# Free Radicals in the Physiological Control of Cell Function

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**Dröge, Wulf.** Free Radicals in the Physiological Control of Cell Function. *Physiol Rev* 82: 47–95, 2002; 10.1152/ physrev.00018.2001.—At high concentrations, free radicals and radical-derived, nonradical reactive species are hazardous for living organisms and damage all major cellular constituents. At moderate concentrations, however, nitric oxide (NO), superoxide anion, and related reactive oxygen species (ROS) play an important role as regulatory mediators in signaling processes. Many of the ROS-mediated responses actually protect the cells against oxidative stress and reestablish "redox homeostasis." Higher organisms, however, have evolved the use of NO and ROS also as signaling molecules for other physiological functions. These include regulation of vascular tone, monitoring of oxygen tension in the control of ventilation and erythropoietin production, and signal transduction from membrane receptors in various physiological processes. NO and ROS are typically generated in these cases by tightly regulated enzymes such as NO synthase (NOS) and NAD(P)H oxidase isoforms, respectively. In a given signaling protein, oxidative attack induces either a loss of function, a gain of function, or a switch to a different function. Excessive amounts of ROS may arise either from excessive stimulation of NAD(P)H oxidases or from less well-regulated sources such as the mitochondrial electron-transport chain. In mitochondria, ROS are generated as undesirable side products of the oxidative energy metabolism. An excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of cancer, diabetes mellitus, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, ischemia/reperfusion injury, obstructive sleep apnea, and other diseases. In addition, free radicals have been implicated in the mechanism of senescence. That the process of aging may result, at least in part, from radical-mediated oxidative damage was proposed more than 40 years ago by Harman (*J Gerontol* 11: 298–300, 1956). There is growing evidence that aging involves, in addition, progressive changes in free radical-mediated regulatory processes that result in altered gene expression.

# I. INTRODUCTION

## A. From Oxidative Damage to Redox Regulation: Historic Background

The presence of free radicals in biological materials was discovered less than 50 years ago (114). Soon thereafter, Denham Harman hypothesized that oxygen radicals may be formed as by-products of enzymic reactions in vivo. In 1956, he described free radicals as a Pandora's box of evils that may account for gross cellular damage, mutagenesis, cancer, and, last but not least, the degenerative process of biological aging (234, 235).

The science of free radicals in living organisms entered a second era after McCord and Fridovich (386) discovered the enzyme superoxide dismutase (SOD) and, finally, convinced most colleagues that free radicals are important in biology. Numerous researchers were now inspired to investigate oxidative damage inflicted by radicals upon DNA, proteins, lipids, and other components of the cell (reviewed in Ref. 49).

A third era began with the first reports describing advantageous biological effects of free radicals. Mittal and Murard (394) provided suggestive evidence that the superoxide anion ($O_2^- \cdot$), through its derivative, the hydroxyl radical, stimulates the activation of guanylate cyclase and formation of the "second messenger" cGMP. Similar effects were reported for the superoxide derivative hydrogen peroxide (615). Ignarro and Kadowitz (272) and Moncada and colleagues (456) discovered independently the role of nitric oxide (NO) as a regulatory molecule in the control of smooth muscle relaxation and in the inhibition of platelet adhesion. Roth and Dröge (472) found that in activated T cells the superoxide anion or low micromolar concentrations of hydrogen peroxide increase the production of the T-cell growth factor interleukin-2, an immunologically important T-cell protein. Keyse and Tyrrell (300) showed that hydrogen peroxide induces the expression of the heme oxygenase (HO-1) gene. Storz and colleagues (551) reported the induction of various genes in bacteria by hydrogen peroxide, and Schreck and Baeuerle (501) reported the activation of the transcription factor nuclear factor $\kappa B$ (NF-$\kappa$B) by hydrogen peroxide in mammalian cells.

At the beginning of the 21st century, there is now a large body of evidence showing that living organisms

have not only adapted to an unfriendly coexistence with free radicals but have, in fact, developed mechanisms for the advantageous use of free radicals. Important physiological functions that involve free radicals or their derivatives include the following: regulation of vascular tone, sensing of oxygen tension and regulation of functions that are controlled by oxygen concentration, enhancement of signal transduction from various membrane receptors including the antigen receptor of lymphocytes, and oxidative stress responses that ensure the maintenance of redox homeostasis (see Table 1). The field of redox regulation is also receiving growing attention from clinical colleagues in view of the role that oxidative stress has been found to play in numerous disease conditions. These pathological conditions demonstrate the biological relevance of redox regulation. The delicate balance between the advantageous and detrimental effects of free radicals is clearly an important aspect of life. The science of biological "redox regulation" is a rapidly growing field of research that has impact on diverse disciplines including physiology, cell biology, and clinical medicine.

## B. About This Review

We are now living in a particularly exciting time of redox research where information from different fields and independent approaches is falling into place and beginning to reveal a meaningful picture. This is a good time for a broad overview that summarizes the main principles of redox regulation. In textbook style, this review describes the current knowledge and paradigms but does not discuss future research directions, historical controversies, or experimental models. Moreover, it was not within the scope of this review to deal with all the details. Even the more than 600 references cited here do not cover all relevant publications in the field. For the interested reader, a number of more detailed and specific reviews on this topic are recommended (see Refs. 13, 20, 45, 66, 122, 125, 183, 211, 212, 251, 294, 333, 337, 397, 446, 506, 510, 512, 632, 659).

## II. MAJOR TYPES OF FREE RADICALS AND THEIR DERIVATIVES IN LIVING ORGANISMS

### A. Reactive Oxygen Species

The superoxide anion is formed by the univalent reduction of triplet-state molecular oxygen ($^3O_2$). This process is mediated by enzymes such as NAD(P)H oxidases and xanthine oxidase or nonenzymically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain (see Fig. 1). SODs convert superoxide enzymically into hydrogen peroxide (130, 187). In biological tissues superoxide can also be converted nonenzymically into the nonradical species hydrogen peroxide and singlet oxygen ($^1O_2$) (549). In the presence of reduced transition metals (e.g., ferrous or cuprous ions), hydrogen peroxide can be converted into the highly reactive hydroxyl radical ($\cdot OH$) (101). Alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase (Fig. 1). In the glutathione peroxidase reaction glutathione is oxidized to glutathione disulfide, which can be converted back to glutathione by glutathione reductase in an NADPH-consuming process (Fig. 1).

Because superoxide and NO are readily converted by enzymes or nonenzymic chemical reactions into reactive nonradical species such as singlet oxygen ($^1O_2$), hydrogen peroxide, or peroxynitrite ($ONOO^-$), i.e., species which can in turn give rise to new radicals, the regulatory effects of these nonradical species have also been included in this review. Most of the regulatory effects are indeed not directly mediated by superoxide but rather by its reactive oxygen species (ROS) derivatives. Frequently, different reactive species coexist in the reactive environment and make it difficult to identify unequivocally which agent is responsible for a given biological effect.

### B. Reactive Nitrogen Species

The NO radical ($NO\cdot$) is produced in higher organisms by the oxidation of one of the terminal guanido-

TABLE 1. *Important physiological functions that involve free radicals or their derivatives*

| Type of Radical | Source of Radical | Physiological Process |
|---|---|---|
| Nitric oxide ($NO\cdot$) | Nitric oxide synthase | Smooth muscle relaxation (control of vascular tone) and various other cGMP-dependent functions |
| Superoxide ($O_2^-\cdot$) and related ROS | NAD(P)H oxidase | Control of ventilation |
| | | Control of erythropoietin production and other hypoxia-inducible functions |
| | | Smooth muscle relaxation |
| | | Signal transduction from various membrane receptors/enhancement of immunological functions |
| Superoxide ($O_2^-\cdot$) and related ROS | Any source | Oxidative stress responses and the maintenance of redox homeostasis |

ROS, reactive oxygen species.
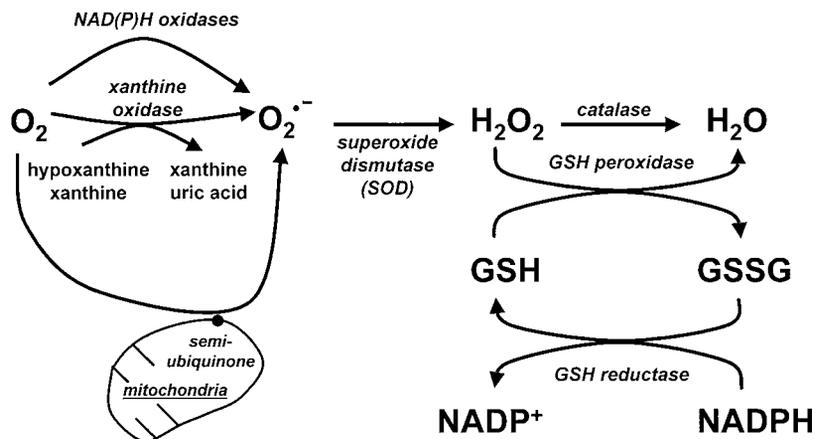
# Pathways of ROS production and clearance



FIG. 1. Pathways of reactive oxygen species (ROS) production and clearance. GSH, glutathione; GSSG, glutathione disulfide.

nitrogen atoms of L-arginine (437). This process is catalyzed by the enzyme NOS. Depending on the microenvironment, NO can be converted to various other reactive nitrogen species (RNS) such as nitrosonium cation ($NO^+$), nitroxyl anion ($NO^-$) or peroxynitrite ($ONOO^-$) (546). Some of the physiological effects may be mediated through the intermediate formation of S-nitroso-cysteine or S-nitroso-glutathione (207).

## C. Key Message From Section II

The most relevant radicals in biological regulation are superoxide and NO (see Table 1). These radicals are formed by two groups of enzymes, i.e., the NAD(P)H oxidase and NOS isoforms, respectively. Many regulatory effects are mediated by hydrogen peroxide and other ROS that are chemically derived from superoxide.

## III. OXIDATIVE STRESS RESPONSE AS A MODEL OF REDOX SIGNALING

### A. Maintenance of "Redox Homeostasis"

The term *redox signaling* is widely used to describe a regulatory process in which the signal is delivered through redox chemistry. Redox signaling is used by a wide range of organisms, including bacteria, to induce protective responses against oxidative damage and to reset the original state of "redox homeostasis" after temporary exposure to ROS.

### 1. Oxidant-antioxidant balance

Free radicals and reactive nonradical species derived from radicals exist in biological cells and tissues at low but measurable concentrations (228, 527). Their concentrations are determined by the balance between their rates of production and their rates of clearance by various antioxidant compounds and enzymes, as illustrated schematically in Figure 2. Halliwell and Gutteridge (228) have defined antioxidants as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus, to significantly delay or inhibit the oxidation of these substrates. This definition includes the enzymes SOD, glutathione peroxidase (GPx), and catalase, as well as nonenzymic compounds such as $\alpha$-tocopherol (vitamin E), $\beta$-carotene, ascorbate (vitamin C), and glutathione.

In addition, there are compounds that have a relatively low specific antioxidative activity, i.e., on a molar basis, but, when present at high concentrations, can contribute significantly to the overall ROS scavenging activity. The most prominent examples of such high-level, low-efficiency antioxidants are free amino acids, peptides, and proteins. Practically all amino acids can serve as targets for oxidative attack by ROS, although some amino acids such as tryptophan, tyrosine, histidine, and cysteine are particularly sensitive to ROS (126, 128, 545). Because the cumulative intracellular concentration of free amino acids is on the order of $10^{-1}$ M, free amino acids are quantitatively important ROS scavengers (see sect. III*B5*).

