). The Mn-dependent enzyme (SOD-2) is localized to the inner mitochondrial membrane [30, 47–55].

SOD-1, a homotrimer of 32 kDa, is an important component of the oxidative stress defense system. Although its gene knockout is not lethal, the animals are more susceptible to paraquat toxicity and are infertile [53]. Furthermore, mutations and/or alterations in the expression of SOD-1 have been suggested to occur in disease states such as familial amyotrophic lateral sclerosis and Down syndrome [54, 55].

SOD-2 is a 22-kDa protein whose function is to remove $O_2^-$ generated in mitochondria and prevent damage [30]. The key role of this enzyme in preventing cell injury is manifested in knockout mice in which newborns die shortly after birth in metabolic acidosis. These mice also have severe cardiomyopathy and lipid accumulation in liver and skeletal muscle [52].

SOD-3 is a tetrameric glycoprotein with an apparent molecular weight of 135,000. It is localized to the extracellular space and has binding affinity for heparan and heparan sulfate proteoglycans and type I collagen [49]. It is produced by a large variety of cells, including fibroblasts and glial cells [48]. Its function in vivo may be to maintain the vasodilatory action of nitric oxide (NO) by inhibiting the accumulation of $O_2^-$. This species, upon reaction with NO, generates peroxynitrite radicals. Indeed, SOD-3 activity is decreased in coronary arteries of patients with atherosclerosis [50]. In the lung, SOD-3 protects against oxygen toxicity that can induce cell death [51].

**Other oxidases**

Lysyl oxidase, the enzyme involved in formation of aldehyde precursors of cross-links in collagen and elastin [36, 56–58] is an oxidase that generates $H_2O_2$. This enzyme binds to collagen and elastin in the extracellular space and is responsible for the oxidation of $\varepsilon$-amino groups of specific lysyl and hydroxylysyl residues to yield aldehydes necessary for intra- and inter-chain cross-linking [36, 56–58]. A co-product of this enzymatic reaction is the formation of $H_2O_2$. In addition to its enzymatic activity, lysyl oxidase induces chemotaxis of vascular smooth muscle cells, an action prevented by $\beta$-aminopropionitrile. Oxidation of lysyl and hydroxylysyl residues in collagen and elastin is associated with an increased production and accumulation of $H_2O_2$ resulting in enhanced stress fiber formation and focal adhesion assembly [34].

Because $H_2O_2$ is a mediator of the fibrogenic actions of acetaldehyde and transforming growth factor (TGF)-$\beta$1 [59, 60], generation of cross-linking precursors could further fibrogenesis by generating additional ROS. Thus, some antifibrogenic actions of $\beta$-aminopropionitrile and penicillamine could be attributed to their capacity to inhibit the enzyme and decrease $H_2O_2$ formation [61–63]. The enzymatic activity of monoamino oxidases involved in metabolizing multiple amines is also blocked by $\beta$-aminopropionitrile. Tyramine degradation by monoamino oxidases (MAOs) in adipocytes generates $H_2O_2$ and cAMP and these effects are blocked by pargyline [34]. Furthermore, in kidney cells transfected with MAO-B, tyramine oxidation induced tyrosine phosphorylation of Shc, ERK and increased DNA synthesis. Similarly, these effects are blocked by pargyline and N-acetyl-cysteine [35]. Thus, the reduction in collagen production by these agents is likely due in part to their capacity to inhibit several oxidases that generate $H_2O_2$ [58, 63].

## Antioxidant defense systems involved in detoxification of $H_2O_2$

There are multiple enzymatic and non-enzymatic systems involved in the elimination of $H_2O_2$. These systems can be divided into three main groups [for a review, see ref. 64]: antioxidant enzymes, which include catalase and glutathione peroxidase (GPX); chain-breaking antioxidants, such as tocopherols and ascorbate, and transition metal-binding proteins, such as transferin and ferritin. Antioxidant enzymes catalyze the breakdown of ROS, in the intracellular environment. On the other hand, chain-breaking antioxidants are powerful electron donors and react preferentially with free radicals before important target molecules are damaged. Lastly, transition metal-binding proteins prevent the interaction of transition metals, such as iron and copper, with $H_2O_2$ and superoxide, thus inhibiting formation of highly reactive hydroxyl radicals.

## Antioxidant enzymes

### Catalase

Catalase (EC 1.11.1.6) is a tetrameric heme-containing enzymatic complex of 240 kDa containing identical subunits of 60 kDa. This enzyme reacts very efficiently with $H_2O_2$ to form $H_2O$ and molecular oxygen and with $H^+$ donors (methanol, ethanol, formic acid, phenols) with peroxidase activity. It is localized mainly in peroxisomes; however, amounts in other subcellular compartments remain unclear because peroxisomes are easily ruptured during cell manipulation procedures [53, 64, 65]. Although catalase is not essential for survival, it plays an important role in the acquisition of tolerance to oxidative stress [66].

### Glutathione peroxidases

GPXs (EC 1.11.1.19) catalyze the oxidation of glutathione at the expense of $H_2O_2$ or other hydroxyperoxides. GPX shares the substrate $H_2O_2$ with catalase; however, unlike the latter, it can also react effectively with lipid and other organic hydroxyperoxides. Thus, these enzymes play an important role in protecting cells against lipid peroxidation. Most GPXs require selenium at the active site and are considered to be one of the most essential antioxidant defense mechanisms in mammals [53, 64, 65]. So far, five GPX isoenzymes have been described and although their expression is ubiquitous, levels of each isoenzyme vary from tissue to tissue [for a review, see ref. 67]. Most GPXs are localized mainly to the cytosol and mitochondria, suggesting that these enzymes are the main scavengers of $H_2O_2$ in these subcellular compartments. An important consideration is that activity of these enzymes is dependent on the availability of reduced glutathione, which in turn depends on the activity of glutathione reductase and $\gamma$-glutamyl cysteine synthase, the rate-limiting enzyme for reduced glutathione (GSH) synthesis [68]. Consistent with these observations, glutathione reductase has a similar distribution to that of GPX [69].

## Chain-breaking antioxidants

Below, we will summarize some of the properties and actions of several chain-breaking antioxidants, including vitamins E and C, carotenoids, flavonoids, thioredoxins and reduced glutathione. Although their properties will be described separately, we would like to emphasize that in vivo, very complex interactions between antioxidants occur, so that predicting their function in a particular setting is difficult. For example, under certain circumstances, antioxidants may exert paradoxical effects and contribute to oxidative damage, e. g., high concentrations of vitamin C plus iron can lead to increased oxidative damage [70, 71]. Similarly, tocopherols in the absence of aqueous-phase antioxidants can promote low-density lipoprotein oxidation [72].

