Mechanisms of Oxidant Stress-Induced Acute Tissue Injury (43885A)

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Abstract. **During the last 25 years, a large body of experimental evidence has accumulated from pharmacological intervention studies that suggests an important role for reactive oxygen species in numerous pathophysiological processes. While a variety of chemical mechanisms of reactive oxygen-induced damage to lipids, proteins, and DNA is fairly well understood, the molecular pathology of oxidant stress-induced tissue injury *in vivo* remains unclear in most cases. Recent advances indicate that the direct destructive potential of reactive oxygen *in vivo* is limited by the extensive detoxification capacity of most cells and may be restricted to a small fraction of cells exposed to a locally high oxidant stress. However, reactive oxygen species can participate in recruitment of inflammatory cells by upregulation of adhesion molecules and generation of chemotactic factors, and are necessary for protease-mediated cell injury *in vivo*. Reactive oxygen species can also scavenge other biologically active molecules (e.g., nitric oxide), thereby modulating indirectly their effector cells. In addition to the discussed effects relevant for acute injury, other oxidant stress-induced mechanisms (e.g., DNA modifications) may be relevant in chronic disease states. A solid mechanistic understanding of the role of reactive oxygen species in the overall pathophysiology is critical for providing a rationale for antioxidant therapy and the targeted development of new antioxidant drugs.** [P.S.E.B.M. 1995, Vol 209]

Up until the 1960s, oxygen free radicals and oxidant stress were not considered particularly relevant for mammalian biology. However, three landmark publications "radically" changed this perception. The first was the discovery by McCord and Fridovich in 1969 that an enzyme exists in essentially every mammalian cell that has superoxide dismutase activity (1), suggesting the continuous formation of superoxide *in vivo*. A few years later, Babior *et al.* showed that the bactericidal activity of neutrophils is associated with production of the superoxide radical (2), linking oxygen free radicals to inflammatory disease states. The third landmark paper was published in 1981 by Granger *et al.* (3) establishing the hypothesis that reperfusion injury after intestinal ischemia may be caused by xanthine oxidase-derived oxygen radicals. This paper triggered numerous investigations into the ischemia-reperfusion syndrome including major human disease states such as myocardial infarction, stroke, and shock. In recent years, many more important diseases were linked to free radical formation (4). However, due to analytical problems in measuring oxidant stress and free radical mechanisms of injury, much of the experimental evidence is circumstantial. Furthermore, free radical processes may be the sole cause of an injury, a part of the pathomechanism, or only an epiphenomenon. Because of the still controversial debate of the role of oxygen radicals in experimental models and the pathogenesis of human diseases, there is a need for a more detailed mechanistic understanding. This review will first briefly define oxidant stress, potential mechanisms of injury, and the cellular defense mechanisms, and then discuss indepth the pathophysiological relevance of reactive oxygen species *in vivo*.

Free Radicals and Reactive Oxygen Species

During aerobic metabolism, cells generate energy by reducing molecular oxygen to water. This reaction, which is catalyzed by the mitochondrial enzyme cytochrome c oxidase, involves the transfer of four electrons to oxygen without formation of intermediates.

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However, other enzymes may form, as a regular end product or accidentally, partially reduced oxygen species. As shown in Figure 1, transfer of one electron to oxygen yields the first reactive intermediate, the superoxide radical ($O_2^{\cdot -}$). Superoxide rapidly dismutates spontaneously to hydrogen peroxide (H_2O_2) and oxygen. Any further reaction requires a reductive cleavage of the bonds between the oxygen atoms, a reaction catalyzed by transition metals (Fenton reaction), to form the extremely reactive hydroxyl radical ($^{\cdot}OH$). Recently, a transition metal-independent pathway to form hydroxyl radicals was proposed (5). Nitric oxide, generated by macrophages, hepatocytes, endothelial cells, etc. (6), can react with superoxide to form peroxynitrite ($OONO^-$), which, after protonation, is unstable and decomposes spontaneously to form the hydroxyl radical and nitrogen dioxide (5). Because of the large energy gain of the reduction of the hydroxyl radical to water, this active oxygen species will react instantaneously with any biological molecule in its immediate environment by abstracting a hydrogen atom:

$$^{\cdot}OH + R\text{–}H \rightarrow H_2O + R^{\cdot}$$

The resulting more stable radicals are less reactive and therefore have a longer half-life. Other reactive oxygen species, which may be of biological relevance, are hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$). Hypochlorous acid is generated enzymatically by neutrophils and is a strong oxidant. Singlet oxygen is an electronically excited form of molecular oxygen and can be generated by the reaction of hypochlorous acid with hydrogen peroxide (7). The pathophysiological importance of singlet oxygen *in vivo* is still controversial (7).

Reactive oxygen species, in particular superoxide and hydrogen peroxide, can be generated by many enzymatic sources (e.g., xanthine oxidase and glucose oxidase), subcellular organelles (e.g., mitochondria and cytochrome P_{450}), or inflammatory cells (e.g., neutrophils and macrophages). Furthermore, environmental factors such as radiation or toxic chemicals can induce the formation of reactive oxygen species either directly or by causing cell damage and thus activating intracellular sources. For a more detailed discussion of potential sources of reactive oxygen formation the reader is referred to a review by Freeman and Crapo (8). The importance of individual sources for the pathophysiology *in vivo* will be discussed later.

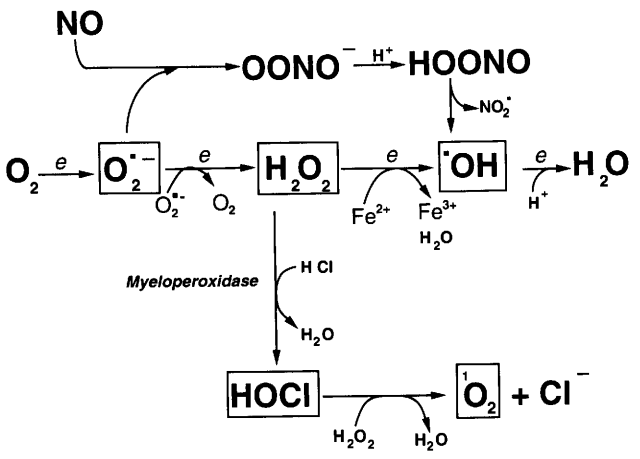

Figure 1. Reactive oxygen species formed by one electron step reductions of molecular oxygen, spontaneous reactions with nitric oxide (NO) or enzymatic reactions.

Damaging Mechanisms

Reactive oxygen species, in particular the hydroxyl radical, can react with all cellular macromolecules (e.g., lipids, proteins, nucleic acids, and carbohydrates) (9). The initial reaction generates a second radical, which can then react with other molecules to continue the radical chain reaction. Because these are spontaneous chemical reactions, thermodynamic laws require that the energy gain from forming a new molecular bond has to be larger than the dissociation energy of the old bond (i.e., the newly formed radical has to be more stable than the original). A consequence of the formation of more and more stable radicals is that the progression of the radical chain reaction focuses increasingly on certain "susceptible" targets (i.e., activated molecular bonds), which require little energy to dissociate. This means that reactive oxygen species cause damage to preferred molecules.

Examples for highly susceptible targets are *bis*-allylic methylene positions of polyunsaturated fatty acids (PUFA) because the presence of the adjacent double bond weakens the carbon-allylic hydrogen bond (10). Abstraction of a hydrogen atom of such a methylene group by a radical initiates the oxidative modification of fatty acids (lipid peroxidation; LPO) (Fig. 2). After rearrangement to the energetically more stable configuration of a conjugated diene, oxygen is added and the radical chain is continued by abstracting another hydrogen atom (propagation reaction). The resulting fatty acid hydroperoxide can be reduced to the hydroxy fatty acids or reductively cleaved in a Fenton-type reaction to yield aldehydes and alkanes (β-scission). Although any fatty acid with two or more double bonds may undergo these peroxidation reactions, the susceptibility to LPO increases exponentially with the number of *bis*-allylic methylene positions (11). These results correlate with findings *in vivo* indicating that predominantly fatty acids with four or more double bonds (e.g., eicosatetraenoic acid [$C_{20:4}$] and docosahexaenoic acid [$C_{22:6}$]) are lost during lipid peroxidation (12). Because of the spontaneous nature of these chemical reactions, numerous LPO products are formed. This causes substantial analytical problems for *in vivo* experiments (13). One has the choice between simple, unspecific assays for classes of com-