### 2. Oxidized proteins as substrates for proteolytic digestion and their contribution to redox homeostasis

Oxygen radicals and other ROS cause modifications of proteins (reviewed in Ref. 220). These oxidative modifications may lead to changes in protein function, chemical fragmentation, or increased susceptibility to proteolytic attack (124, 543, 631). Proteolytic degradation is

## Mechanisms of redox homeostasis.
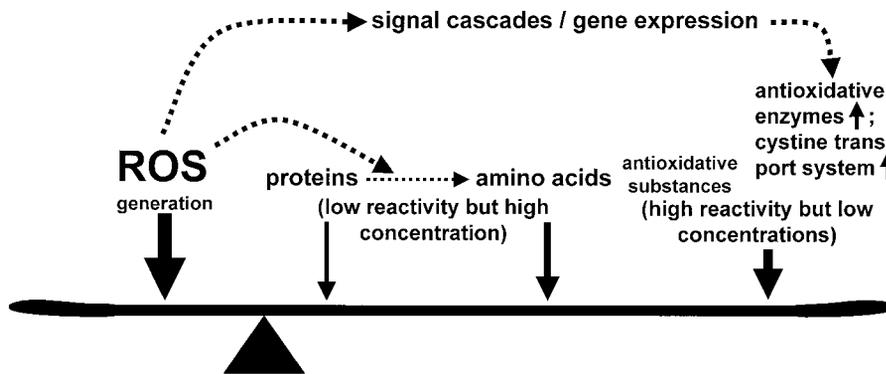## Balance between ROS production and various types of scavengers



FIG. 2. Mechanisms of redox homeostasis. Balance between ROS production and various types of scavengers. The steady-state levels of ROS are determined by the rate of ROS production and their clearance by scavenging mechanisms. Certain antioxidative enzymes including superoxide dismutase (SOD), glutathione peroxidase, catalase, and thioredoxin are potent ROS scavengers but occur in cells only at relatively low concentrations. The same is true for nonenzymic antioxidants. Amino acids and proteins are also ROS scavengers. Amino acids are less effective than the classical antioxidants on a molar basis, but their cumulative intracellular concentration is >0.1 M.

executed mainly by proteasomes (219). In one of the studies, proteolysis was estimated to increase more than 11-fold after exposure to superoxide or hydrogen peroxide (127). Proteolysis is enhanced by $20-400$ $\mu$M hydrogen peroxide, whereas millimolar concentrations inhibit proteolysis and may lead to the intracellular accumulation of oxidized proteins (220, 544).

The proteins may differ strongly in their susceptibility to oxidative damage. The redox-sensitive amino acids of bovine serum albumin, for example, were shown to be oxidized about twice as fast as those of glutamine synthase (54), and intact proteins are less sensitive to oxidation than misfolded proteins (161). These findings implicate that *1*) phylogenetic evolution has selected for protein structures that are relatively well-protected against oxidation and *2*) ROS scavenging activities of intact proteins are weaker than those of misfolded proteins or equivalent concentrations of their constituent amino acids. Protein oxidation and enhanced proteolytic degradation cause, therefore, a net increase in ROS scavenging capacity as schematically illustrated in Figure 2. Preliminary experiments showed that treatment of human skeletal muscle cells with proteasome inhibitors causes a substantial increase in intracellular ROS levels and that this increase is reversed by the addition of free amino acids (R. Breitkreutz and W. Dröge, unpublished observations). More systematic studies are needed to determine the relative contribution of proteins, free amino acids, and classical antioxidant compounds and enzymes to the total ROS scavenging capacity of different cells and tissues.

*3. Changes in oxidant-antioxidant balance as a*
*trigger for redox regulation: the theory of redox*
*homeostasis and the existence of different*
*quasi-stable states*

Living cells and tissues have several mechanisms for reestablishing the original redox state after a temporary

exposure to increased ROS or RNS concentrations. The production of NO (NO·), for example, is subject to direct feedback inhibition of NOS by NO (see sect. IIIB1). Elevated ROS concentrations induce in many cells the expression of genes whose products exhibit antioxidative activity (Fig. 2). A major mechanism of redox homeostasis is based on the ROS-mediated induction of redox-sensitive signal cascades that lead to increased expression of antioxidative enzymes or an increase in the cystine transport system, which, in turn, facilitates in certain cell types the increase in intracellular glutathione (see Fig. 2; for details see sect. III, *B2–B4*). Moreover, because proteins generally provide less ROS scavenging activity than an equivalent amount of the free amino acids contained in them, it is reasonable to assume that oxidative enhancement of proteolysis also contributes, at least to some extent, to the maintenance of redox homeostasis (see Fig. 2 and sect. IIIB5).

Cells or tissues are in a stable state if the rates of ROS production and scavenging capacity are essentially constant and in balance (see Fig. 2 and baseline level in Fig. 3). Redox signaling requires that this balance is disturbed, either by an increase in ROS concentrations or a decrease in the activity of one or more antioxidant systems (Fig. 3). In higher organisms, such an oxidative event may be induced in a regulated fashion by the activation of endogenous RNS- or ROS-generating systems (see sect. IVA). However, similar responses may be induced by oxidative stress conditions generated by environmental factors (see sect. IIIA4). If the initial increase in ROS is relatively small, the antioxidative response may be sufficient to compensate for the increase in ROS and to reset the original balance between ROS production and ROS scavenging capacity. Thus physiological manifestations of redox regulation involve typically a temporary increase and/or a temporary shift of the intracellular thiol/disulfide redox state toward more oxidative conditions, as illustrated in

## Regulatory events and their dysregulation depend on the magnitude and duration of the change in ROS and/or RNS concentration
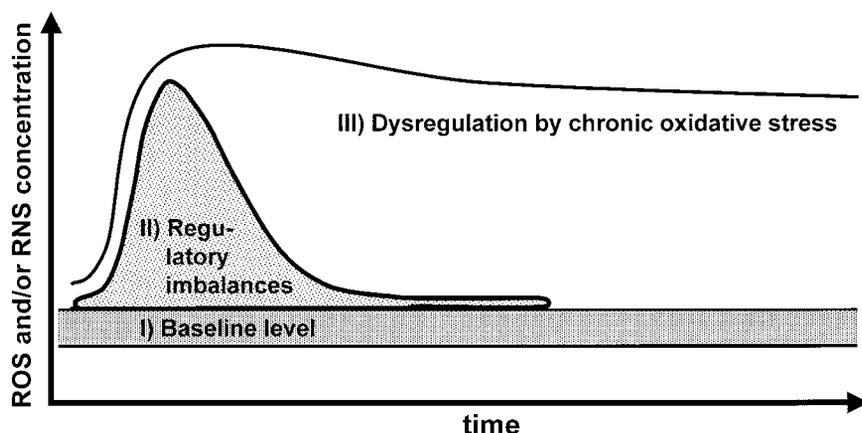


FIG. 3. Regulatory events and their dysregulation depend on the magnitude and duration of the change in ROS or reactive nitrogen species (RNS) concentration. ROS and RNS normally occur in living tissues at relatively low steady-state levels. The regulated increase in superoxide or nitric oxide production leads to a temporary imbalance that forms the basis of redox regulation. The persistent production of abnormally large amounts of ROS or RNS, however, may lead to persistent changes in signal transduction and gene expression, which, in turn, may give rise to pathological conditions.

Figure 3. In the long run, these mechanisms tend to maintain a stable state called redox homeostasis.

Under certain conditions, however, ROS production is increased more strongly and persistently, and the anti-oxidative response may not be sufficient to reset the system to the original level of redox homeostasis. In such cases, the system may still reach an equilibrium according to the model in Figure 2, but the resulting quasi-stable state may now be associated with higher ROS concentrations and different levels of free amino acids and/or different patterns of gene expression due to redox-sensitive signaling pathways. Indications for such a shift to more oxidative conditions have been seen in the process of aging (see sect. VIC), implying that a pro-oxidative shift may not always be associated with an overtly pathological condition.

Pathological conditions (see sect. VI, *H–P*) may develop in more extreme cases of persistently high ROS levels (see Fig. 3). Again, these conditions do not necessarily involve a loss of homeostasis but rather a chronic shift in the level of homeostasis. Accordingly, pathological symptoms may result from both the damaging effects of ROS and from ROS-mediated changes in gene expression.

### 4. Redox regulation by changes in the thiol/disulfide redox state

In several cases (see Table 2), changes in the intracellular thiol/disulfide redox state have been shown to trigger the same redox-responsive signaling proteins and pathways as those triggered by hydrogen peroxide (28, 192, 248, 323). Bacterial OxyR (see sect. IIIB2) is a model case of a redox-sensitive signaling protein that may be activated either directly by hydrogen peroxide or, alternatively, by changes in the intracellular glutathione redox state (28). Conversely, protein tyrosine phosphatases (see sect. vB) are inactivated either by ROS or by pro-oxidative changes in the intracellular thiol/disulfide redox state. Accordingly, this review also addresses the role of the thiol/disulfide redox state in the control of cell function.

### 5. Effects of carcinogens and allergens on redox-responsive signaling pathways

Certain alkylating agents play an important role as environmental carcinogens by exerting effects on redox-sensitive signaling pathways similar to those induced by ROS (621). There is a strong possibility that the resulting dysregulation of signaling cascades may contribute to the process of carcinogenesis (see sect. VII).

Certain heavy metal compounds are important environmental allergens. A point in case is the sulfhydryl-reactive compound mercury dichloride ($HgCl_2$). Treat-

TABLE 2. *Signaling mechanisms that respond to changes in the thiol/disulfide redox state*

| Example | Reference No. | Section |
|---|---|---|
| AP-1 transcription factor in human T cells | 192 | v*H* |
| NF-$\kappa$B transcription factor in human T cells | 192 | v*I* |
| yAP-1 transcription factor in *S. cerevisiae* | 323 | III*B3* |
| Control of $K^+$ channel activity in the carotid body | 9 | IV*C* |
| Human insulin receptor kinase activity | 494 | v*C* |
| Bacterial OxyR | 28 | III*B2* |
| Protein tyrosine phosphatases | 43 | v*B* |
| Src family kinases | 248 | v*D* |
| JNK and p38 MAPK signaling pathways | 248 | v*E* |
| Amplification of immunologic functions | 248 | IV*F* |
| Signaling in replicative senescence | 536 | VI*D* |

AP-1, activator protein 1; NF-$\kappa$B, nuclear factor $\kappa$B; JNK, c-Jun $NH_2$-terminal kinase; MAPK, mitogen-activated protein kinase.

ment of murine lymphocytes with $HgCl_2$ was found to induce strong aggregation and activation of the protein tyrosine kinase p56[lck]. These findings suggest that the oxidation of redox-reactive sulfhydryl groups of certain signaling proteins leads to a dysregulation of lymphocytes and may contribute thereby to the development of allergies (411).

These examples illustrate that the mechanisms of redox regulation may be targets for hazardous environmental agents. However, a more detailed discussion of this point would exceed the scope of this review.

## B. Examples of Redox Signaling in the Maintenance of Redox Homeostasis

The maintenance of redox homeostasis involves regulatory mechanisms that are capable of sensing NO or ROS. NO, for example, inhibits directly the NO-producing enzyme. ROS stimulate in many cells the expression of compensatory gene products. Some but not all of these regulatory mechanisms are well characterized at the molecular level.

### 1. NO-mediated feedback inhibition of NOS

Many of the enzymes that utilize a heme prosthetic group in catalysis are inactivated by NO. This applies to the heme-containing cytochrome P-450-related enzyme NOS, leading to feedback inhibition of NO production by NO (6, 79, 215). The inhibition of NOS was shown to involve the formation of a ferrous-nitrosyl complex (6).

### 2. Oxidative stress-inducible gene expression in bacteria as a model for ROS-responsive signaling pathways

The protective responses of bacteria against ROS are among the best investigated examples of redox-regulated gene expression (reviewed in Ref. 658). Prokaryotes have several different signaling pathways for responding to ROS or to alterations in the intracellular redox state (28, 45, 176, 551, 634, 657). Studies in *Escherichia coli* re-

vealed that low levels of ROS activate the expression of several gene products involved in the antioxidant defense including Mn-SOD (238), catalase (649), hydroperoxidase I (katG), an alkylhydoperoxide reductase (ahpCF), glutathione reductase (gorA), glutaredoxin 1 (grxA), and a regulatory RNA (oxyS).

At least nine proteins that are synthesized in *Salmonella typhimurium* and *E. coli* after exposure to hydrogen peroxide are under the control of the oxyR locus (45, 108, 138). The OxyR protein controls protective responses against normally lethal doses of hydrogen peroxide or against killing by heat. In addition, it negatively autoregulates its own expression (109, 489). Hydrogen peroxide does not stimulate the synthesis of OxyR but converts the reduced form of OxyR into its oxidized and regulatory competent form (Fig. 4) (45, 551, 657). Exposure of the OxyR protein to hydrogen peroxide results initially in the conversion of Cys-199 to a sulfenic acid derivative which subsequently forms an intramolecular disulfide bond with Cys-208, as illustrated schematically in Figure 4 (657). Because the redox potential of OxyR is $-185$ mV, and the thiol-disulfide redox state of the normal bacterial cytoplasm is typically $-260$ to $-280$ mV, the OxyR is normally in the reduced form and exemplifies proteins that can be activated either through direct oxidation by hydrogen peroxide or by an oxidative shift in the thiol/disulfide redox status, as illustrated in Figure 4 (28). Both oxidized and reduced OxyR are able to bind to the oxyR-oxyS promoter region but exhibit different binding characteristics (578). The formation of disulfide bonds can be reversed by glutaredoxin 1 and by thioredoxin (Trx) (657). Because OxyR controls the induction of glutathione reductase and glutaredoxin 1, the OxyR response is part of an autoregulatory circuit.