### Vitamin E

Vitamin E belongs to a family of naturally occurring lipid-soluble compounds with different antioxidant properties of which $\alpha$-tocopherol is the most abundant in the human body. It is a physiological membrane-bound chain-breaking antioxidant that protects cell membrane lipids from oxidant damage by free radicals. The resulting tocopheroxyl radical is relatively stable, since the excess charge associated with the extra electron gets dispersed across the chromanol ring. This resonance-stabilized rad-

ical can be subsequently oxidized to form tocopherol quinone, or react with another $\alpha$-tocopheroxyl radical to form stable dimers. Alternatively, $\alpha$-tocopherol can be regenerated by reaction with other antioxidants, such as ascorbate or GSH [64].

**Carotenoids**

Carotenoids are a group of approximately 600 lipid-soluble antioxidants structurally related to vitamin A. In human plasma and tissues, a wide range of carotenoids have been identified including cyclic (e.g., $\beta$-carotene, $\alpha$-carotene) and acyclic carotenes (e.g., lycopene, phytoene) together with a number of xanthophylls (e.g., zeaxanthin, lutein and $\beta$-cryptoxanthin), all derived from dietary sources. These compounds have multiple antioxidant activities, including the ability to scavenge singlet oxygen, an excited state of a partially reduced form of $O_2$, and the hydroperoxyl radical, which can attack fatty acids, peroxidizing them directly. Thus, carotenoids play a key role in preventing lipid peroxidation. Singlet oxygen-scavenging activity of carotenoids can occur in two ways: (i) by a physical transfer of the excitation energy from singlet oxygen to the carotenoid with subsequent dissipation of this energy as heat, without a concomitant loss of the carotenoid molecule and (ii) by a chemical reaction between singlet oxygen and carotenoids that results in the irreversible destruction of the antioxidant molecule. However, with regard to chemical mechanisms whereby carotenoids scavenge hydroperoxyl radicals, these are still not well understood. They combine with hydroperoxy radicals to form a large resonance-stabilized radical, a reaction that may take place in different sites of the molecule. On the other hand, there is also evidence to suggest that quenching of hydroperoxyl radicals by carotenoids can lead to the release of carotene epoxides, or other compounds including apocarotenals, apocarotenones and carbonyl chain cleavage products. As expected, the ability of carotenoids to scavenge hydroperoxyl radicals and inhibit lipid peroxidation is dependent on the length of their conjugated double-bond structure [64, 73, 74].

**Flavonoids**

Flavonoids are a large family of low molecular-weight polyphenolic compounds found in vascular plants. They have multiple biological activities including antioxidant, antiinflammatory, antiviral and anticarcinogenic activities [for a comprehensive review, see ref. 75]. Due to their chemical nature, they are powerful chain-breaking antioxidants that can act as potent metal chelators and free radical scavengers. Kandaswami and Middleton [76] have reviewed the free radical-scavenging and antioxidant activity of plant flavonoids. ROS that can be scavenged or whose formation can be inhibited by flavonoids include superoxide anion, $H_2O_2$, singlet $O_2$ and perhydroxy, hydroxyl, alkoxyl and peroxyl radicals.

**Vitamin C**

Vitamin C (ascorbate) is a potent water-soluble antioxidant that is essential for many enzymatic activities (e.g., prolyl- and lysyl-hydroxylases) and also acts as a free radical scavenger. This compound scavenges hydroxyl and peroxyl radicals, $O_2^-$ and nitrogen dioxide, as well as hypochlorous acid, ozone, singlet oxygen, nitrosating species, nitroxide and peroxynitrite. In addition, it can regenerate other small-molecule antioxidants, such as $\alpha$-tocopherol, glutathione, ureate and $\beta$-carotene from their respective radical species. During its antioxidant action, ascorbate undergoes a two-electron reduction, first to the semidehydroascorbyl radical and subsequently to dehydroascorbate. The semidehydroascorbyl radical is relatively stable since the excess electron charge disperses over three oxygen atoms. In contrast, dehydroascorbate is relatively unstable and hydrolyzes rapidly to diketogulonic acid, which is subsequently degraded into oxalic acid. On the other hand, dehydroascorbate can also be reduced back to ascorbate by enzyme-dependent and -independent pathways involving the selenoenzyme thioredoxinreductase or GSH respectively [64, 70, 71, 77].

**Thioredoxins and GSH**

Two key antioxidant players involved in intracellular redox regulation are GSH and thioredoxins [64, 78]. Thioredoxins are a family of small proteins (approximately 12 kDa) that undergo NADPH-dependent reduction by thioredoxin reductase and in turn reduce oxidized cysteine groups on proteins. Due to this dithiol to disulfide exchange activity, these proteins act as hydrogen donors and determine the oxidation state of protein thiols. Thioredoxins have a characteristic conserved catalytic site containing two cysteine residues, which can be oxidized reversibly to form disulfide bridges. Two main thioredoxins have been described, thioredoxin-1 and thioredoxin-2. Thioredoxin-1 has many biological actions including the supply of reducing equivalents to thioredoxin peroxidases and ribonucleotide reductase, the regulation of transcription factor activity, and the regulation of enzyme activity by heterodimer formation. Interestingly, thioredoxin-1 stimulates cell growth and inhibits apoptosis [79].

GSH (L-$\gamma$-glutamylcysteinylglycine), a cysteine-containing tripeptide, is the principal thiol responsible for maintaining intracellular redox status and protecting cells against oxidative/nitrosative stresses. It is found at relatively high concentrations (1–10 mM) in virtually all mammalian cells, mainly in the cytoplasm. Close to 15% of cytosolic GSH is translocated to the mitochondria through a specific mitochondrial carrier. This tripeptide has several functions, including providing a storage and transport form of cysteine, conjugation with xenobiotics and electrophilic intermediates, maintenance of sulfhydryl groups and transfer of reducing equivalents. This

latter function is critical for protecting cells against ROS toxicity, particularly hydrogen peroxide. GSH is an essential factor for the activity of glutathione peroxidase, but can also scavenge ROS directly. GSH is oxidized nonenzymatically by $H_2O_2$ or enzymatically by glutathione peroxidase, and this is reduced back to GSH by the flavoenzyme glutathione reductase and NADPH [64, 80–82].

**Transition metal-binding proteins**

Metalloproteins, such as ceruloplasmin, metallothionein, ferritin, transferrin and lactoferrin are well known for their critical role in metal homeostasis and function as storage reservoirs and/or chaperones for essential trace metals, such as copper and iron. Evidence indicates that these proteins are induced during the acute-phase response and under oxidative stress conditions. These proteins ameliorate the deleterious effects of ROS, by sequestering the redox-active metals iron and copper, thus minimizing their capacity to catalyze ROS production via the Fenton reaction [64]. In addition, ceruloplasmin can catalyze oxidation of the ferrous ions to the less reactive ferric state (ferroxidase activity), thus functioning as an important plasma antioxidant when redox-active iron is involved in molecular damage [83, 84]. Moreover, this protein was recently demonstrated to catalytically remove $H_2O_2$ and lipid hydroperoxides at physiologically relevant concentrations of GSH [85]. Thus, this glutathione peroxidase-like activity of ceruloplasmin, together with its ferroxidase activity could play an important role in protecting cells against oxidative stress. On the other hand, there is also evidence suggesting that under certain circumstances, ceruloplasmin enhances oxidative stress [86]. $H_2O_2$ can release $Cu^+$ from ceruloplasmin in a time-dependent manner and this is accompanied by formation of $HO^-$ radicals and fragmentation of the protein [87, 88].