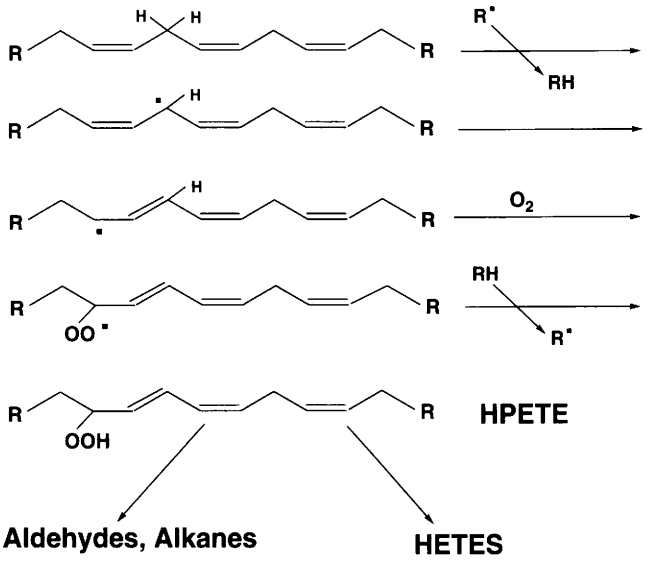

Figure 2. Scheme of lipid peroxidation. Polyunsaturated fatty acids undergo hydrogen abstraction, diene conjugation, and reaction with molecular oxygen to form a hydroperoxy fatty acid (e.g., hydroperoxy eicosatetraenoic acid [HPETE]). This intermediate can be reduced to the respective hydroxy fatty acid (HETE) or reductively cleaved (β-scission) to form aldehydes and alkanes. During this reaction sequence new radicals are generated.

pounds (e.g., thiobarbituric acid for aldehydes) and more specific but less sensitive assays (e.g., conjugated dienes), or specific and highly sensitive but expensive, labor-intensive methods, which require special instrumentation (e.g., mass spectrometric analysis of hydroxy fatty acids and F_2-isoprostanes). A problem frequently overlooked is the fact that there may be only a shift in the product profile without affecting overall lipid peroxidation (14). For example, availability of traces of iron may favor β-scission over reduction and therefore cause enhanced formation of aldehydes and alkanes without changes in the hydroperoxide formation. Furthermore, the oxygen concentration in the tissue may affect how much of an alkyl radical is reduced to the alkane or forms the respective alcohol (14). Analysis of only one product (e.g., aldehydes or alkanes) may then be misleading. This means that, particularly *in vivo,* several representative products should be measured. Unfortunately, analytical problems hinder the widespread use of many sensitive and specific assays. Consequences of lipid peroxidation are dependent on the extent of these reactions and can range from affecting membrane fluidity and the activity of membrane proteins (carriers, receptors) to disruption of cell membranes with total loss of ion homeostasis (15–17).

Proteins are another target for reactive oxygen. Several mechanisms have been identified by which reactive oxygen species modify protein structure and function (18): (i) reactive oxygen species can cause metal-catalyzed protein oxidation with introduction of carbonyl groups into the molecule, fragmentation or crosslinking (19); (ii) aldehyde lipid peroxidation products may react with sulfhydryl (cysteine) or amino groups (lysine, histidine) and covalently bind to these amino acids. Loss of protein sulfhydryl groups may also occur through direct oxidation, excessive formation of glutathione disulfide (GSSG), or arylation by a reactive metabolite. While the latter mechanism of toxicity was shown to be involved in the pathogenesis of acetaminophen-induced liver necrosis (20), the pathophysiological relevance of excessive GSSG formation or direct oxidation was questioned because reactive oxygen-mediated liver injury *in vivo* occurred without significant loss of protein sulfhydryl content (21).