Protective responses against superoxide are controlled by the sox locus (255, 581), which regulates the induction of ~10 proteins, including Mn-SOD, NADPH:ferredoxin oxidoreductase, and glucose-6-phosphate dehydrogenase (reviewed in Ref. 45). One of the gene products from the sox locus, the SoxR protein, exists in solution as a homodimer containing two stable (2Fe-2S)
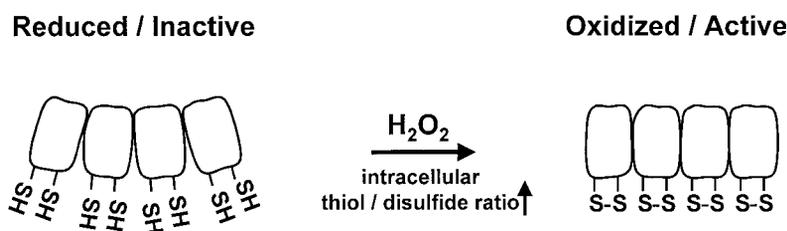
# Schematic Model of OxyR activation



FIG. 4. Schematic model of OxyR activation. The regulatory protein OxyR is activated by the formation of disulfide bridges. This process is mediated either by hydrogen peroxide ($H_2O_2$) or by oxidative changes in the intracellular thiol/disulfide redox state.

centers that are anchored to four cysteine residues near the COOH terminals (67, 255, 634). Under normal physiological conditions, these iron-sulfur centers are in the reduced state. They are readily oxidized, however, under oxidative stress (149). The oxidative process is reversible if the oxidative stress conditions are removed. Only the oxidized form of SoxR stimulates transcription of soxS (67, 149, 195). Both the oxidized and the reduced forms of SoxR can bind to DNA but interact differently with RNA polymerase.

The redox-sensitive proteins OxyR and SoxR have been described here as two particularly prominent and well-investigated cases of bacterial redox regulation. Additional examples of redox-responsive regulatory mechanisms in prokaryotic cells have been reviewed by Bauer et al. (45).

### 3. The oxidative stress response of the budding yeast Saccharomyces cerevisiae

Treatment of *S. cerevisiae* with hydrogen peroxide activates the yAP-1 transcription factor that binds specifically to the AP-1 site of the eukaryotic AP-1 family of transcription factors (323). Similar activation can be induced by diamide or diethyl maleate, i.e., two thiol oxidants that modulate the intracellular reduced glutathione (GSH) state. The yAP-1 transcription factor is involved in protective responses against oxidative stress, inducing Trx production from the TRX2 gene (323). Overexpression of yAP-1 on a multicopy plasmid was also found to increase the expression of SOD, glutathione reductase, and glucose-6-phosphate dehydrogenase, whereas yAP-1 mutant strains showed greatly decreased levels of these enzymes (498). Oxidative activation of yAP-1 operates at the posttranslational level and involves the translocalization of yAP-1 from the cytoplasm to the nucleus. This translocation is controlled by a cysteine-rich domain at the COOH terminus. Three conserved cysteine residues in this region are believed to be important in sensing the redox state (324). The transcription factor SKN7 cooperates with yAP-1 and is, therefore, also critically involved in oxidative stress response (399).

The response of *S. cerevisiae* to hydrogen peroxide (0.4 mM for 15 min) was studied by two-dimensional gel electrophoresis of total cell proteins, revealing an increase in the expression of 115 proteins and a decrease in the expression of another 52 proteins (200). The induced proteins include Mn-SOD, Cu/Zn-SOD, glutathione reductase, catalase, Trx reductase, cytochrome-*c* peroxidase, several proteasome subunits, and heat shock proteins. In addition, carbohydrate metabolism is rapidly redirected to the regeneration of NADPH at the expense of glycolysis.

### 4. Protective responses in higher organisms

The redox-responsive signal cascades in mammalian cells show certain similarities to the oxidative stress response of *S. cerevisiae*. While activated macrophages and neutrophils in inflamed tissue of higher organisms generate massive amounts of ROS to kill environmental pathogens (see sect. IV*A2*), other host cells must be protected against this oxidative burst. Lymphocytes recruited into the inflammatory environment initiate antigen-specific immunological effector mechanisms and can function only because they are able to activate powerful protective mechanisms against the oxidative stress. Exposure to ROS or changes in the intracellular thiol redox state modulate various signal transduction cascades and increase the activities of several transcription factors (reviewed in Refs. 12, 13, 183, 337, 397, 446, 506, 512).

The oxidoreductase Trx is one of the proteins that is inducibly expressed in lymphocytes and other cells by hydrogen peroxide, ultraviolet (UV) irradiation, and other conditions of oxidative stress (373, 480, 565). Together with the glutathione system, Trx plays a key role in the maintenance of a reducing intracellular redox state in higher organisms. The $5'$-upstream sequence of the human Trx gene contains putative binding sites for the redox-responsive transcription factors AP-1 and NF-$\kappa$B (407, 408).

Exposure of macrophages to low levels of ROS or other inducers of oxidative stress induces the expression of peroxyredoxin I (i.e., a Trx peroxidase), heme oxygenase-1 (HO-1), and the cystine transporter $x_c^-$ (278, 486). Because the plasma concentration of reduced cysteine is relatively low, the cystine transporter plays a limiting role in the cellular supply of cyst(e)ine and in the biosynthesis of glutathione in macrophages and lymphocytes (reviewed in Ref. 160). ROS and various other oxidants were also found to induce MnSOD mRNA levels to a moderate extent in several cell types (522).

The redox control of the heme oxygenase-1 (HO-1) gene is one of the best studied models of redox regulation in mammalian cells. HO-1 induction in skin fibroblasts may serve as an inducible defense pathway to remove heme liberated by oxidants. The HO-1 protein and mRNA are strongly induced by ROS, physiological doses of UVA irradiation, and various other inducers of oxidative stress, including NO (237, 300, 585, 586). The UVA response is synergistically enhanced by depletion of intracellular glutathione. The sustained induction of HO-1 mRNA and its inducibility in many tissues and various mammalian species has rendered HO-1 mRNA a useful marker for cellular oxidative stress at the mRNA level. In murine macrophages, HO-1 expression is induced by hydrogen peroxide via AP-1 (16, 91, 92). Activation of ERK and p38 MAPK was implicated in HO-1 expression in chicken hepatoma cells (167).

## C. Key Message From Section III

Free radicals and their derivatives exist in living tissues at low but measurable concentrations that are determined by the balance between the rates of radical production and their corresponding rates of clearance.

The relatively high intracellular concentrations of glutathione and other antioxidative compounds provide a strong basal scavenging capacity.

The ROS-mediated oxidation of proteins and the resulting increase in proteolytic degradation is expected to cause an increase in intracellular ROS scavenging capacity and may thereby contribute to the maintenance of redox homeostasis.

A wide variety of living organisms including bacteria have the capacity to respond to increased levels of ROS with an increase in intracellular glutathione or with increased expression of proteins/enzymes with ROS scavenging capacity. This process is known as "oxidative stress response." The resulting increase in ROS clearance capacity allows cells and tissues to maintain redox homeostasis.

The inducibility of HO-1 mRNA in many tissues and various mammalian species has rendered HO-1 mRNA a useful marker for cellular oxidative stress at the mRNA level.

The regulation of physiological responses by free radicals (see sects. IV and V) is embedded in these basic mechanisms of redox homeostasis.

Oxidative stress responses provide some of the best studied examples of redox-responsive signaling pathways.

Changes in the intracellular thiol/disulfide redox state cause in many cases chemical modifications of redox-sensitive signaling compounds similar to the modifications caused by ROS. The corresponding signal pathways may become sensitive to systemic changes in the thiol/disulfide redox state (see sect. VI, *C* and *H*).

## IV. NITRIC OXIDE AND REACTIVE OXYGEN SPECIES AS REGULATORY MEDIATORS OF PHYSIOLOGICAL RESPONSES

The following sections describe cases of redox regulation that serve physiological functions other than protection against oxidative stress and redox homeostasis. These cases typically involve the regulated production of NO or ROS and the resulting effects on defined signaling cascades.

### A. Regulated Production of Free Radicals in Higher Organisms

#### 1. Regulated production of NO

The enzyme NOS exists in three isoforms, i.e., neuronal NOS (nNOS; type I) (69), inducible NOS (iNOS; type II) (639), and endothelial NOS (eNOS; type III) (330). Many tissues express one or more of these isoforms. The isoforms nNOS and eNOS are constitutively expressed, but their activity is regulated by the intracellular calcium concentration. The isoform iNOS is inducibly expressed in macrophages after stimulation by cytokines, lipopolysaccharides, and other immunologically relevant agents (reviewed in Ref. 59). Expression of iNOS is regulated at the transcriptional and posttranscriptional level by signaling pathways that involve agents such as the redox-responsive transcription factor NF-$\kappa$B or mitogen-activated protein kinases (MAPKs) (366). The rate of NO synthesis is also determined to some extent by the availability of the substrate L-arginine and by the cofactor tetrahydrobiopterin (BH$_4$).

#### 2. ROS production by phagocytic NADPH oxidase: the oxidative burst

Activated macrophages and neutrophils can produce large amounts of superoxide and its derivatives via the phagocytic isoform of NADPH oxidase. This enzyme is a heme-containing protein complex illustrated schematically in Figure 5. In an inflammatory environment hydrogen peroxide is produced by activated macrophages at an estimated rate of $2–6 \times 10^{-14}$ mol·h$^{-1}$·cell$^{-1}$ and may reach a concentration of $10–100$ $\mu$M in the vicinity of these cells (299, 335, 412). The massive production of antimicrobial and tumoricidal ROS in an inflammatory environment is called the "oxidative burst" and plays an important role as a first line of defense against environmental pathogens. The physiological relevance of NADPH oxidase as a defense agent is suggested by the observation that mice lacking the NADPH oxidase components gp91$^{phox}$ or p47 exhibit reduced resistance to infection (132, 148, 169, 400, 448, 466, 521). The combined activities
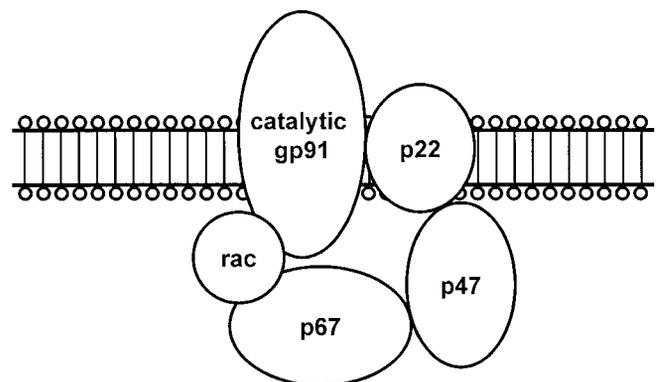
## Structure of neutrophil NAD(P)H oxidase



FIG. 5. Structure of neutrophil NAD(P)H oxidase. The enzyme consists of the membrane-bound cytochrome $b_{558}$ complex comprising gp91$^{phox}$ and p22$^{phox}$, the cytosolic proteins p47 and p67, and a low-molecular-weight G protein of the rac family.

of NADPH oxidase and myeloperoxidase in phagocytes leads, in addition, to the production of hypochlorous acid (HClO), one of the strongest physiological oxidants and a powerful antimicrobial agent (229, 500). Stimulated neutrophils and macrophages generate also singlet oxygen by reactions that involve either myeloperoxidase or NADPH oxidase (549). Importantly, however, physiologically relevant ROS concentrations can also modulate redox-sensitive signal cascades and enhance immunological functions of lymphocytes (see sect. IV*F*).

Phagocytic NADPH oxidase becomes activated upon translocation of cytosolic p47, p67, and a G protein of the rac family to the membrane-bound cytochrome $b_{558}$ complex that contains gp91$^{phox}$ and p22 (Fig. 5). The catalytic moiety gp91$^{phox}$ is a plasma membrane-associated complex protein containing a flavin-adenine dinucleotide component and two hemes (for details see Refs. 211, 333). The activation of phagocytic NADPH oxidase can be induced by microbial products such as bacterial lipopolysaccharide, by lipoproteins, or by cytokines such as interferon-$\gamma$, interleukin-1$\beta$, or interleukin-8 (60). The activation of NADPH oxidase is mainly controlled by the rac isoform rac2 in neutrophils and rac1 in macrophages and monocytes (567, 346).

### 3. ROS production by NAD(P)H oxidases in nonphagocytic cells

The production of ROS by nonphagocytic NAD(P)H oxidase isoforms plays a role in the regulation of intracellular signaling cascades in various types of nonphagocytic cells including fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes, and thyroid tissue (36, 163, 210, 211, 288–290, 354, 387, 553, 557, 559, 571, 572, 660). In most of these cases, rac1 is involved in the induction of NAD(P)H oxidase activity (289, 290, 660). Muscle cells and fibroblasts account for most of the superoxide produced in the normal vessel wall.