**Rofe of $H_2O_2$ in physiological processes**

Formation of ROS is only one part of the equation leading to oxidative stress damage. Many enzymatic and nonenzymatic mechanisms are involved in the elimination and/or neutralization of ROS. Thus, a perfect balance of systems involved in $H_2O_2$ production and elimination is necessary to maintain homeostasis. This is illustrated by the fact that small amounts of SOD can protect against radiation injury. However, when SOD is overexpressed, production of $H_2O_2$ or OH radicals can surpass the capacity of the cell to eliminate them, thus inducing oxidative stress [89]. The role of the delicate balance involved in maintaining $H_2O_2$ levels is further illustrated in the kidney. Oxygen consumption varies greatly in the three regions of the kidney (cortex > medulla > papilla), and as expected, this gradient correlates with the amount of $H_2O_2$ formed. However, because levels of SOD and catalase correlate with actual levels of $H_2O_2$ formed, the steady-state concentration of this ROS is the same in the three zones of the kidney. Nonetheless, the papilla is more resistant to reperfusion/ischemia damage than the cortex and medulla, perhaps due to the higher levels of glutathione peroxidase and $\alpha$-tocopherol in this region [90–92].

**$H_2O_2$ as a second messenger and modulator of gene expression**

$H_2O_2$ is involved in signal transduction pathways and thus alters the activity of multiple protein kinases and phosphatases. It modifies the activity of enzymes and transcription factors oxidizing free SH groups in cysteine residues to form disulfide bridges and also by oxidizing methionine residues to sulfoxides and sulfones. Moreover, $H_2O_2$ induces formation of NO and this ROS in turn modifies protein conformation via nitrosylation of specific tyrosine and cysteine residues [93–95]. Because NO enhances oxygen consumption in mitochondria and this in turn is coupled to the rate of $H_2O_2$ formation, NO further enhances formation of this ROS [96]. On the other hand, NO reacts with $O_2^-$ to form peroxynitrate, a highly reactive species. In addition to its effect on proteins, $H_2O_2$ induces DNA damage and can alter binding of transcription factors to cis-regulatory elements in gene promoters. Accordingly, through a complex set of molecular events, $H_2O_2$ activates and/or represses gene expression (see below) [97–99].

**Regulation of gene expression by ROS-induced alterations in protein conformation**

Because proteins contain reactive SH groups, they are susceptible to chemical modification by $H_2O_2$ or any other ROS [97]. Although disulfide exchange occurs at alkaline pH values, reactive SH groups can be readily oxidized to form disulfide cross-links with other SH groups of the same protein or they can form mixed disulfides with the SH group of glutathione. Some of these chemical modifications of proteins, although important in preventing further damage by ROS, could result in conformational changes of the protein leading to its accumulation in cells and or its rapid degradation by the proteasome. Indeed, studies performed with ribonuclease A revealed that exposure to $H_2O_2$ induces conformational rearrangements of the protein and modifications of some amino acid side chains leading to degradation by the proteasome [98]. Similarly, actin function is impaired by oxidative stress. $H_2O_2$ oxidizes Cys374 of monomeric actin, followed by the oxidation of several methionine residues to methionine sulfoxides. Because these changes occur in

domains required for polymerization and binding of specific proteins, oxidative stress can induce important changes in actin organization, polymerization and distribution. Thus, many actin-dependent activities could be altered as well [99].

**Redox regulation of transcription factors**

Many environmental stimuli including several cytokines and growth factors, ultraviolet radiation and chemical agents can generate high levels of ROS which can potentially perturb the normal redox balance and shift cells into a state of oxidative stress. This response in turn triggers numerous signaling pathways aimed at restoring cellular homeostasis and is accompanied by significant alterations in the pattern of gene expression. Although the molecular events leading to changes in gene expression are not well understood, many of them are mediated through functional alterations in the activity of transcription factors. Indeed, a large number of redox-responsive transcription factors have been identified [for reviews see refs 100–104]. ROS can enhance or repress their biological activity either directly, through modifications by oxidation on sulfur-containing residues (i.e., cysteine and methionine), or via other signals, such as alterations in their state of phosphorylation/dephosphorylation. Indeed, ROS induce the activation of several signaling pathways including the ERK, JNK, p38 MAPK and PI3K/Akt cascades, and this effect is cell specific [105–106]. The sensitivity of a given transcription factor to changes in redox state is variable and will depend on its conformation and cysteine/methionine content. Most transcription factors contain strategic cysteine/methionine residues which play important roles in their ability to bind and/or recognize DNA, interact with other transcription factors and/or transactivate (or repress) gene expression [for reviews see refs 100–104]. Below, we will briefly describe some representative transcription factors whose biological activity is modulated by oxidative stress.

**Activator protein-1**

Activator protein-1 (AP-1) is a member of a family of sequence-specific transcription factors that play important roles in regulating cell proliferation, differentiation, apoptosis, inflammation and stress response [for reviews, see refs 102, 107]. This family of proteins controls the expression of many genes that play key roles during the wound-healing response, including several matrix metalloproteinases [108, 109], type I collagen [110] and TGF-$\beta$1 [111]. AP-1 is a dimeric factor composed of either homo- or heterodimers between members of the c-Fos (c-Fos, FosB, Fra-1 and Fra-2) and c-Jun (c-Jun, c-JunB and c-JunD) families. These subunits contain a basic leucine zipper (bZIP) domain that plays an important role in dimerization (leucine zipper) and DNA binding (basic region), as well as a transcriptional activation domain located at the amino terminus [107]. Different AP-1 dimers regulate different cellular responses, although the mechanisms controlling their assembly, targeting and functional specificity remain unclear. The activity of AP-1 increases rapidly in cells exposed to a variety of extracellular signals that alter the cellular redox status, such as mitogens, $H_2O_2$ and ultraviolet light, through transcriptional, post-transcriptional and/or post-translational mechanisms [103, 112]. Redox regulation of AP-1 transactivation potential can be exerted via two major mechanisms, including changes in AP-1 phosphorylation and reversible oxidation and reduction of Fos/Jun proteins. AP-1 remains inactive until c-Jun is phosphorylated on specific serine residues localized in its activation domain. Work from several laboratories has demonstrated that phosphorylation of c-jun can be controlled by redox activation of ERK/JNK, members of the MAPK family [103, 107, 112]. On the other hand, the activity of AP-1 can be regulated by reversible oxidation of a conserved cysteine residue in the DNA-binding domain of c-Fos and c-Jun. Thus, chemical reduction of either of these transcription factors has been demonstrated to strongly increase their DNA-binding activity [103, 112]. Likewise, naturally occurring cysteine to serine mutations on both of them significantly enhance their activity [113]. Interestingly, the DNA-binding activity of AP-1 can be facilitated by Ref-1 (also known as APE), a redox factor that in co-operation with thioredoxin regulates the redox status of critical cysteine residues of c-Fos (Cys154), and c-Jun (Cys272) [114]. Ref-1 is a ubiquitously expressed multifunctional protein that plays a key role in protecting cells from the toxic effects of oxidative stress, mainly through its ability to repair DNA damage and regulate gene transcription. While the former activity is mediated through its properties as a class II hydrolytic apurinic/apyrimidinic endonuclease, the latter are exerted by reversibly altering the redox state of specific cysteine residues located in the DNA-binding domain of several transcription factors including AP-1, nuclear factor-$\kappa$B (NF-$\kappa$B) and Egr-1 [115]. The mechanisms whereby Ref-1 exerts its actions have been recently reviewed [116].