Reactive oxygen can cause modification of nucleotides, single strand breaks of DNA, and crosslinking (9). However, at least in cultured hepatocytes, DNA damage could be dissociated from oxidant stress-induced acute cell injury (22). DNA damage by reactive oxygen species may be more relevant for chronic diseases.

Defense Mechanisms Against Reactive Oxygen

Because of the serious damaging potential of reactive oxygen species, cells depend on elaborate defense mechanisms to rapidly metabolize these toxic intermediates and to prevent significant free radical injury (Fig. 3) (23). The first line of defense against superoxide are superoxide dismutases, which catalyze the diffusion-limited dismutation of superoxide to hydrogen peroxide and oxygen. The cytosolic enzyme contains copper, the mitochondrial enzyme has manganese as redox-active metal ion. Hydrogen peroxide

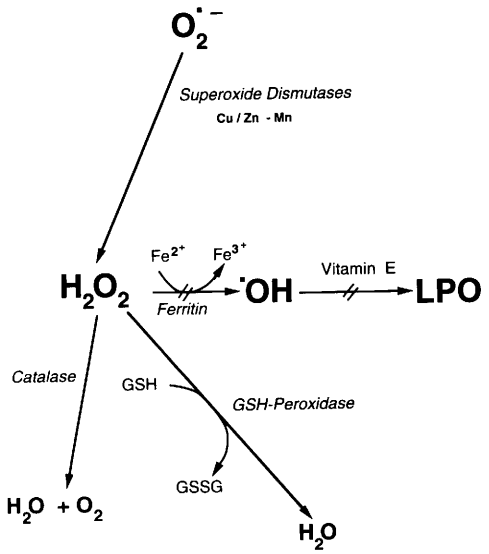

Figure 3. Scheme of cellular defense mechanisms, which includes enzymes for the rapid metabolism of reactive oxygen species, high-affinity storage proteins for transition metals (e.g., ferritin) and antioxidants (e.g., vitamin E).

formed in peroxisomes is metabolized completely by the high content of catalase in these organelles (24). Because hydrogen peroxide can easily diffuse through membranes, peroxisomal catalase can also participate in the detoxification of hydrogen peroxide generated in other cell compartments (24). Most important for the metabolism of cytosolic and mitochondrial hydrogen peroxide is the selenium-containing enzyme glutathione-peroxidase. This enzyme reduces hydrogen peroxide to water, requiring reducing equivalent from glutathione (GSH). Another part of this second line of defense is the general strategy of cells to keep any potentially redox-active metal tightly bound to transport and storage proteins to minimize the possibility of hydroxyl radical formation. Thus, iron is transported bound to transferrin or stored as ferric iron bound to ferritin (25). Other defense mechanisms include chain-breaking antioxidants, e.g., α-tocopherol (vitamin E), which interrupts the progression of free radical chain reactions by forming stable radicals. In a case in which all defense mechanisms fail and macromolecules are damaged, repair process are activated (e.g., phospholipases) or turnover is stimulated to correct the modification or to replace the damaged molecule without affecting cellular functions.

Pathophysiological Relevance of Oxidant Stress *in Vivo*

Extensive research efforts during the last 25 years clearly demonstrated the potential danger of reactive oxygen and cellular adaptation processes to survive the continuous formation of these toxic intermediates. Nevertheless, reactive oxygen is suspected to be involved in numerous diseases, however, the experimental evidence is mainly circumstantial and the exact role of reactive oxygen *in vivo* remains unclear in most cases. Because of the potential benefit of nutritional antioxidant supplements as preventive measure as well as antioxidant drugs for acute and chronic disease states, it is necessary to focus attention on mechanistic investigations to understand the role of reactive oxygen *in vivo*. The next few sections will point out some of the fundamental questions that will have to be addressed in order to understand the mechanism of a disease process.