The NAD(P)H oxidase isoforms of the cardiovascular system are membrane-associated enzymes that appear to utilize both NADH and NADPH (211). The rate of superoxide production in nonphagocytic cells is only about one-third of that of neutrophils. Vascular smooth muscle cells, in contrast to neutrophils, endothelial cells, or fibroblasts, generate superoxide and hydrogen peroxide mainly intracellularly.

The cardiovascular NAD(P)H oxidase isoforms are induced by hormones, hemodynamic forces, or by local metabolic changes (211). Angiotensin II increases NAD(P)H-driven superoxide production in cultured vascular smooth muscle cells and fibroblasts. Thrombin, platelet-derived growth factor (PDGF), and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) stimulate NAD(P)H oxidase-dependent superoxide production in vascular smooth muscle cells. Interleukin-1, TNF-$\alpha$, and platelet-activating factor

increase NAD(P)H-dependent superoxide production in fibroblasts. Mechanical forces stimulate NAD(P)H oxidase activity in endothelial cells. Reoxygenation stimulates NAD(P)H oxidase activity in cardiac myocytes.

Whereas the gp91$^{phox}$ of phagocytic cells (see Fig. 5) has also been found in endothelial cells, other nonphagocytic cells appear to utilize structural homologs of gp91$^{phox}$. Several novel homologs of gp91$^{phox}$ have recently been identified and are the subject of intense research. The gp91$^{phox}$ homolog p138$^{tox}$ is (the catalytic unit of) the NADPH oxidase that supports thyroid hormone biosynthesis (163). An NAD(P)H oxidase with low affinity for oxygen and high affinity for cyanide is believed to act as one of the sensors for oxygen tension in the carotid body; these sensors control the rate of ventilation (8). The function of oxygen sensing is apparently shared by several proteins, including a nonmitochondrial cytochrome $b_{558}$, a mitochondrial protein, and possibly a third heme protein (329, 659). A similar group of proteins was suggested to be involved as oxygen sensors in the regulation of erythropoietin production in human hepatoma cells (659). A microsomal NADH oxidase was implicated as an oxygen sensor in bovine pulmonary and coronary arteries, where changes in oxygen tension regulate vascular relaxation through changes in superoxide production and cGMP formation (632).

There is a strong possibility that rac-like proteins also occur in plants (5, 624), where they may be involved in the induction of NAD(P)H oxidase-like enzymes (569). The oxidative burst in plants is an effective bactericidal mechanism.

### 4. ROS production in lymphocytes by 5-lipoxygenase

The enzyme 5-lipoxygenase (5-LO) has been identified as an inducible source of ROS production in lymphocytes (60, 359), but the evidence for its physiological role in redox signaling is still scarce. Lipoxygenases are non-heme-containing dioxygenases that oxidize polyunsaturated fatty acids at specific carbon sites to give hydroperoxy fatty acid derivatives with conjugated double bonds. The numbers in specific enzyme names such as 5-LO, 12-LO, or 15-LO refer to the arachidonic acid site that is predominantly oxidized (644). 5-LO is best known for its role in the biosynthesis of the leukotrienes $A_4$, $B_4$, $C_4$, $D_4$, and $E_4$. The oxidized metabolites generated by 5-LO were found to change the intracellular redox balance and to induce signal transduction pathways and gene expression. 5-LO was shown to be involved in the production of hydrogen peroxide by T lymphocytes after ligation of the CD28 costimulatory receptor (359) and in response to interleukin-1$\beta$ (60). Hydrogen peroxide production in response to CD28 stimulation was found to be decreased by specific inhibitors of 5-LO or lipid peroxidation but not by inhibitors of NADPH oxidase or cyclooxygenase. The

physiological role of 5-LO in CD28-mediated signal transduction remains to be confirmed by more detailed studies.

### 5. ROS production by cyclooxygenase

Cyclooxygenase-1 has been implicated in ROS production in cells stimulated with TNF-$\alpha$, interleukin-1, bacterial lipopolysaccharide, or the tumor promoter 4-$O$-tetradecanoylphorbol-13-acetate (TPA) (178, 406). A role for cyclooxygenase in the inflammatory response of 5-LO-defective mice was also suggested (206); however, the evidence for the participation of cyclooxygenase in redox signaling is still scarce.

## B. Regulation of Vascular Tone and Other Regulatory Functions of NO

The most prominent cases of NO-mediated regulation are in the control of vascular tone and platelet adhesion. The mechanism of these processes is particularly well characterized at the molecular level.

### 1. Signals involving guanylate cyclase

Guanylate cyclase belongs to the family of heterodimeric heme proteins and catalyzes the formation of cGMP, which is utilized as an intracellular amplifier and second messenger in a large range of physiological responses (197). NO binds to the heme moiety of guanylate cyclase, disrupting the planar form of the heme iron. The resulting conformational change activates the enzyme (273). Its product cGMP modulates the function of protein kinases, phosphodiesterases, ion channels, and other physiologically important targets (497). The most important examples include the regulation of smooth muscle tone (272) and the inhibition of platelet adhesion (456). Vascular smooth muscle relaxation is mediated by a cGMP-dependent protein kinase that phosphorylates and activates a calcium-sensitive potassium channel (27). Other examples have been reviewed by Deora and Lander (140).

Superoxide and hydrogen peroxide may also play a role in the activation of guanylate cyclase (82, 394, 615). Since micromolar concentrations of hydrogen peroxide stimulate NOS (190), there is a strong possibility that the activation of guanylate cyclase by superoxide and hydrogen peroxide may be mediated, at least in part, indirectly via NOS. Other studies suggest that compound I, a form of catalase, may play a critical role in the activation of guanylate cyclase by hydrogen peroxide (82). After interacting with hydrogen peroxide, catalase is converted into an oxidized heme intermediate called compound I, which is normally converted back into its reduced form by a second molecule of hydrogen peroxide.

### 2. RNS-mediated switch of function: aconitase and iron-regulatory protein-1

In iron-sulfur proteins, iron is bound simultaneously to inorganic sulfide groups and cysteine thiolate groups of the proteins. Such proteins are sensitive to both ROS and RNS. Oxidation usually results in dissolution of the iron-sulfur cluster and loss of function (85, 97, 250).

A special case is mammalian (4Fe-4S) aconitase, which is involved in the citric acid cycle. RNS inhibit the aconitase activity by disrupting the Fe-S clusters but expose simultaneously an RNA-binding site with specificity for the iron-response elements of the transferrin receptor and ferritin mRNAs. In this form the protein is called iron-regulatory protein-1 and is involved in iron homeostasis. In this case, the RNS-induced loss of one enzyme function is associated with the gain of or switch to another function for the same protein.

### 3. NO-mediated activation of the GTP-binding protein p21 Ras and protein kinase cascades

In human peripheral blood mononuclear cells and endothelial cells, NO was found to activate all three MAPK pathways (89, 336, 339, 445). The effect has been attributed to the NO-mediated stimulation of a membrane-associated protein tyrosine phosphatase activity which may lead to the dephosphorylation and activation of the Src family protein tyrosine kinase p56$^{lck}$ (339). Another Src family protein kinase, p60$^{c\text{-}src}$, was also found to be activated by NO in fibroblasts or immunoprecipitates (15). The activation was associated with autophosphorylation at Tyr-416 and S-S bond-mediated aggregation of the kinase molecules. NO may also activate Ras by $S$-nitrosylation of cysteine-118 (335–338).

## C. ROS Formation as a Sensor for Changes in Oxygen Concentration: Control of Ventilation

Oxygen homeostasis is maintained in higher organisms by a tight regulation of the red blood cell mass and respiratory ventilation. Carotid bodies are sensory organs that detect changes in arterial blood oxygen. They are composed of glomus type I chemoreceptor cells that release neurotransmitters in response to hypoxia. This process changes the level of electrical activity in the efferent fibers of the carotid sinus nerve, thus relaying the sensory information to the brain stem neurons that regulate breathing. A growing body of evidence indicates that changes in oxygen concentration are sensed independently by several different ROS-producing proteins including a b-type cytochrome with properties similar to those of the cytochrome $b_{558}$ in the NADPH oxidase complex in neutrophils (reviewed in Ref. 7). Other studies suggest that changes in the rate of mitochondrial ROS

production may play a major role in oxygen sensing by the carotid body (80, 449). Furthermore, it was found that the $\beta$-subunit of the potassium channel resembles the structure of NADPH-oxidoreductase (221). It is widely accepted that transduction of the sinus nerve signal involves changes in the $K^+$-channel conductivity of type I cells in response to changes in oxygen tension (356). Exactly how changes in ROS production are translated into changes in $K^+$ channel activity remains to be established. Acker and Xue (9) implicated ROS-mediated changes in the glutathione redox state in the control of $K^+$ efflux and the corresponding $Ca^{2+}$ influx.

### D. The Oxygen Sensor in the Regulation of Erythropoietin Production: Redox Regulation Through the Transcription Factor Hypoxia-Inducible Factor 1

The red blood cell mass is regulated by the hormone erythropoietin, which is mainly produced by kidney and liver cells, following stimulation by hypoxia. The oxygen-sensing mechanisms are still unclear and the subject of controversy. It is clear, however, that changes in oxygen tension are sensed by changes in ROS production (174, 264, 291, 413). Expression of erythropoietin protein or mRNA in hepatoma cell lines or perfused kidneys was found to be strongly repressed by hydrogen peroxide. Treatment of normoxic cells with exogenous catalase stimulated erythropoietin production (94). A cytochrome $b$-like NAD(P)H oxidase is believed to play a role as a major oxygen sensor and ROS producer. However, the role of a mitochondrial mechanism of ROS production has also been reported (7, 103, 173, 659).

The erythropoietin gene is controlled by the transcription factor hypoxia-inducible factor 1 (HIF-1) (608) (as illustrated schematically in Fig. 6). HIF-1 is a heterodimeric protein composed of the subunits HIF-1$\alpha$ and HIF-1$\beta$ (Fig. 6). The HIF-1$\alpha$ and HIF-1$\beta$ genes are constitutively expressed, and changes in oxygen tension fail to affect the concentration of the HIF-1$\beta$ subunit. In contrast, under normoxic conditions, HIF-1$\alpha$ is rapidly degraded by proteasomes in an ROS-dependent manner (265). Hypoxia decreases the ROS-mediated degradation of HIF-1$\alpha$ and enhances thereby the formation of the heterodimeric complex (Fig. 6) (510, 659).

HIF-1$\alpha$ and HIF-1$\beta$ mRNAs are expressed in most if not all human and rodent tissues (510). The number of target genes activated by HIF-1 continues to increase, and targets include genes whose protein products are involved in angiogenesis, energy metabolism, erythropoiesis, cell proliferation and viability, vascular remodeling, and vasomotor responses (reviewed in Ref. 510).

The same transcription factor is now known to control the production of a variety of hypoxia-regulated hor-
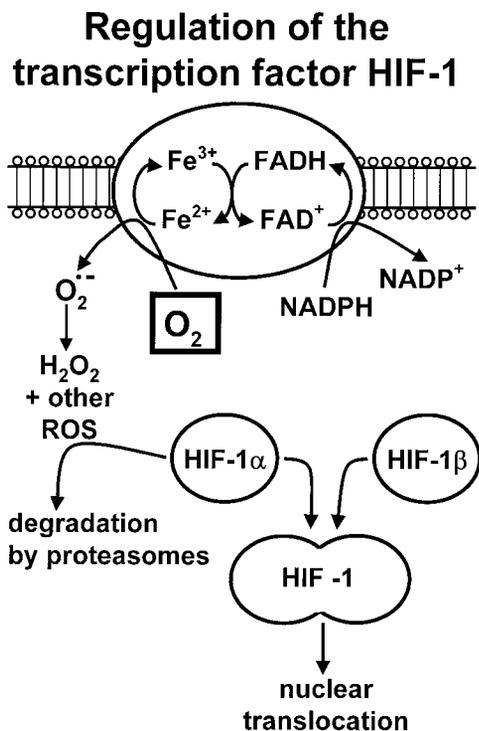


FIG. 6. Regulation of the transcription factor hypoxia-inducible factor 1 (HIF-1). HIF-1 is a heterodimeric protein composed of the subunits HIF-1$\alpha$ and HIF-1$\beta$, the genes of which are both constitutively expressed. Changes in oxygen tension fail to affect the concentration of HIF-1$\beta$, whereas HIF-1$\alpha$ is rapidly degraded under normoxic conditions by proteasomes in a ROS-dependent fashion. In these cells, ROS production is tightly linked to oxygen concentrations and, therefore, serves as a sensor for oxygen tension. The figure illustrates ROS production by a membrane-bound NAD(P)H oxidase.

mones and proteins including the vascular endothelial growth factor (VEGF) that stimulates the formation of new blood vessels (80) and the tyrosine hydroxylase (TH) that facilitates the control of ventilation by the carotid body (reviewed in Refs. 510, 659).