**Nuclear factor $\kappa$B**

NF-$\kappa$B is a family of transcription factors involved in key reactions of inflammatory, acute-phase, wound-healing and immune responses. This family comprises at least five well-characterized proteins referred to as p50, p52, p65 (RelA), c-Rel and RelB that can form various homo- and heterodimeric combinations with different transcriptional activities. All these proteins have a common N-terminal region of approximately 300 amino acids termed the Rel homology domain, which contains sequences critical for DNA binding, protein dimerization and intracellular localization. The subunit components of NF-$\kappa$B

are maintained in the cytoplasmic compartment bound to the inhibitory protein I-κB. Upon induction by a variety of agents including ROS, cytokines and mitogens, I-κB becomes phosphorylated and rapidly degraded by the proteasome in a redox status-dependent manner and released from NF-κB. This dissociation masks nuclear localization signals in the NF-κB molecule, which in turn leads to its translocation into the cell nucleus. After its nuclear transport, NF-κB interacts with specific promoter regions of target genes leading to changes in the expression of a wide variety of genes encoding inflammatory mediators, cell adhesion molecules, regulators of the cell cycle and apoptosis and extracellular matrix components [100, 103, 104, 112, 117–119].

The physiological role of ROS in activation of NF-κB is controversial [for reviews see refs 120–122]. Several reports in the literature have clearly demonstrated that treatment of various cell types/lines with hydrogen peroxide stimulates NF-κB DNA-binding activity. Conversely, treatment with antioxidants, such as N-acetyl-cysteine and dithiocarbamates, blocks NF-κB activation [123–126]. Additional evidence for the role of hydrogen peroxide as a mediator of NF-κB activation was obtained using cell lines that overexpress either catalase or SOD. In the former, treatment with hydrogen peroxide failed to increase NF-κB activation, whereas in the latter, a significant increase in NF-κB DNA-binding activity after treatment with this ROS was observed [126–129]. At the present time, the molecular basis for hydrogen peroxide-mediated NF-κB activation is largely unknown, although data in the literature appear to indicate that I-κB phosphorylation and degradation may be one of the steps targeted [122]. However, we would emphasize that evidence from several laboratories has clearly established that these effects are cell type specific and that many antioxidants may inhibit NF-κB activation and subsequent gene expression by non-antioxidant actions [121]. Moreover, emerging evidence clearly suggests that the mechanisms whereby NF-κB becomes activated are very complex and involve many steps other than its release from the inhibitor I-κB. For example, in addition to degradation of I-κB, phosphorylation of NF-κB subunits has been shown to be critical for the transcriptional activation/repression of many NF-κB-regulated genes [122]. Thus, $H_2O_2$ conceivably modulates one or several of the kinases/phosphatases responsible for NF-κB phosphorylation. Identification of these critical enzymes, as well as the sites of phosphorylation/dephosphorylation may help to answer this issue.

**Sp1**

Sp1 is a ubiquitous transcription factor that binds to GC-rich DNA sequences. This protein is involved in the regulation of many genes, including those encoding type I collagen [100, 130–135]. The Sp1 protein contains three zinc finger motifs of the Cys2His2 type that are essential for DNA-binding activity. This feature provides a structural basis for redox regulation of Sp1. Indeed, its DNA-binding activity is impaired in vitro by hydrogen peroxide and thiol-modifying reagents [136]. Moreover, low levels of GSH within the cell decrease Sp1 DNA-binding activity [137]. Likewise, in vivo studies have revealed that oxidative stress alters the transactivation potential of Sp1 [138]. Interestingly, the chronic shift in the intracellular redox status to more oxidant conditions observed during aging is associated with decreased Sp1 DNA-binding activity [138, 139]. Because Sp1-binding sites are present in the promoter regions of many genes, redox regulation of this transcription factor has a broad influence on gene expression and consequently on cell phenotype.

**Nuclear factor-1**

Nuclear factor-1 (NF-1) proteins are a family of ubiquitous transcription factors encoded by four different genes: NF1-A, NF-1B, NF-1C/CTF and NF-1X. All members of this family share a highly conserved DNA-binding domain that recognizes a palindromic TTGG-$CN_5$GCCAA consensus sequence. NF-1 isoforms form homo- and heterodimers that regulate a wide variety of gene promoters [101, 140]. Redox regulation of NF-1 is complex and affects both its DNA-binding and transactivating functions. With regard to the former, the NF-1 DNA-binding domain contains three cysteines, whose in vitro oxidation impairs its DNA-binding activity [141]. Moreover, NF-1 DNA-binding activity decreases in glutathione-depleted cells, or in those treated with hydrogen peroxide [142]. The transactivating domain of NF-1 has recently been demonstrated. The transactivating domain of NF-1 is susceptible to modulation by changes in redox potential. This effect is exerted through oxidation of Cys427, located at the N-terminal part of the NF-1 transactivating domain [143].

**c-Myb**

The c-Myb gene encodes a helix-turn-helix transcription factor that regulates proliferation, differentiation and transformation of several cell types including hematopoietic and hepatic stellate cells [144–147]. This protein binds to the PyAACT/GG consensus DNA sequence through three homeo domain-like regions and activates the transcription of target genes [144–147]. Work from several laboratories has clearly established that the binding and transcriptional activities of c-Myb can be regulated by changes in the redox status. In vitro studies have revealed that the DNA-binding activity of c-Myb can be enhanced by addition of the reducing agent dithiothreitol. Conversely, oxidation by diamide or alkylation with N-ethylmaleimide inactivates c-Myb. These effects appear to be mediated through Cys130 in the DNA-binding domain of c-MybN [148]. On the other hand, others have es-

tablished that oxidation of Cys43, also located in the DNA-binding region, abolishes c-Myb DNA binding [149]. These results suggest that Cys43 and/or Cys130 of the c-Myb protein can function as molecular sensors of the cells redox state, thereby regulating gene expression.