Quantitative Aspects of Reactive Oxygen Formation. Quantitative considerations (e.g., how much reactive oxygen is necessary to cause cell damage) are frequently overlooked aspects of free radical damage *in vivo*. In an attempt to address this aspect of reactive oxygen-induced liver injury, chemically generated superoxide was quantified and correlated with the onset of parenchymal cell damage. The liver could detoxify about 60–72 μmole O_2^-/g liver without significant injury (26). These findings were similar to the direct infusion of *tert*-butyl hydroperoxide in liver (27) and heart (28). Such an intracellular oxidant stress, although subtoxic, is several orders of magnitude higher than can be seen generated by intracellular sources under pathophysiological conditions (29). Because of the effective defense systems, the tolerance of viable cells to enhanced reactive oxygen formation is relatively high. This conclusion could be substantiated in postischemic cells (30) or in cells with impairment of individual detoxification pathways (26). Thus, with only increased formation of reactive oxygen, it is difficult to cause acute cell injury *in vivo*. On the other hand, if the pathophysiological conditions also allow the mobilization and effective reduction of iron, even a low oxidant stress can be extremely toxic, in particular in vitamin E-depleted animals (12, 31, 32). Allyl alcohol and acetaminophen do not cause a relevant increase of reactive oxygen formation (33–35). In control animals, liver injury develops over 4–24 hr without significant lipid peroxidation (33–35). In striking contrast, massive lipid peroxidation occurs in vitamin E-depleted animals within 0.5–2 hr, followed by severe liver cell damage (12, 31, 32). Lipid peroxidation and injury is strictly iron dependent and can be attenuated by vitamin-E treatment (12, 32, 36). Thus, even without excessive intracellular oxidant stress, serious free radical-mediated injury can occur by iron mobilization in susceptible animals. In summary, quantitative investigations indicated that extremely high levels of intracellular reactive oxygen are needed to cause acute cell injury, however, enhanced availability of transition metal ions in combination with severe deficiency of cellular antioxidants can drastically increase the susceptibility to oxidant injury.

Sources of Reactive Oxygen. Fundamental for the understanding of the role of reactive oxygen in the pathophysiology is to identify the actual sources of free radical formation. Generally, a well-characterized inhibitor may be used to identify a particular source. However, most chemicals are not very specific and may have additional properties, which may affect the pathogenesis independent of the hypothesized pathway. For example, the beneficial effect of the xanthine oxidase inhibitor allopurinol against hepatic ischemia-reperfusion injury was generally interpreted as evidence that xanthine oxidase-derived intracellular reactive oxygen is involved in the injury (37, 38). However, the postulated conversion of xanthine dehydrogenase to the reactive oxygen-forming oxidase was very slow (39) and attempts to quantify the postischemic oxidant stress indicated that only very little reactive oxygen was generated intracellularly in hepatocytes during reperfusion (30), suggesting that xanthine oxidase may not be a relevant source of the oxidant stress. Moreover, even if xanthine oxidase contributes to intracellular reactive oxygen formation, the oxidant stress caused by this enzyme is rather moder-

ate and occurs only during a limited time period (40). On the other hand, the postischemic liver could still detoxify large quantities of reactive oxygen without additional injury (30). The lack of a relevant intracellular oxidant stress in hepatocytes drew attention to the extracellular space (41). Careful studies revealed evidence for a substantial oxidation of plasma glutathione, indicative of a vascular oxidant stress (41, 42). Inhibition or activation of Kupffer cells before ischemia attenuated or enhanced the oxidant stress, respectively, (42) indicating that resident hepatic macrophages are the main source of reactive oxygen. Direct isolation of Kupffer cells confirmed these findings (43). Kupffer cells are activated during ischemia (44) and further stimulated by activated complement factors during reperfusion (45). Based on the localization of the oxidant stress in the vasculature, the beneficial effects with conjugated desferoxamine (46) and conjugated dismutase (47), compounds restricted to the vascular space, could now be explained.