### E. Redox Regulation of Cell Adhesion

Controlled changes in the adhesive properties of cells and tissues play an important role in many biological processes. Adhesion of leukocytes to endothelial cells in postcapillary venules, for example, is an early step in chronic inflammation and depends on the expression of cell-surface receptors known as cell adhesion molecules (17). Cell adhesion molecules are also implicated in embryogenesis, cell growth, differentiation, and wound repair (186). The expression of cell adhesion molecules is stimulated by bacterial lipopolysaccharides and by various cytokines such as TNF, interleukin-1$\alpha$, and interleukin-1$\beta$ (17).

The adherence of leukocytes to endothelial cells is also induced by ROS (478, 509). This effect is abolished by catalase but not by superoxide dismutase, suggesting that

hydrogen peroxide and not superoxide is the effective agent (509). Moreover, the oxidant-induced adherence of neutrophils is inhibited by hydroxyl radical scavengers or iron chelators, suggesting that the induction of adherence may be mediated by hydroxyl radicals generated from hydrogen peroxide within the cell.

Adhesion of neutrophils to endothelial cells involves the intercellular adhesion molecule-1 (ICAM-1), CD11b/CD18, and L-selectin (509). In addition, ROS treatment of endothelial cells induces the phosphorylation of the focal adhesion kinase pp125$^{FAK}$, a cytosolic tyrosine kinase that has been implicated in the oxidant-mediated adhesion process (477, 488).

## F. Redox-Mediated Amplification of Immune Responses

Lymphocytes are the carriers of immunological specificity and, therefore, play an important role in the defense against environmental pathogens. A sophisticated combination of regulatory mechanisms ensures that even minute amounts of pathogen activate highly aggressive responses without causing major damage to host tissue. The immune response typically involves the lymphocyte receptor for antigen, receptors for costimulatory signals, and various types of cytokine (507, 516). The response is also subject to regulation by redox processes. The functional activation of T lymphocytes is strongly enhanced by ROS and/or by a shift in the intracellular glutathione redox state (248). Superoxide and/or physiologically relevant concentrations of hydrogen peroxide were shown to augment the production of interleukin-2 by antigenically or mitogenically stimulated T cells in various experimental systems (358, 472). Low micromolar concentrations of hydrogen peroxide were also shown to induce the expression of the interleukin-2 receptor in a mouse T-cell lymphoma line (358).

T-cell functions such as interleukin-2 production can be readily induced in vitro without the addition of ROS simply by exposure of T cells to relatively high concentrations of antigen or antibodies that bind to the T-cell receptor and the costimulatory receptor CD28 (352). In T cells, strong activation of the costimulatory receptor CD28 causes a significant decrease in intracellular glutathione levels and the endogenous production of hydrogen peroxide (359). An intact organism, however, is typically infected by relatively small concentrations of pathogens, and ligand concentrations for the T-cell receptor and the costimulatory CD28 receptor are expected to be suboptimal, at least in the initial phases of the infection.

Exposure of T lymphocytes to physiologically relevant concentrations of environmental ROS or other inducers of moderate oxidative stress does not bypass the requirement for signaling cascades initiated by specific cell membrane receptors, but such exposure can amplify signaling cascades after relatively weak receptor stimulation (248). Signaling cascades from the various cell membrane receptors are differentially regulated by ROS. Transcription from the interleukin-2 promoter is strongly enhanced in Jurkat T cells by exposure to 50 $\mu$M hydrogen peroxide in combination with but not without anti-CD28 ligands, indicating that the redox effect can enhance the stimulatory signal from the antigen receptor but cannot replace the signal from the CD28 costimulatory receptor. Thus hydrogen peroxide from the inflammatory environment thus appears to decrease the triggering thresholds of the antigen receptor-dependent signal cascades as illustrated schematically in Figure 7. It is often critical for the survival of the infected host that a specific immune response be induced before optimal antigen doses have accumulated.

The importance of the oxidative microenvironment in the activation of immune responses by small concentrations of antigen in vivo is exemplified by studies of the generation of cytotoxic T lymphocytes in mice after in vivo immunization with small numbers of syngeneic tumor cells or with cells expressing foreign minor histocompatibility antigens (474). The simultaneous injection of glutathione was found to enhance immunization with relatively large numbers of stimulator cells but suppress in vivo immunization by small numbers of stimulator cells (i.e., small amounts of antigen). The glutathione-dependent enhancement of the response to high doses of antigen is in line with the requirement for reducing conditions in the execution phase of the signaling cascade and for the DNA synthesis (see sect. v$K$) and suggests that this requirement may become a limiting factor under conditions of high antigen concentrations.

Last but not least, there is evidence that the intracellular redox state also modulates the immunological functions of macrophages. Hamuro et al. (230) reported that macrophages vary strongly in their release of prostaglandins, interleukin-6, and interleukin-12, depending on the intracellular content of glutathione. The balance between "reductive" and "oxidative" macrophages regulates thereby the ratio of helper T cells of type 1 versus type 2 (TH1/TH2).

## G. Role of ROS in Programmed Cell Death

### 1. Induction and execution of apoptosis

Apoptosis is a special form of programmed cell death that plays an indispensable role in the development and homeostasis of multicellular organisms (636). An increase in cellular ROS production is often observed in apoptotic processes triggered by various stimuli including APO-1/Fas/CD95 ligands (41, 171, 257, 287, 317, 588, 623, 655). However, some authors found that triggering of the APO-1/Fas/CD95 receptor does not induce ROS production (268), and some authors observed membrane changes typical of apoptosis in the absence of ROS (96, 281), indicating that pro-oxidative conditions are not a general prerequisite for apoptotic cell

# Function of ROS in the immunological response against environmental pathogens
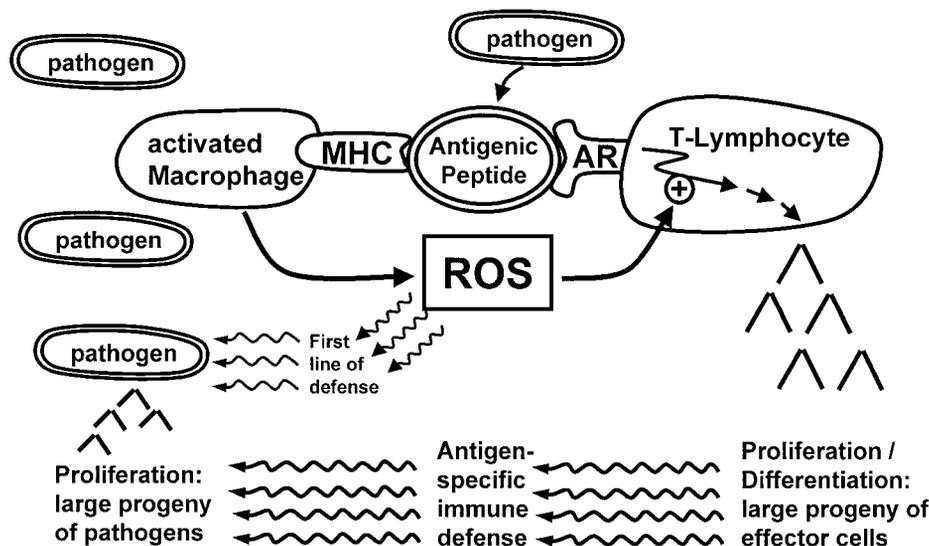


FIG. 7. Functions of ROS in the immunological response against environmental pathogens. The massive production of ROS (oxidative burst) by activated macrophages in the inflammatory environment provides a first line of defense against environmental pathogens. A certain fraction of pathogens, however, may escape this rapid but moderately effective manifestation of "innate immunity" and may generate within a few days a large progeny of pathogens. Antigenic peptides generated within the activated macrophages by the breakdown of pathogens are presented by major histocompatibility complex (MHC) determinants to the antigen receptors (AR) of T lymphocytes. This interaction triggers the proliferation and differentiation of the T cells and leads within a few days to a large progeny of immunological effector cells. The effector cells provide a highly effective and antigen-specific immunological defense. ROS that are concomitantly produced by the activated macrophages in the inflammatory environment enhance the AR-mediated signal cascades and decrease thereby the activation threshold of the T cells. Without this effect, the T lymphocytes would require relatively large concentrations of antigenic peptides and would lose valuable time in their "race" with the proliferating pathogens. In this situation time may be a matter of life or death for the organism.

death. Nevertheless, high ROS concentrations induce apoptotic cell death in various cell types (reviewed in Refs. 162, 535), suggesting that ROS contribute to cell death whenever they are generated in the context of the apoptotic process. Exposure of T lymphocytes to relatively moderate concentrations of hydrogen peroxide was found to induce a CD95-independent apoptotic process that requires mitochondrial ROS production and the activation of NF-$\kappa$B (162). This underscores the need to transfer lymphocytes to more reducing conditions for the development of immunological effector functions (see sect. v$K$).

## 2. NO-dependent apoptosis

NO-dependent apoptosis has been observed in several experimental models and certain clinical pathologies (10, 18, 76, 132, 575). Induction of apoptosis by NO is associated with a decrease in the concentration of cardiolipin, decreased activity of the mitochondrial electron transport chain, and release of mitochondrial cytochrome $c$ into the cytosol (589, 592). However, some cell types, such as endothelial cells from the microvasculature, are extremely resistant to the induction of apoptosis by NO (357), and low concentrations of NO provide protection

from apoptotic cell death in various cell types by inhibiting certain caspases (113, 304, 349, 479). High intracellular glutathione levels are associated with increased resistance to NO-mediated apoptosis (589).

## 3. Induction of cell death by TNF-$\alpha$

TNF-$\alpha$ induces cell death in many types of tumor cells and has been used in model systems for studies of the molecular mechanisms of cell death. In transformed cell lines TNF-$\alpha$ induces endogenous ROS production by mitochondria (423, 504). Whether and how these ROS contribute to the induction of cell death depends on the signaling and execution pathways that are activated (145). In leukocytes and fibroblasts, TNF-$\alpha$ induces the release of superoxide by the activation of membrane-bound NADPH oxidases. This process induces proliferation or cell death depending on the condition of the ROS-producing cell (249, 310, 387, 504, 515).

## H. Regulatory Role of ROS in Plants

The attempted infection of plants by an avirulent pathogen was found to induce a protective "hypersensi-

tive response" (HR) that involves the generation of superoxide and hydrogen peroxide (331). These ROS induce a number of defense genes or drive cells into apoptosis (279, 331, 347). A study of the infection of *Arabidopsis* leaves by avirulent *Pseudomonas syringae* revealed that the local primary oxidative burst leads to subsequent secondary microbursts in distant leaves and contributes thereby to the development of a state of systemic acquired resistance (SAR) (21). Activation of SAR is associated with the expression of several gene families, i.e., the pathogenesis-related (PR) genes (587). Salicylic acid acts as a signaling molecule in the system (617). The receptor for salicylic acid has catalase activity (105). Binding of salicylic acid causes the downregulation of catalase activity and leads thereby to a significant increase in the intracellular hydrogen peroxide concentration at sites distant to the original insult (105). The distant accumulation of (secondary) low levels of hydrogen peroxide induces the expression of various defense genes and gene products including chitinase and peroxidase (633). Two stress MAPKs of *Arabidopsis*, i.e., AtMPK3 and AtMPK6, are activated by hydrogen peroxide through the MAPK kinase ANP1 (318).

## I. Key Messages From Section IV

Numerous physiological functions are controlled by redox-responsive signaling pathways. These cases of redox regulation typically involve the regulated production of NO or ROS by NOS or NAD(P)H oxidase, respectively, and the effects of these compounds on specific signaling cascades.

The redox-sensitive target molecules of these signaling cascades and their chemical modification by oxidative agents are, in most cases, not yet well understood. A few well-studied examples show that for a given redox-responsive signaling protein NO or ROS may induce a gain of function, a loss of function, or a switch from one function to another.

The surprisingly large number of NAD(P)H oxidase isoforms and NO synthetases is by itself a strong indication for the physiological relevance of redox signaling in biological regulation.

A particularly active field of research deals with the role of ROS in the regulation of cardiovascular functions. Practically all cell types in the vascular wall produce ROS. Upon stimulation by growth factors or cytokines, vascular NAD(P)H oxidases produce superoxide and other ROS that activate multiple intracellular signaling pathways. Thus ROS play a decisive role in the normal functioning of cardiac and vascular cells (211, 559).

The regulation of vascular tone by cGMP is a special case. The enzyme guanylate cyclase was found to be activated by both NO and hydrogen peroxide (272, 394,

615, 632). This effect of hydrogen peroxide is believed to play a role in the oxygen-dependent regulation of cGMP levels and vascular contraction.