### $H_2O_2$ and energy metabolism

$H_2O_2$ inhibits glycolysis via a transient inactivation of glyceraldehyde-3-phosphate dehydrogenase, which undergoes ADP ribosylation. This inhibition protects the cells from apoptosis. When ADP ribosylation is blocked by the administration of 3-aminobenzamide, apoptosis occurs [150]. Low levels of $H_2O_2$ (<50 μM) also inhibit the Krebs cycle enzyme, aconitase. Nonetheless, because glutamate can fuel the cycle, generation of NADH is normal and the respiratory chain generates ATP. At higher concentrations of $H_2O_2$, $\alpha$-ketoglutarate dehydrogenase is inhibited and, thus, the entrance of glutamate to the cycle is prevented and NADH formation is inhibited [151]. This inhibitory effect could result in a lower accumulation of $H_2O_2$ because NADH fuels the respiratory chain in mitochondria. However, an important side-reaction of this regulatory process is the inhibition of ATP formation, which could result in cell death [151].

### Role of $H_2O_2$ in pathological processes

The data summarized above clearly indicate that $H_2O_2$ is a mediator of multiple physiological events. Therefore, because pathological events are the result of disturbances in the molecular mechanisms that maintain cell and organ homeostasis, $H_2O_2$ is also a key mediator of multiple pathological processes. However, we must emphasize that although excess formation of $H_2O_2$ has been documented in many pathological situations, further work is needed to establish whether this ROS is the primary cause of the disease or whether its production occurs secondary to injurious or inflammatory events. Below, we will attempt to summarize some pathological conditions in which $H_2O_2$ is suggested to play a role as a mediator of disease processes. Particular emphasis will be given to scar formation and liver fibrosis, since these areas are of main interest to us.

### Inflammation, immune responses and collagen deposition

Organ fibrosis and wound healing are generally associated with inflammation, neovascularization and migration of inflammatory and mesenchymal cells to the injured sites resulting in excess collagen deposition. The cell type responsible for excess collagen deposition is derived from fibroblasts that have been activated by multiple factors produced during inflammation [152–156]. As discussed previously, inflammatory cells produce $H_2O_2$ via their plasma membrane NADPH oxidase. Therefore, this ROS could be responsible for several events occurring during inflammation and excess deposition of collagen, including the following.

1) Chemotactic migration of various cell types, including macrophages and vascular smooth muscle cells [36, 157].

2) Development of an autocrine loop whereby $H_2O_2$ induces expression of TGF-$\beta$1 in fibroblasts and this cytokine in turn enhances accumulation of $H_2O_2$ [59, 158]. In this regard, there is recent evidence to suggest that in some cells (mouse proximal tubular cells), antioxidant therapy could have an opposite effect and thus induce TGF-$\beta$1 [159].

3) Excess deposition of collagen by myofibroblasts. $H_2O_2$ is one of the mediators involved in acetaldehyde- and TGF-$\beta$1-mediated up-regulation of the *col1a1* gene [59, 60].

4) Induction of mutagenesis. Inflammatory cells can generate transhalogenation reactions at the sites of inflammation by inducing bromination (and chlorination) of nucleotides in the presence of $H_2O_2$, myeloperoxidase and $Br^-$ ($Cl^-$). These brominated (chlorinated) nucleotides are mutagenic and cytotoxic at sites of inflammation [160–162].

5) Facilitation of cell adhesion. Recent studies have revealed that $H_2O_2$ enhances human eosinophil adhesion to human umbilical cord endothelial vein cells and induces the expression of $\beta$2 integrin, CD 11b and CD18 [163]. The expression of these cell surface receptors is a key event in healing responses that favors the attachment of inflammatory cells to vascular endothelial cells and extracellular matrix components that contain arginine-glycine-asparartic acid (RGD) sequences. Indeed, competing for binding of inflammatory cells to extracellular matrix with stable derivatives of the RGD sequence can prevent inflammation and scarring occurring after injury [164].

6) Alterations in immune responses. $H_2O_2$ is immunosuppressive by inhibiting phosphorylation of p38MAPK and JNK induced by lipopolysaccharide in mouse splenic lymphocytes [165].

7) Induction of cytokines and growth factors. $H_2O_2$ induces the expression of cytokines including the acute-phase cytokine interleukin (IL)-6 [166], TGF-$\beta$ [59, 60, 167] and connective tissue growth factor [168]. Likewise, several cytokines and growth factors exert their biological activities by mechanisms involving $H_2O_2$ [169, 170]. Therefore, autocrine and paracrine loops are established in which one reactive oxygen intermediate induces the formation of other ROS [171]. However, depending on the cell type, the same ROS may exert opposite effects. While $H_2O_2$ induced by TGF-$\beta$1 inhibits proliferation of epithelial cells [172], as a second messenger induced by

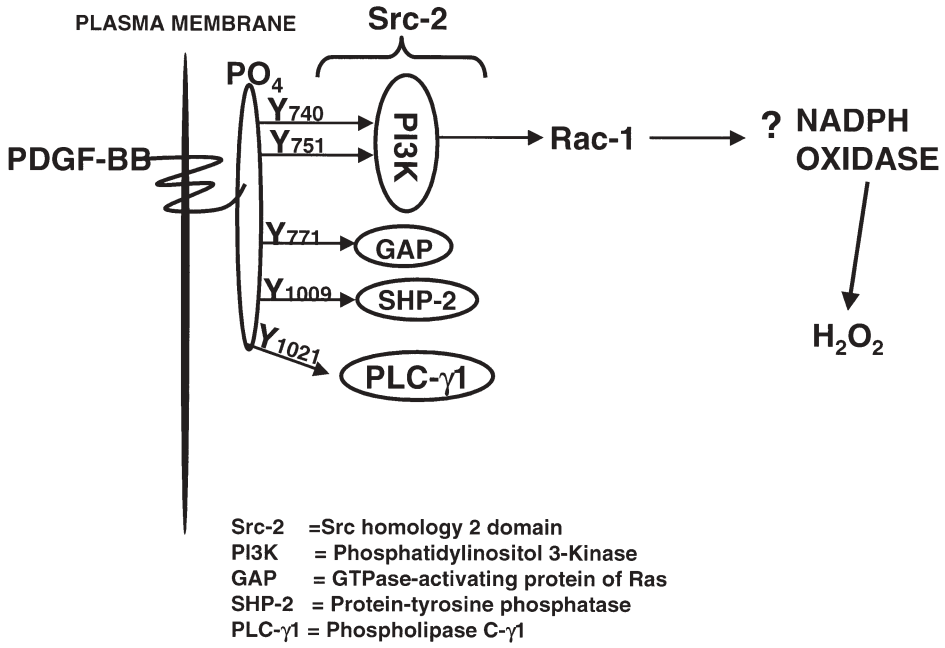


Figure 2. Possible mechanisms whereby PDGF-BB induces expression of $H_2O_2$, an important mediator of PDGF-BB-dependent fibroblast proliferation. Upon binding of the growth factor to its receptor, there are multiple events resulting in phosphorylation of several tyrosine residues and recruitment of protein kinases. Of these, PI3kinase has been suggested to activate NADPH oxidase by a mechanism that requires Rac-1.