The initial Kupffer cell-induced oxidant stress and tissue injury causes continuous neutrophil infiltration and activation of these cells (48), which leads to a neutrophil-induced injury several hours later (49). Functional inactivation of neutrophils to inhibit reactive oxygen formation attenuated reperfusion injury in the liver (50). The interesting aspect of these investigations was that a relatively short period of ischemia is enough to activate and prime tissue macrophages, which means that these cells will respond to additional mediators (e.g., complement factors) with a potentiation of the oxidant stress (51). A second finding was the sequence of events: the initial oxidant stress was caused only by Kupffer cells and later complemented by neutrophils (48). This suggests that inactivation of Kupffer cells has to occur early (42), while inhibition of neutrophils can be effective well into the reperfusion phase (50). Antioxidants (tocopherol acetate, Trolox) reduce the injury directly (52, 53) but also indirectly by reducing neutrophil infiltration (54).

These studies demonstrated a postischemic oxidant stress in the vasculature with inflammatory cells as the main source of reactive oxygen. How can the beneficial effects of allopurinol be explained? Allopurinol is recognized to be an antioxidant (55) and most therapeutically effective doses are considerably higher than would be needed to inhibit xanthine oxidase (35). Alternatively, xanthine oxidase can be released from hepatocytes, bind to the surface of endothelial cells and may contribute to reactive oxygen formation in the extracellular space (56). Immunohistochemical localization of xanthine oxidase on endothelial cells *in vivo* supports this idea (57). The hypothesis is consistent with the documentation of an oxidant stress in the vasculature (41); thus, xanthine oxidase could be a third extracellular source of reactive oxygen during reperfusion. However, one has to keep in mind that this mechanism requires a substantial hepatocellular release of xanthine oxidase, which may only occur after longer ischemic periods. Under these conditions, where there is ischemic parenchymal cell injury, intracellular reactive oxygen formation by xanthine oxidase and mitochondria could be detected during reperfusion (40). This means that there are not only several cell types (Kupffer cells and neutrophils) involved in generating reactive oxygen during different times of reperfusion, but also, depending on the length of the ischemic period, additional intracellular (xanthine oxidase, mitochondria) and extracellular sources (xanthine oxidase). Thus, experimental conditions determine the relative importance of a source of reactive oxygen in a given experimental model and therefore decide whether or not a certain pharmacological intervention may be successful. Because experimental conditions determine the results, it is critical to select conditions relevant to human pathophysiology. For example, after 3 hr of warm ischemia, which causes serious ischemic damage to hepatocytes, mitochondria were shown to be a source of reactive oxygen (58). After only 20–45 min of ischemia, there is no evidence for an intracellular oxidant stress, instead, Kupffer cells generate reactive oxygen in the vasculature space (42). During human liver surgery involving hepatic ischemia (tumor resection, transplantation), the ischemic time will be restricted to well below the time of hepatocellular injury, suggesting that relevant experimental conditions for the pathophysiology are those without ischemic parenchymal cell injury.

Molecular Mechanisms of Oxidant Stress-induced Injury *in Vivo*. One of the most controversial topics of free radical pathology is the question of how reactive oxygen is involved in the injury process *in vivo*. It is generally implied that a protective effect of an antioxidant in combination with evidence for a free radical process (e.g., enhanced levels of malondialdehyde) indicates that lipid peroxidation may be the major mechanism of injury. However, this conclusion is not necessarily justified. The problem can only be answered by quantitatively comparing injury and lipid peroxidation in the unknown pathophysiology with experimental models, where lipid peroxidation is the known cause of injury. For example, there is extensive evidence for lipid peroxidation during reperfusion after hepatic ischemia. Reports (reviewed in Ref. 59) indicate a protective effect of vitamin E and a significant increase of malondialdehyde levels, ethane exhalation, hydroxy eicosatetraenoic acid content (HETEs), and diene conjugation during the early phase of reperfusion. However, very consistent with all LPO products measured, the increase in liver tissue was at most between 50% and 200% above baseline values. Do these findings mean that LPO is the major