Other responses to changes in oxygen tension include the control of ventilation by the carotid body and the regulated production of certain hormones such as erythropoietin, VEGF, and insulin-like growth factor II (IGF-II). All of these hormones are under transcriptional control of the transcription factor HIF-1 (510, 659).

One of the well-studied cell types with respect to redox-responsive signaling cascades is the lymphoid cell. The redox sensitivity of antigen-induced signaling cascades strongly suggests that massive ROS production by phagocytic cells in the inflammatory environment (i.e., the oxidative burst) serves not only as a first line of defense against environmental pathogens but also enhances the response of lymphocytes to (small amounts of) antigen.

## V. REDOX-SENSITIVE TARGETS IN SIGNALING CASCADES

### A. Role of ROS in Receptor-Mediated Signaling Pathways: the EGF Receptor as a Case in Point

There are various examples of growth factors, cytokines, or other ligands that trigger ROS production in nonphagocytic cells through their corresponding membrane receptors (see sect. IV*A3*). Such ROS production can mediate a positive feedback effect on signal transduction from these receptors since intracellular signaling is often enhanced by ROS or by a pro-oxidative shift of the intracellular thiol/disulfide redox state, as illustrated in Figure 8 (36, 39, 50, 115, 301, 354, 355, 525, 554, 557, 558, 571, 606, 635). The molecular details of the oxidative enhancement are not entirely clear. However, work on different receptors and signaling pathways revealed certain consistent patterns that have important physiological implications.

For example, the role of ROS has been demonstrated for nerve growth factor (NGF) signaling in neuronal cells (558), for epidermal growth factor (EGF) signaling in human epidermoid carcinoma cells (36), and for PDGF (37, 246). Stimulation by any of these growth factors results in a transient increase in intracellular ROS through the signaling protein Rac1. Elimination of hydrogen peroxide by catalase was shown to inhibit EGF- and NGF-induced tyrosine phosphorylation of various cellular proteins, including phosphorylation of the growth factor receptor itself (see Fig. 8). In the case of the EGF receptor, induction of ROS production was reported to require the kinase activity of the receptor but not the phosphorylation of the four autophosphorylation sites at the COOH terminus of the EGF

# Role of ROS in
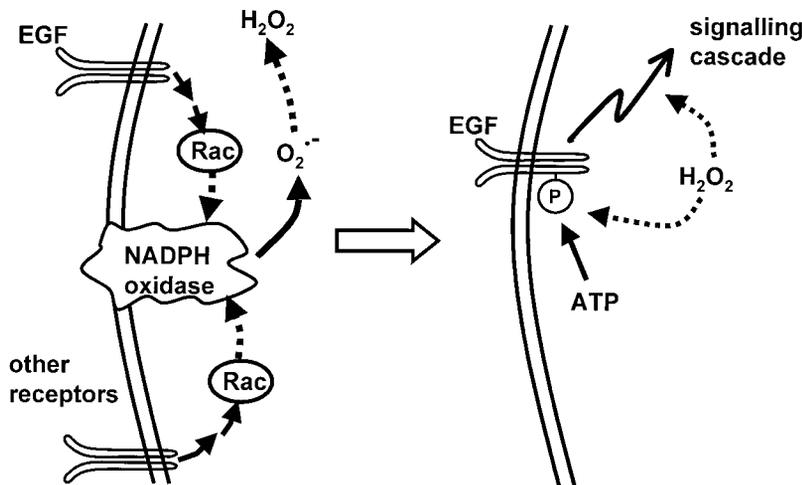# EGF receptor-mediated signalling



FIG. 8. Role of ROS in epidermal growth factor (EGF) receptor-mediated signaling. The interaction of the EGF receptor or other related membrane receptors with corresponding ligands leads to activation of NAD(P)H oxidase and the production of superoxide and hydrogen peroxide. Hydrogen peroxide, in turn, facilitates the autophosphorylation of the membrane receptor and the induction of the signal cascade. The activation of NAD(P)H oxidase by other membrane receptors such as the angiotensin II receptor can provide a cooperative effect that contributes to the autophosphorylation of the EGF receptor and to the activation of the EGF-dependent signaling cascade.

receptor (36). This unexpected finding remains to be confirmed. In view of the many growth factors, cytokines, or other ligands that trigger endogenous production of ROS, there is a strong possibility that the redox dependency of the signal transduction process may facilitate synergistic interactions between different types of membrane receptors, as illustrated in Figure 8. This physiologically advantageous cooperativity between different receptors may have been a major driving force in the phylogenetic evolution of the rather complex mechanism of redox regulation. The enhancing effect of ROS on tyrosine phosphorylation and catalytic activation, as exemplified by the EGF receptor, applies in a similar manner to various other protein components of intracellular signaling pathways.

A particularly well-studied case of cooperativity is the interaction between the angiotensin II type 1 receptor and the EGF receptor or PDGF-$\beta$ receptor. The angiotensin II type 1 receptor is a G protein-coupled receptor that mediates growth effects in vascular smooth muscle cells, cardiomyocytes, and cardiac fibroblasts. In addition, this receptor was shown to mediate responses that are normally activated by tyrosine kinase-linked receptors as exemplified by those for EGF and PDGF. The transactivation of these two growth factor receptors by angiotensin II is mediated by ROS and inhibited by antioxidants such as *N*-acetylcysteine (212, 246, 458, 607). Preliminary evidence suggests that angiotensin II-mediated transactivation of the EGF receptor involves the intermittant activation of the Src family kinase p60$^{c-src}$ by hydrogen peroxide (591), which can directly activate p60$^{c-src}$ in mouse fibroblasts (4; see sect. v*D*).

## B. Enhancement of Signaling Cascades by Oxidative Inhibition of Protein Tyrosine Phosphatases

Exposure to high concentrations of hydrogen peroxide on the order of 1 mM or strong pro-oxidative changes in the intracellular thiol/disulfide redox state will generally lead to increased tyrosine phosphorylation in numerous proteins (232, 409, 491–493, 541). This effect is to some extent, albeit not exclusively, the consequence of the oxidative inhibition of protein tyrosine phosphatases. Massive inhibition associated with increased net phosphorylation of receptor tyrosine kinases is induced by various types of strong oxidative stress, including high doses of ROS, UV irradiation, or alkylating agents (43, 44, 139, 217, 245, 251, 313, 555). Protein tyrosine phosphatases counteract the effect of protein tyrosine kinases and reset membrane receptors after ligand-induced autophosphorylation. The EGF receptor, for example, is normally dephosphorylated at all tyrosine residues in <1 min after ligand-induced autophosphorylation (313), but this dephosphorylation is retarded by high concentrations of hydrogen peroxide on the order of 1 mM or other inducers of oxidative stress. A protein tyrosine phosphatase was also shown to regulate the activation of the EGF receptor (454).

All protein tyrosine phosphatases share a common sequence motif with a catalytically essential cysteine residue in the active center (42). Biochemical evidence suggests that inhibition of catalytic activity may proceed in either of two ways (Fig. 8). Hydrogen peroxide at concentrations on the order of 1 mM converts this cysteine residue into cysteine sulfenic acid (Cys-SOH) and thereby

inactivates the enzyme, as demonstrated with three distinct protein tyrosine phosphatases (139). This effect is facilitated by vanadate and its derivative pervanadate. Alternatively, the redox-sensitive cysteine residue may be converted by glutathione disulfide into a mixed disulfide with concomitant loss of catalytic activity (43). Cysteine sulfenic acids are highly reactive and are expected to react with glutathione at its relatively high intracellular concentration. Therefore, it is reasonable to assume that ROS-induced oxidation will also lead to the rapid glutathionylation of the redox-sensitive cysteine moiety (Fig. 9). Collectively, these findings indicate that protein tyrosine phosphatase is a typical example of a regulatory protein that responds either to changes in ROS concentrations or changes in the intracellular thiol/disulfide redox state, as was also found in the case of the bacterial OxyR protein (see Table 2). The cysteine-to-serine mutation at the catalytic site inactivates catalytic activity but not substrate binding activity of the phosphatases (182, 191, 285, 556). The physiological relevance of the oxidative inhibition of the tyrosine phosphatases, however, is still controversial in view of the relatively high ROS concentrations that are typically required to inhibit the tyrosine phosphatases.

## C. Role of ROS in the Regulation of Insulin Receptor Kinase Activity

The most important insulin-responsive tissues are liver, skeletal muscle, and adipose tissue. In these tissues insulin controls several physiologically important functions, including the rate of glucose uptake, intracellular glucose metabolism, lipid metabolism, and the synthesis of proteins at the transcriptional and translational level (421).

Signaling by insulin requires autophosphorylation of the insulin receptor kinase at Tyr-1158, Tyr-1162, and Tyr-1163 (146, 184, 266, 471, 612, 616). In intact cells high concentrations of hydrogen peroxide on the order of 1 mM pervanadate and thiol-reactive agents induce insulin-like effects in the absence of insulin (121, 175, 240, 247, 564, 620). Whenever tested, these effects were found to involve the insulin-independent tyrosine phosphorylation of the insulin receptor $\beta$-chain (175, 247, 564). In view of the marked inhibition of protein tyrosine phosphatases by hydrogen peroxide at concentrations on the order of 1 mM (see sect. $vB$), it is likely that activation of the insulin receptor by similarly high concentrations of hydrogen peroxide may be mediated, at least in part, by inhibition of tyrosine phosphatases (247).

Lower and physiologically relevant concentrations (<0.1 mM) of hydrogen peroxide are not sufficient to trigger the autophosphorylation of the insulin receptor in the absence of insulin, but do enhance the response to 100 nM insulin (496), indicating that the redox signal has a coregulatory function in insulin receptor activation under physiologically relevant conditions. This property is shared by other ROS-responsive signaling pathways. Because hydrogen peroxide production can be induced by insulin (321, 380, 403), the redox effect appears again to be part of a positive feedback regulation (see Fig. 10) that is reminiscent of the redox control of the EGF receptor. Krieger-Brauer et al. (321) reported that insulin-mediated stimulation of NADPH oxidase may not require the kinase activity of the insulin receptor. This finding remains to be confirmed. Regardless of this detail, the stimulation of ROS production through the insulin receptor and the redox sensitivity of insulin receptor kinase activity both suggest a certain degree of cooperativity between the in-

## Alternative pathways of protein tyrosine phosphatase inhibition by ROS or by changes in the thiol/disulfide redox state
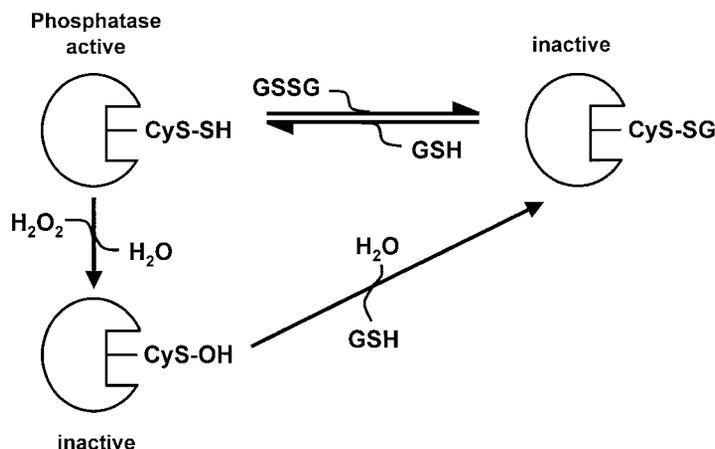


FIG. 9. Alternative pathways of protein tyrosine phosphatase inhibition by ROS or by changes in the thiol/disulfide redox state. A cysteine residue in the catalytic site of the phosphatase is critical for its catalytic activity. Inactivation can occur either by reaction with hydrogen peroxide to form a sulfenic acid derivative or by reaction with glutathione disulfide, resulting in glutathiolation of the critical cysteine residue. Reactivation may occur by reaction with reduced glutathione or other thiol compounds.

# Role of ROS in insulin receptor kinase activation

sulin receptor and other membrane receptors, as illustrated in the case of the EGF receptor (Fig. 8).