platelet-derived growth factor (PDGF)-BB, it plays a role in cell proliferation [169] (see fig. 2). In other instances, such as occurs during tumor necrosis factor (TNF)-$\alpha$-induced apoptosis, $H_2O_2$ is an important mediator of cell death [173].

**Liver fibrosis**

Oxidative stress in general and $H_2O_2$ in particular play key roles in the development of liver cirrhosis in humans and animals [2, 10, 11] (see fig. 3). In addition to previous studies suggesting that antioxidant therapy could ameliorate the disease, cell culture studies have also shown that antioxidants can prevent collagen gene expression by cultured fibroblasts and hepatic stellate cells. In alcohol-fed rats, evidence for an oxidative stress response by the liver has been demonstrated prior to the development of fibrosis. In choline-deficient rats in which a high incidence of liver fibrosis and hepatocarcinomas have been described, there is evidence to suggest mitochondrial malfunction manifested by impaired respiratory function with a 70% decrease in NADH-dependent oxygen consumption and excess production of $H_2O_2$ [174].

There are several mechanisms whereby ethanol induces an oxidative stress response in the liver.

1) Ethanol metabolism by the liver results in changes in NAD/NADH ratios that alter the redox state of the cells [175]. Through this process there is activation of several transcription factors acting as redox sensors that trigger an oxidative stress response. Moreover, changes in redox are accompanied by an increase in the ratio of lactate/pyruvate. Because pyruvate has strong antioxidant properties and prevents lymphocyte death induced by $H_2O_2$ [176], as well as $H_2O_2$-induced damage in mesothelial cells [177], this could be an additional mechanism whereby ethanol enhances oxidative stress. In this regard, pyruvate has been shown to protect cultured hepatocytes from ethanol-induced damage [178].

2) Acetaldehyde, the metabolite of ethanol generated by alcohol dehydrogenase, the main enzyme involved in its metabolism, induces accumulation of $H_2O_2$ by an as yet unknown mechanism. This ROS is involved in activation of both type I collagen genes by cultured hepatic stellate cells [60] (see fig. 3). Moreover, $H_2O_2$ induces the expression of TGF-$\beta$1 thus creating a fibrogenic autocrine loop [158] (see fig. 4).

3) In the presence of $Fe^{2+}$ or $Cu^{+}$, $H_2O_2$ is converted to $^{\bullet}OH$ radicals and this in turn induces lipoperoxidation with formation of multiple aldehydes including malonyldialdehyde and 4-hydroxynonenal. These aldehydes, similar to acetaldehyde, stimulate formation of $H_2O_2$ and induce the expression of collagen genes by cultured fibroblasts and hepatic stellate cells [179–181].

4) CYP2E1, the microsomal ethanol-oxidizing system involved in ethanol metabolism, produces acetaldehyde and generates $O_2^{\cdot-}$, $H_2O_2$ and 1-hydroxyethyl radicals [182, 183]. The latter could trigger lipid peroxidation and

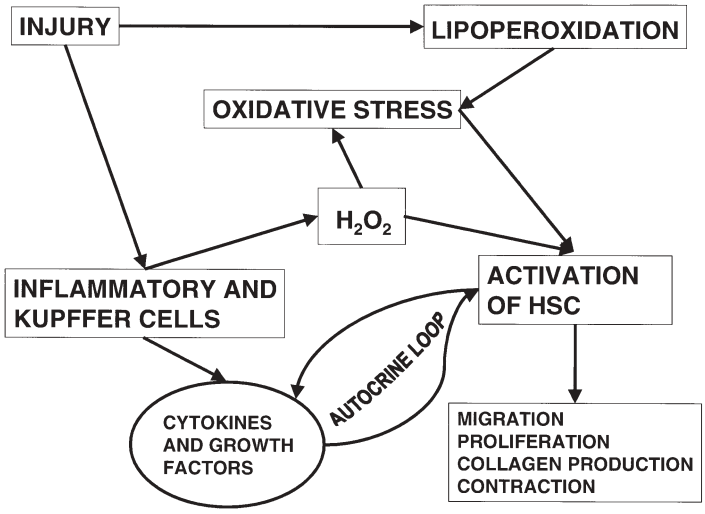


Figure 3. Possible mechanisms whereby liver injury results in activation of hepatic stellate cells (HSC) and excess collagen deposition. Liver injury results in the formation of lipoperoxides. The reactive aldehydes formed generate $H_2O_2$ and, thus, contribute to oxidative stress. On the other hand, liver injury results in chemotactic migration of inflammatory cells and activation of Kupffer cells. These in turn will produce $H_2O_2$ and further contribute to the oxidative stress response of the cell. The cytokines and growth factors produced by inflammatory cells, as well as the $H_2O_2$ released by them, will activate HSCs and these in turn will migrate to the injured site, proliferate and produce fibrous scar tissue. Because HSCs also produce cytokines and growth factors, an autocrine loop is established whereby these factors sustain HSC activation.

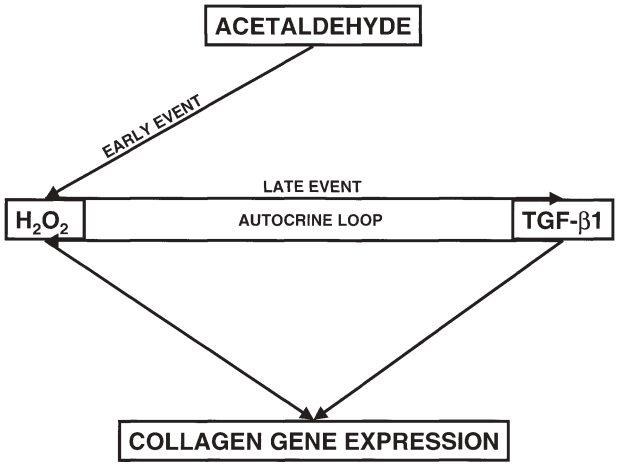


Figure 4. Autocrine loop involved in acetaldehyde-mediated up-regulation of the type I collagen genes. An early acetaldehyde event is to induce accumulation of $H_2O_2$. This ROS induces the expression of the *col1a1* gene by a mechanism dependent on the nuclear translocation and DNA binding of members of the C/EBP$\beta$ family of transcription factors. This event is TGF-$\beta$1 independent. On the other hand, $H_2O_2$ is known to induce the expression of TGF-$\beta$1 in cultured HSCs and, therefore, production of this cytokine could further up-regulate type I collagen gene expression at late points after acetaldehyde administration. Because TGF-$\beta$1 also induces the expression of the *col1a1* gene by an $H_2O_2$-dependent mechanism, an autocrine loop is established. This could be a possible mechanism of perpetuation of liver fibrogenesis upon discontinuation of the fibrogenic stimulus.