mechanism of injury for parenchymal cells? To address this question, LPO was initiated *in vivo* by intraportal infusion of *tert*-butyl hydroperoxide and LPO products (HETEs), and injury were quantified. The results indicated that a similar or slightly higher increase of HETE content in the liver as during reperfusion was not associated with liver damage (27). Moderate liver injury was only observed after severe LPO as indicated by a 20 to 50-fold increase of HETE levels above control values (27). These data suggest that, despite a serious oxidant stress and the beneficial effects of antioxidants, LPO is unlikely the major mechanism of liver cell injury. How can we reconcile these apparent conflicting results? First, the identification of Kupffer cells as the main source of reactive oxygen during the early phase (42) would suggest that a potential free radical injury should occur mainly in vascular cells (e.g., endothelial cells). Indeed, there is evidence for LPO in nonparenchymal cells (60) and early endothelial cell damage (46, 61). This means that LPO may occur in a small compartment of the tissue and determination in the whole organ may underestimate the changes in endothelial cells. Because the major reperfusion injury in the liver is caused by neutrophils (49), the initial vascular injury induced by Kupffer cells (42) is critical for continuous neutrophil recruitment and activation, and thus the later, severe neutrophil-induced parenchymal injury. LPO products may act as chemotactic factors and/or LPO-induced injury to vascular cells may activate neutrophils. Is reactive oxygen and lipid peroxidation involved in the neutrophil-induced reperfusion injury? There is again only a minor increase of LPO products during the late reperfusion phase (27) and *in vitro* experiments using hepatocyte-neutrophil coculture systems showed that protease inhibitors but not reactive oxygen scavenger attenuated cell injury (62, 63). These data suggest that neutrophil-induced parenchymal cell damage is a protease-mediated process. However, in an *in vivo* situation, proteases are rapidly neutralized by antiproteases and reactive oxygen species generated by neutrophils are necessary to inactivate the more susceptible anti-proteases and facilitate protease-induced injury (64). This would explain why *in vivo* free radical scavenger and protease inhibitors are effective in protecting against inflammatory damage (37, 47, 65).

In addition to the effects already discussed, reactive oxygen species were shown to induce in the absence of endothelial cell injury, a transient, reversible, platelet-activating factor-dependent adherence of neutrophils to perfused blood vessels (66) and endothelial cells (67). The mechanism depends on intact endothelium and involves adhesion molecules on both neutrophils and endothelial cells. Furthermore, inhibition of nitric oxide formation was shown to increase neutrophil adhesion in postcapillary venules (68). Enhanced superoxide formation in the vasculature can lower nitric oxide levels by forming peroxynitrite and thus supporting neutrophil accumulation. Reactive oxygen species can also facilitate neutrophil-induced tissue injury through these indirect mechanisms. Reactive oxygen species can impair directly the receptor-dependent nitric oxide production by endothelial cells and thus induce vasospasm and thrombosis (69), a major complication of reperfusion after myocardial infarction.

Summary

Reactive oxygen can cause serious cell damage. However, the excessive amounts necessary and the effective intracellular detoxification mechanisms limit the destructive potential of these reactive intermediates *in vivo*. Nevertheless, pharmacological evidence strongly supports a role of reactive oxygen in many disease processes. As discussed, using the mechanism of hepatic reperfusion injury as an example, reactive oxygen species seem to be involved at various levels. Reactive oxygen is necessary for protease-mediated cell injury by inflammatory cells *in vivo;* reactive oxygen can also be involved indirectly in the injury process by upregulation of adhesion molecules and generation of chemotactic factors, thus contributing to recruitment and activation of neutrophils. The direct damaging effect of reactive oxygen species may be restricted to a small fraction of cells exposed to a locally high oxidant stress (e.g., endothelial cells). In addition to the discussed effects relevant for acute injury, other oxidant stress-induced mechanisms (e.g., DNA modifications) may be relevant in chronic disease states. A solid mechanistic understanding of the role of reactive oxygen species in the overall pathophysiology is critical for providing a rationale for antioxidant therapy and the targeted development of new antioxidant drugs.

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