A combination of molecular modeling and functional studies indicated that the ROS-mediated enhancement of insulin receptor autophosphorylation involves cysteine residues within the receptor kinase domain itself (495, 496). The crystal structures of several protein kinase species, including the three times phosphorylated insulin receptor kinase domain (IRK-3P) (266), cAPK (656), Lck (643), c-Src (642, 622), Hck (523), and FGFRK (395), show structurally similar kinase domains with similar ATP binding sites. The crystal structure of the nonphosphorylated insulin receptor kinase domain (IRK-0P) differs from other kinase structures and exhibits an atypical position of the activation loop, which prevents productive ATP binding (267). However, autophosphorylation can be mediated through binding of phosphate donors to different binding sites in a process requiring the synergistic action of hydrogen peroxide. Three-dimensional models of the nonphosphorylated insulin receptor kinase domain revealed that conversion of any of the four cysteine residues 1056, 1138, 1234, and 1245 into sulfenic acid results in structural changes that render the well-known catalytic site at Asp-1132 and Tyr-1162 accessible for a phosphate donor from a direction different from that of the known ATP binding site. In addition, Tyr-1158 is brought into close contact with Asp-1083, suggesting that Tyr-1158 is using Asp-1083 rather than Asp-1132 as the catalytic amino acid for its autophosphorylation (495). Conversion of one of the four cysteine residues 1056, 1138, 1234, or 1245 into an S-nitroso derivative, i.e., the putative product after reaction with NO, leads to similar structural changes. Functional studies showed, however, that insulin-induced autophosphorylation of the insulin receptor $\beta$-chain is strongly inhibited by various NO donors (496). This effect can be tentatively explained by the assumption that S-ni-

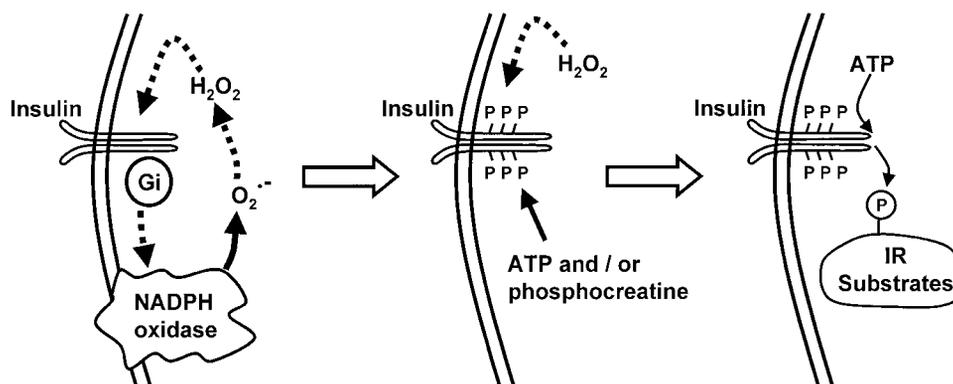trosylation prevents the further processing needed for activation of the insulin receptor domain. Taken together, these findings suggest that the apparent redundancy of functionally relevant cysteine residues in distinct regions of the insulin receptor protein may have resulted from an evolutionary process that increased the probability of a regulatory interaction with ROS.

As in the case of several other signaling processes (see Table 2), insulin responsiveness of insulin receptor kinase activity is amplified not only by exposure to ROS but also by a pro-oxidative shift in the intracellular glutathione redox state (494). Antioxidants such as butylated hydroxyanisol or the glutathione precursor N-acetylcysteine were found to inhibit the induction of insulin receptor kinase activity (494).

A decrease in intracellular glutathione levels resulting from treatment with buthionine sulfoximine has by itself little or no effect on insulin receptor activation but augments the effect of moderate concentrations of hydrogen peroxide (E. Schmid and W. Dröge, unpublished observations) or the stimulatory effect of the glutathione reductase inhibitor 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (494). Higher concentrations of hydrogen peroxide, however, tend to inhibit rather than enhance insulin receptor signaling and autophosphorylation (231; Schmid and Dröge, unpublished observations). With the use of glucose oxidase, it was also shown that prolonged exposure to moderate concentrations of hydrogen peroxide decreases insulin receptor signaling (576).

## D. Activation of Cytoplasmic Protein Kinases by ROS

In line with the strong inhibition of protein tyrosine phosphatases by hydrogen peroxide at high concentra-

tions on the order of 1 mM, several authors reported a massive increase in tyrosine phosphorylation of various cellular proteins under similar highly oxidative conditions (232, 409, 491–493). The activity of several protein tyrosine kinases such as Lck, Fyn, Syk, and ZAP70 is enhanced under such conditions (75, 239, 409, 499–501). The physiological relevance of these strongly oxidizing conditions is still unclear. At concentrations of <0.2 mM hydrogen peroxide or after moderate sulfhydryl oxidation, the increase in phosphorylation is restricted to one or a few prominent proteins (409). Exposure of T cells to 0.15 or 0.5 mM hydrogen peroxide yields a single major phosphorylated protein, the Src family protein tyrosine kinase $p56^{lck}$. This phosphorylation is associated with the induction of $p56^{lck}$ kinase activity (409). Analogous to this finding, hydrogen peroxide was found to stimulate JAK2, a critical mediator for the activation of the Ras/Raf/ERK pathway, in normal murine fibroblasts, in fibroblasts derived from transgenic mice deficient in $p60^{src}$, but not in fibroblasts deficient in $p59^{fyn}$ (3). Moreover, Ras activation by hydrogen peroxide was found to be impaired in $p59^{fyn}$-deficient cells but not in $p60^{src}$-deficient cells. Studies on a human T cell line showed that the MAPK species c-Jun $NH_2$-terminal kinase (JNK) and p38 are activated by mild intracellular thiol oxidation in a tyrosine kinase-dependent manner (248). It was also demonstrated that the kinase activity of $p59^{fyn}$ is activated by low micromolar concentrations of glutathione disulfide and that the Src family kinases $p59^{fyn}$ and $p56^{lck}$ are strongly activated by a mild oxidative shift of the intracellular thiol/disulfide redox state (248). Analogous findings were obtained in studies of rat vascular smooth muscle cells and fibroblasts where the protein tyrosine kinase Src was found to be critically involved in JNK activation by 0.03 or 0.1 mM hydrogen peroxide (651). The molecular details of the activation of Src family kinases by mildly oxidizing conditions, however, are not clearly understood.

### E. Oxidative Activation of MAPK Cascades

MAPK signaling cascades are regulated by phosphorylation and dephosphorylation on serine and/or threonine residues and respond to activation of receptor tyrosine kinases, protein tyrosine kinases, receptors of cytokines and growth factors, and heterotrimeric G protein-coupled receptors. Numerous studies with various experimental systems show that in particular the MAPK species JNK and p38 are strongly activated by ROS or by a mild oxidative shift of the intracellular thiol/disulfide redox state (2, 20, 222, 248, 355). The extracellular signal-regulated kinase 1 (ERK-1) and ERK-2 were found to be activated in vascular smooth muscle cells by superoxide but not by hydrogen peroxide (35). Angiotensin II induces the induction of superoxide and hydrogen peroxide and

activates ERK-1, ERK-2, and p38 MAPK (2, 590). PDGF was found to induce the activation of ERK-1 and ERK-2 (557). JNK and p38 MAPK were shown to be activated by hydrogen peroxide in perfused rat hearts (112). As described in section vD, the redox sensitivity of JNK and p38 MAPK is mediated at least to some extent by the oxidative activation of upstream tyrosine kinases of the Src family (452).

In addition, the JNK/glutathione S-transferase $P_i$ (GSTp) complex has also been identified as a redox-responsive signaling element. In normal growing cells of the mouse fibroblast cell line 3T3–4A, JNK is associated with and catalytically inhibited by glutathione-S-transferase (GSTp) (12). Complex formation between GSTp and JNK limits the degree of Jun phosphorylation under nonstressed conditions. Exposure to low micromolar hydrogen peroxide concentrations causes the oligomerization of GSTp and the dissociation of the GSTp-JNK complex, indicating that JNK inhibition requires monomeric GSTp.

The apoptosis signaling-regulating kinase 1 (ASK1) plays a role in the activation of MKK3/6, MKK4/MKK7, and the MAPK species p38 and JNK (270). This leads ultimately to the phosphorylation of ATF2, c-Jun, and p53 (11, 189, 275). Screening for ASK1-associated proteins has led to the identification of Trx as the redox-sensitive target molecule (481). Under normal conditions, Trx binds to the $NH_2$-terminal domain of ASK1 and inhibits its kinase activity. Deletion of the Trx-binding $NH_2$-terminal residues of ASK1 renders it constitutively active and no longer responsive to the inhibitory effect of Trx. ROS induce the dimerization of Trx and its dissociation from ASK1, followed by multimerization of ASK1 and activation of its kinase activity (205, 353).

### F. Oxidative Activation of Protein Kinase C Isoforms

The serine/threonine kinase protein kinase C (PKC)-$\alpha$ is involved in signal transduction to various effector pathways that regulate transcription and cell cycle control. Certain PKC isoforms, including PKC-$\alpha$ are typically activated by the lipid second messenger diacylglycerol (416, 430). This effect is mimicked by phorbol esters (57). The binding site for diacylglycerol or phorbol esters is located in a conserved cysteine-rich region within the $NH_2$-terminal C1 domain (83, 430, 455, 457).

Alternatively, PKC-$\alpha$ and certain other PKC isoforms can be activated by hydrogen peroxide in a phospholipid-independent process that involves tyrosine phosphorylation in the catalytic domain (204, 316). The oxidative activation of PKC-$\alpha$ is significantly enhanced by vitamin A and certain derivatives such as 14-hydroxy-retroretinol which bind to a zinc finger domain in the conserved

cysteine-rich region of the regulatory domain (263). The serine/threonine kinase cRaf contains an analogous cysteine-rich domain that binds phosphatidylserine instead of diacylgylcerol (185). Like PKC-$\alpha$, cRaf is also activated by ROS in a retinol-dependent fashion (263, 274). Vitamin A or its derivatives 14-hydroxy-retroretinol and 13,14-dihydroxyretinol are strictly required for lymphoid cell growth and viability (78, 141, 193, 627).

$\alpha$-Tocopherol (vitamin E), in contrast, was found to inhibit the translocation of PKC into the membrane and the phosphosrylation of its 80-kDa protein substrate in smooth muscle cells (34, 62). This effect may be involved in the $\alpha$-tocopherol-mediated inhibition of vascular smooth mucle cell proliferation (62) and of NF-$\kappa$B activation in Jurkat T cells (561). RRR-$\beta$-tocopherol, in contrast, does not inhibit PKC activation but prevents the effect of RRR-$\alpha$-tocopherol (34).

## G. ROS-Induced Changes in Cytosolic Ca$^{2+}$ Concentrations

Changes in the cytosolic Ca$^{2+}$ level play a role in the modulation of several intracellular signal pathways, including PKC-$\alpha$ and calmodulin-dependent signal pathways (111). These pathways have also been implicated in apoptotic processes. The cytosolic Ca$^{2+}$ level can be increased by ROS in various cell types through the mobilization of intracellular Ca$^{2+}$ stores and/or through the influx of extracellular Ca$^{2+}$ (150, 155, 226, 327, 427–429, 476). The ROS-mediated increase in the cytosolic Ca$^{2+}$ concentration contributes to the oxidative stress-mediated activation of PKC-$\alpha$ (340) and to the transcriptional induction of the AP-1 proteins c-Fos and c-Jun (118, 367, 520).

## H. Activation of the Transcription Factor AP-1

The transcription factor AP-1 is typically composed of c-Fos and c-Jun proteins and is implicated in differentiation processes. In T lymphocytes AP-1 is involved in the expression of the interleukin-2 gene and other immunologically relevant genes (372, 552). Many different oxidative stress-inducing stimuli, e.g., relatively low concentrations of hydrogen peroxide, UV light, $\gamma$-irradiation, and interleukin-1, lead to AP-1 activation by different mechanisms (23, 144, 389, 419, 548).

The mRNAs of c-Fos and c-Jun are induced by relatively small amounts of hydrogen peroxide, superoxide, NO, and other inducers of oxidative stress (117, 283, 389, 401, 402, 419, 520). If Jurkat T lymphocytes are treated with 200 $\mu$M hydrogen peroxide in the absence of a mitogenic stimulus, expression of c-Jun and transient expression of c-Fos are enhanced without subsequent induction of interleukin-2 production (52). In contrast,

treatment of the murine T cell line ESb with 200 $\mu$M hydrogen peroxide causes an increase in AP-1 transcription factor activity and the expression of c-Jun and interleukin-2 (358). The oxidative activation of AP-1 transcription factor activity is based on oxidative activation of JNK. This MAPK phosphorylates serine residues 63 and 73 of the NH$_2$-terminal transactivation domain of c-Jun, a domain which is required for functional activation (295, 297).

As for the redox-sensitive bacterial OxyR protein (see sect. IIIB2) and activation of the human insulin receptor kinase (see sect. VC), the activation of AP-1 and its upstream signaling cascades can also be enhanced by a change in the intracellular glutathione redox state (192, 248). A mild oxidative shift of the redox state by three different oxidants resulted in a strong enhancement of JNK and p38 MAPK activity but not so for ERK-1 and ERK-2 (248). JNK and p38 MAPK are also activated by UVA irradiation and by singlet oxygen (311, 312). High concentrations of hydrogen peroxide ($\sim$1 mM) also result in the activation of ERK-1 and ERK-2 in Jurkat T cells and in cardiac myocytes (14, 213). The differential activation of JNK, p38 MAPK, and ERK by various types of ROS, or RNS or by UV irradiation in different cell types has been reviewed by Klotz et al. (312).