accumulation of fibrogenic aldehydes. Nonetheless, the role of CYP2E1 in inducing liver injury is still controversial and needs to be further investigated. CYP2E1 is mainly induced in chronic alcoholics and, thus, this mechanism of ROS generation could be insignificant in early alcoholics but could play a key role with increased alcohol consumption and/or with the induction of this enzymatic system by other chemicals. A similar extent of liver injury has been reported in CYP2E1 –/– as compared with CYP2E1 +/+ mice chronically fed ethanol [184].

5) Ethanol and its metabolite acetaldehyde produce hepatocyte injury that triggers an inflammatory response of the host. This involves the activation of Kupffer cells and the attraction of inflammatory cells to injured sites. These inflammatory cells generate ROS via the NADPH oxidase and produce TGF-$\beta$1, the fibrogenic cytokine known to use $H_2O_2$ as a second messenger in many of its biological activities [59, 172, 185–187].

6) Although early fibrogenic actions of acetaldehyde on cultured hepatic stellate cells are not mediated by TGF-$\beta$1 [60], there is strong evidence to suggest that some of its late events are mediated and/or enhanced by this cytokine. Because TGF-$\beta$1 induces accumulation of $H_2O_2$ by inhibiting the expression of enzymes involved in its catabolism, this additional mechanism of ROS formation is an important contributor to the state of oxidative stress induced by ethanol [188, 189].

7) Ethanol converts xanthine dehydrogenase to xanthine oxidase, an $H_2O_2$-forming system by a mechanism dependent on the oxidation of SH groups [190–192].

8) Chronic liver injury leads to increased iron stores which in the presence of $H_2O_2$ generated during ethanol metabolism contribute further to liver damage [10].
9) Chronic ethanol consumption induces a hypermetabolic state with a significant increase in oxygen consumption. Because $H_2O_2$ is generated as a side-product of the mitochondrial respiratory chain, increased $O_2$ consumption could result in excess formation of $H_2O_2$. This toxicity could be significantly enhanced by a decrease in mitochondrial glutathione levels induced by ethanol [2, 193, 194].
10) Although the molecular mechanisms whereby ethanol induces perivenular damage are yet to be fully established, two important pathogenic mechanisms have been suggested. CYP2E1 levels are higher in perivenular hepatocytes, thus increasing formation of 1-hydroxyethyl radicals. Perivenular hepatocytes are less oxygenated and, thus, may be more susceptible to oxidative damage secondary to reperfusion occurring by hemodynamic changes. GSH levels in perivenular hepatocytes are lower and, therefore, their antioxidant defense systems may be weaker [195, 196].
11) TGF-$\beta$1 is the main fibrogenic cytokine and its expression correlates with the degree of hepatic fibrosis in humans and animal models of this disease [197–199]. Our studies have established that at least some fibrogenic actions of TGF-$\beta$1 are mediated by accumulation of $H_2O_2$ [59]. In cultured hepatic stellate cells, the accumulation of this ROS results in activation and nuclear translocation of members of the c/EBP family of transcription factors and these, in turn, bind to the –370 to –341 region of the $\alpha$1(I) collagen promoter inducing the transcription of the *col1a1* gene. This effect is mimicked by the addition of $H_2O_2$ to cultured cells and is abrogated by the addition of catalase [59]. Altogether, these findings indicate that irrespective of whether TGF-$\beta$1 is the main fibrogenic cytokine in liver fibrosis or acetaldehyde is the contributing factor, both systems induce an oxidative stress response with accumulation of $H_2O_2$.

**Wound contraction**

Wound contraction is an important event in all healing responses, irrespective of whether scar formation occurs in the skin during wound healing or in a coronary vessel during atherosclerosis. Myofibroblasts are the cells responsible for contracting the wound [200–208] and play a key role in contractures such as that observed in Dupuytre's syndrome [209]. In chronically injured tissues, as occurs after liver damage leading to cirrhosis, myofibroblasts are also present and are responsible for the contraction of the scar that produces the nodular appearance of the cirrhotic liver [204, 208]. Moreover, this contraction together with that of hepatitic stellate cells that modulate portal blood flow, is responsible in part for an increase in portal blood pressure, an important complication of liver scarring [210, 211]. Myofibroblasts contain myosin and actin [200, 202, 203, 210–212] and contract in culture in response to various agonists and TGF-$\beta$1 [213–225]. The molecular mechanisms whereby this process is triggered remain to be elucidated. However, in other cell types, mainly vascular endothelial cells, researchers have clearly established, using endothelium-denuded rat aortic rings, that their contraction is mediated by $H_2O_2$. This contraction is blocked by catalase and suramine, the inhibitor of purinergic receptors [216], but not by SOD or dimethylsulfoxide/mannitol, an inhibitor of OH radicals. Contraction of rat vascular smooth muscle cells by angiotensin II was shown to be $H_2O_2$ mediated, and the angiotensin-induced phosphorylation of light-chain myosin was preventable by catalase. This effect was specific because catalase had no effect on the angiotensin-dependent formation of inositol 1,4,5-trisphosphate [217].

**Aging**

Oxidative stress in general and $H_2O_2$ in particular play an important role in aging. However, the direct role of $H_2O_2$ in its pathophysiology remains to be firmly established. The implication of $H_2O_2$ as a causative agent of aging is derived in part from multiple studies with cultured cells in which many changes occurring as the cells age in culture can be mimicked and/or accelerated by ROS. When human diploid fibroblasts are exposed to subtoxic doses of $H_2O_2$, within 3 days, cells express biochemical and morphologic changes that mimic the aging process, as characterized by an increase in stress fibers and alterations in the distribution of paxillin and vinculin. These proteins, instead of being localized to the edge of the cells, become randomly distributed. These changes in morphology that resemble aging have been suggested to be TGF-$\beta$ mediated, Rb dependent and require de novo protein synthesis [218].
Studies performed with human endothelial cells revealed that $H_2O_2$ induces changes in cytoskeletal organization and formation of membrane blebs. This latter effect of $H_2O_2$ may be mediated via tyrosine phosphorylation of cortactin, the actin-associated protein induced by Src [219]. Human umbilical vein endothelial cells treated with $H_2O_2$ undergo the following changes: F-actin rearrangement, filamin translocation from the membrane to the cytosol, intercellular gap formation, a rapid decrease in cAMP and a $Ca^{2+}$ dependent increase in PIP2. The $H_2O_2$-dependent changes in the cytoskeleton are prevented by inhibitors of phospholipase C, phosphoinositide turnover or by binding PIP2 with a synthetic peptide [220]. Filamin translocation and actin reorganization are associated with a decrease in filamin phosphorylation and are prevented by activation of the PKA pathway. This

phosphorylation occurs at the C-terminal domain of filamin, a known cAMP-dependent phosphorylation site [221].

As indicated previously, oxidation of cysteine and methionine residues in proteins by $H_2O_2$ results in conformational changes with concomitant alterations in their biological activity. In general, many of these proteins are eliminated by phosphorylation, ubiquitinylation and degradation by the proteasome. However, in aged MRC-5 fibroblasts, there is a significant decrease in proteosomal activity that results in accumulation of oxidized proteins [222]. This mechanism, mainly the inefficient elimination of altered proteins by proteosomal degradation, could play a key role in neurological diseases occurring in elderly individuals, in which abnormal proteins accumulate and alter cellular function.

**Other pathological processes**

One of the most common lesions involving oxidative stress is excess sun exposure resulting in UVB damage of skin and development of cancerous lesions. Thus, Uvb-induced DNA damage is perhaps the alteration that has been most widely investigated regarding the accumulation of ROS, their role in disease and the effect of antioxidants in protecting against oxidative stress. Indeed, the concept of oxidative injury was derived, in part, from studies in this field. Various aspects of ROS and UVB-induced DNA damage have been reviewed recently [223] and we will therefore not discuss this topic. However, $H_2O_2$ is an important mediator of UVB skin damage and this ROS induces the expression of VEGF in keratinocytes [224] and play a role in phosphorylation of EGFR, an event prevented by over-expression of catalase and mediated by p44/42 MAP kinase [225].

ROS are also involved in hypertension, atherosclerosis and cardiovascular disease [226–228]. LOX 1, the endothelial cell receptor that binds oxidized low-density lipoproteins, important mediators of atherosclerotic lesions, is redox sensitive [229]. Moreover, this receptor is up-regulated by $H_2O_2$ and homocysteine and down-regulated by the antioxidant N-acetyl-cysteine [229]. There is a significant correlation between $H_2O_2$ and renin plasma levels, thus suggesting a possible genetic predisposition to develop increased blood levels of $H_2O_2$ and its association with atherosclerosis and hypertension [230].

Another disease in which ROS have been implicated is Parkinson's. This is based on evidence suggesting that several parameters involved in modulating ROS in neurons and/or astrocytes are altered. Part of the information concerning oxidative stress and Parkinson's disease has been summarized in several publications [231–235]. First, brain GSH levels decrease and levels of $Fe^{2+}$, a metal enhancing the conversion of $H_2O_2$ to $OH^-$, are elevated in these patients. Second, lipid peroxidation increases and there is mitochondrial complex I impairment and a decrease in $\alpha$-ketoglutarate-positive cells in affected areas of the brain. Depletion of GSH in cultured astrocytes results in increased accumulation of NO, $H_2O_2$ and glutamate. Thus, formation of peroxynitrite could induce neuronal damage. However, a gene named Parkin, associated with Parkinson's disease, has been cloned. The gene is a ubiquitin protein ligase (E3) for which CDC rel-1, a synaptic vesicle-associated protein serves as a substrate [236]. Thus, alterations in the mechanisms involved in degradation of proteins, whose conformation was altered by oxidative stress, by the ubiquitin system could also play a role in Parkinson's disease.

$H_2O_2$ may also play a role in cystic fibrosis and in the development of cataracts. In the former, levels of $H_2O_2$ in exhaled air are increased but decrease after treatment with antibiotics [237]. In the latter, $H_2O_2$ may be responsible for inducing the secretion of matrix metalloproteinases by lens cells and these in turn will be responsible for lens capsule opacification and cataract formation [238].

In addition to the conditions in which oxidative stress plays a role in pathophysiology of the disease, there are other instances in which a lack of production of reactive oxygen intermediates could cause the disease. In this regard, in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, production of superoxide, $H_2O_2$ and NO is impaired. Moreover, they do not respond to lipopolysaccharide and phorbol myristate acetate with increased production of ROS. However, G6PD-deficient granulocytes have normal levels of inducible NO synthase. In granulocytes from normal controls, formation of NO is increased significantly compared to unstimulated levels. Therefore, neutrophil dysfunction and increased susceptibility to infection in patients with severe G6PD deficiency could be due to a blunted oxidative stress response of granulocytes to endotoxin [239].

**Concluding remarks**

A decrease in the generation and/or the effects of ROS could be beneficial in a variety of experimental models of disease including liver fibrosis, systemic sclerosis, cardiovascular disease, cancer and diabetes. Numerous antioxidants have been used including e. g., vitamin E, vitamin C and S-adenosyl-methionine, with variable degrees of success [240–249]. Moreover, several antioxidant therapies are currently undergoing clinical trials to determine their benefit in a number of diseases. During the past few years, our understanding of the role of oxidative stress in disease and of the mechanisms whereby ROS induce cell damage have allowed the development of more rational approaches to antioxidant therapy while, at the same time, exposing the limitations of some of them. An

inherent requirement for an effective antioxidant therapy is that such therapy is effective against the radicals being generated and that sufficient quantities of the antioxidants used reach all sites of radical generation in time to limit tissue injury. Moreover, there is also a need to find methods to deliver antioxidants to specific cell types or to subcellular organelles in which excess ROS are being produced. Unfortunately, so far, few, if any, of the currently known individual antioxidants can adequately meet these goals. Combination therapy or the development of novel compounds with higher specificity and/or enhanced biological activity may prove useful. A new lipophylic triphenylphosphonium cationic derivative of ubiquinone has been developed that can be selectively targeted to mitochondria of cultured cells where it prevents $H_2O_2$-induced oxidative damage, lipid peroxidation and apoptosis. This ubiquinone derivative is recycled because it is reduced to ubiquinol by the respiratory chain and further oxidized after detoxification. This effect is specific, because this antioxidant has no effect on staurosporine- or TNF-$\alpha$-induced apoptosis [250, 251].

During the past few years, significant progress has been made identifying oxidative stress damage as a major contributor of disease. Our challenge for the years to come will be to develop and test the efficacy of novel agents with the ability to prevent and/or block the deleterious effects of ROS. Those that survive the test of time will be incorporated into our therapeutic arsenal. All others will be eliminated and/or substituted for new ones with the promise to become 'magic bullets' in the treatment of oxidative damage-related diseases. The quest is just beginning.

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