## I. Activation of the Transcription Factor NF-$\kappa$B

NF-$\kappa$B is involved in the induced expression of the interleukin-2 gene and in a wide variety of biological responses. In particular, it is implicated in inflammatory reactions, growth control, and apoptosis (38, 39) and is the first eukaryotic transcription factor shown to respond directly to oxidative stress in certain types of cells (501). NF-$\kappa$B activation was shown to be inhibited by antioxidants such as cysteine (389, 391, 490, 505, 542). In various cell types such as the Würzburg subclone of T cells, L6 skeletal muscle myotubes, human breast MCF-7 cells, and 70 Z/3 pre-B cells, NF-$\kappa$B is activated by micromolar concentrations of hydrogen peroxide (368, 391, 501, 502, 512). A moderate pro-oxidative shift in the glutathione redox state was also found to enhance NF-$\kappa$B activation in Molt-4 and Jurkat T cell lines (192, 248). However, NF-$\kappa$B activation is not strictly dependent on ROS. Several signal cascades have recently been identified that can activate NF-$\kappa$B without involvement of ROS, and hydrogen peroxide does not induce NF-$\kappa$B activation in all cell types (65, 66, 350, 560). In Molt-4 cells, NF-$\kappa$B activity was found to be enhanced by 30 $\mu$M hydrogen peroxide if cultured in NCTC 135 medium, i.e., a culture medium with relatively low concentrations of free amino acids, but not in RPMI 1640 medium (160).

Taken together, the large body of experimental evidence now suggests that ROS comodulates the activation

of NF-$\kappa$B in certain cell types under certain conditions. At least two mechanisms contribute to this effect. The first one involves the ROS-mediated enhancement of I$\kappa$B degradation. Treatment of HeLa cells with hydrogen peroxide induces the appearance of a modified form of the inhibitor I$\kappa$B$\alpha$ which is rapidly degraded upon appropriate stimulation unless proteolysis is inhibited by a proteasome inhibitor (319). Treatment with antioxidants was found to block I$\kappa$B$\alpha$ degradation after stimulation with TNF, TPA, or lipopolysaccharide (53, 320, 368, 580). Also, overexpression of glutathione peroxidase was found to abolish the TNF-mediated accumulation of the modified form of I$\kappa$B$\alpha$ (320), and treatment with the antioxidant $N$-acetyl-cysteine was shown to inhibit NF-$\kappa$B activation and I$\kappa$B$\alpha$ degradation but not I$\kappa$B$\alpha$ phosphorylation (350). In view of the role of ROS in the regulation of proteosome-mediated protein catabolism (see sect. IIC5), it is reasonable to assume that the illusive mechanism of the redox control of NF-$\kappa$B is mediated at least partly by the more general mechanism of ROS-mediated enhancement of proteolysis according to the model in Figure 2. A second mechanism involves the oxidative enhancement of upstream signal cascades. Increased phosphorylation of I$\kappa$B$\alpha$ was found in EL4 T cells after exposure to 300 $\mu$M hydrogen peroxide (499), and a similar induction of I$\kappa$B$\alpha$ phosphorylation and activation of the I$\kappa$B kinase IKK$\alpha$ was seen in Jurkat T cells after a moderate pro-oxidative shift in the intracellular glutathione redox state (248).

## J. Importance of the Intracellular Glutathione Level

In all cases examined, the redox regulation of membrane receptor-dependent signals was found to be strongly dependent on the intracellular glutathione level. The enhancement of insulin receptor kinase activation by inhibition of glutathione biosynthesis (see sect. VC) is an example. Similarly, interleukin-2 production by resting small T cells of the mouse was found to be enhanced by superoxide or hydrogen peroxide only if the cells were exposed simultaneously to physiologically relevant millimolar concentrations of L-lactate (472), a macrophage product (33) known to decrease intracellular glutathione levels in lymphocytes (473, 475). Because the lactate derivative pyruvate is an effective scavenger of the glutathione precursor cysteine (159, 286) and because the addition of glutathione abolishes the lactate effect (475), it is reasonable to assume that the lactate-dependent enhancement of interleukin-2 production is due to glutathione depletion.

The biosynthesis of glutathione is mainly determined by the concentrations of the precursor amino acids (465) and competes with protein synthesis for the available amino acids. A redox mechanism that links receptor-mediated signals to low intracellular glutathione levels favors therefore those cells that are engaged in highly active protein synthesis.

## K. Differential Redox Requirements in the Induction and Execution of Signal Cascades

### 1. Signal amplification by a late shift to reducing conditions

Whereas the induction of signaling cascades leading to the activation of the transcription factors NF-$\kappa$B and AP-1 is typically enhanced by pro-oxidative conditions, the final execution of the signaling process requires relatively reducing conditions. To induce the expression of specific genes, the transcription factors must bind to specific sequences of highly acidic, negatively charged DNA. Therefore, the binding region of the transcription factors typically contains an accumulation of positively charged amino acids (reviewed in Ref. 160). This accumulation of positive charges can stabilize deprotonated thiol groups, making any cysteine group in the DNA-binding region highly susceptible to oxidation. For example, the transcription factor NF-$\kappa$B and other members of the NF-$\kappa$B family share a characteristic sequence motif with one cysteine and three arginine residues in the DNA-binding region (198, 302, 326, 374). The DNA-binding activity of NF-$\kappa$B is, therefore, easily inhibited in vitro by oxidation and reactivated by thiols such as 2-mercaptoethanol, dithiothreitol, and thioredoxin (Trx) (192, 577). Physiologically relevant concentrations of glutathione disulfide inhibit DNA binding of NF-$\kappa$B, even in the presence of a severalfold excess of thiols, and DNA-binding activity was found to be restored by physiologically relevant concentrations of reduced Trx (192). NMR studies have demonstrated the direct binding of a Trx peptide to the DNA-binding domain of NF-$\kappa$B (453).

Similarly, oxidation of a conserved cysteine residue in the DNA-binding region adjacent to the leucine zipper in AP-1 abolishes its binding to DNA (637). The DNA-binding activity of AP-1 was found to be restored by Trx and the nuclear signaling protein redox factor-1 (Ref-1) (1, 637, 638) and enhanced by transient overexpression of Trx in vivo (490). Compared with NF-$\kappa$B, AP-1 is less sensitive to oxidative inhibition by glutathione disulfide in vitro but more sensitive to oxidized Trx (192). Thus, in intact cells, the elevation of intracellular oxidized glutathione (GSSG) levels by inhibition of glutathione reductase inhibits selectively the DNA-binding activity of NF-$\kappa$B and not that of AP-1 (192).

To reconcile these seemingly conflicting redox requirements for the induction and execution phases of the signaling process, a delicately balanced intermediate redox state is needed (160). The oxidative enhancement of interleukin-2 production in macrophage-depleted T-cell populations is a

case in point. Interleukin-2 production is strongly enhanced by 10 $\mu$M hydrogen peroxide but completely inhibited by higher concentrations of hydrogen peroxide (100 $\mu$M) (52, 436, 472). Analogous to the inhibition of insulin receptor signaling by excessive or prolonged ROS exposure, prolonged exposure to weak oxidative stress also suppresses anti-CD3-induced interleukin-2 production, protein tyrosine phosphorylation, and other parameters of cell activation (180). Moreover, the induction of NF-$\kappa$B in Molt-4 cells is enhanced by moderate concentrations of the glutathione reductase inhibitor BCNU but inhibited at higher concentrations (192). A study on healthy human subjects showed that persons with intermediate intracellular glutathione levels (20–30 nM/mg protein) had higher $CD4^+$ and $CD8^+$ T-cell numbers than persons with either lower or higher glutathione levels (307).

One of several mechanisms that satisfies the different redox requirements for induction and execution of redox-sensitive signal cascades is based on the redox-mediated nuclear translocation of Trx. Ionizing radiation, which causes oxidative stress, was found to increase AP-1 DNA-binding activity in Jurkat T cells and HeLa cells by increasing the nuclear level of Trx.

In addition, lymphocytes are extremely mobile, and their migration from an oxidative (i.e., inflammatory) into a more reducing environment is expected to help the cells to meet differential redox requirements during the induction and execution phases and to enhance thereby the immunological response. Experiments with cultured T cells showed that activation of AP-1 and NF-$\kappa$B transcription factors can be markedly enhanced if the cells are stimulated under relatively oxidative conditions and shifted after 1 h to more reducing conditions for optimal DNA binding (192). In the in vivo setting lymphocytes experience such a shift whenever they move out of the inflammatory environment into more reducing tissues as illustrated schematically in Figure 11. This requirement for a shift in the redox condition may be viewed as an additional fail-safe mechanism against inappropriate activation of (auto)immune reactions.

Lymphocytes require in the execution phase and for the subsequent DNA synthesis response large amounts of the glutathione precursor cysteine. Even a moderate glutathione deficiency was found to impair various immunological functions (reviewed in Ref. 160). Because lymphocytes have only weak transport activity for cystine, they take advantage of certain types of macrophages that are capable of taking up cystine and releasing large amounts of cysteine into the extracellular space (199). The importance of this process is illustrated by the fact that lamina propria macrophages lack the ability to release cysteine, thus ensuring the physiological unresponsiveness of the lamina propria T lymphocytes to antigen exposure in the intestinal microenvironment (524). The hyperresponsive-

## Distinct levels of redox regulation in the induction and execution of signal cascades
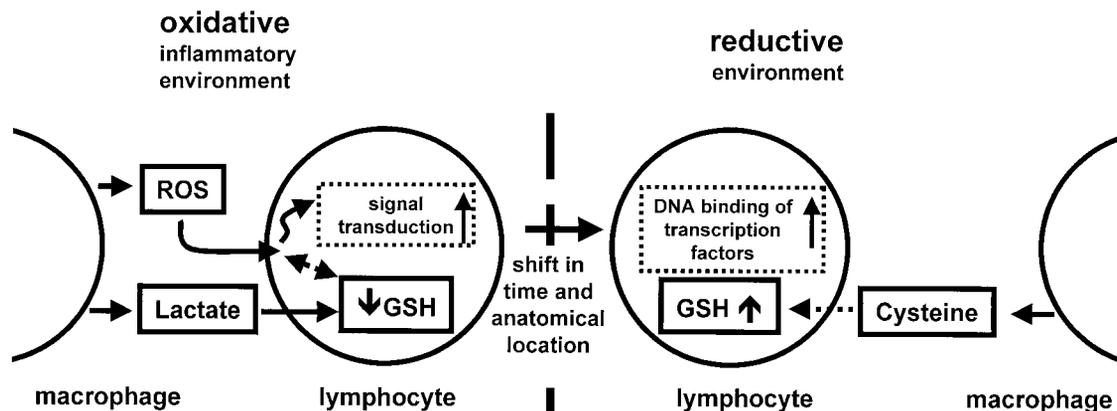


FIG. 11. Distinct redox requirements in the induction and execution of signal cascades: hypothetical model. The antigen-responsive signaling cascades of lyphocytes are strongly enhanced by the ROS produced in large quantities by macrophages in the inflammatory environment. Glycolytically active macrophages produce also large amounts of lactate, which, in turn, induces a decrease in the intracellular glutathione level of the lymphocyte. Experiments with Molt-4 cells have shown that the 4-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced activation of activator protein 1 (AP-1) and nuclear factor $\kappa$B (NF-$\kappa$B) DNA-binding activity is inhibited if the cells are subjected to reducing conditions (1 mM dithiothreitol) before TPA treatment but strongly enhanced if the cells are subjected to reducing conditions 1 h after TPA stimulation. Moreover, the proliferative response and the induction of many types of immunological responses are strongly dependent on relatively high intracellular glutathione levels. The delivery of reduced cysteine by certain types of macrophages to the lymphocytes has been shown to play an important role in the maintenance of adequate glutathione levels in lymphocytes.

ness of lamina propria T lymphocytes in patients with inflammatory bowel disease is associated with the invasion of blood monocytes into the inflamed area.

In the execution phase, pyruvate can serve also as a major endogenous scavenger for hydrogen peroxide (151, 159). Activated and cycling T cells cover a large proportion of their energy demand by glycolytic metabolism, which generates substantial amounts of pyruvate (68, 209, 609).

### 2. Induction of the cell cycle inhibitor p21 by ROS

The cell cycle inhibitor p21 (WAF1, CIP1, or sdi1) plays a central role in the control of the cell cycle by interacting with multiple targets including cyclin-dependent kinases (236, 640). ROS were shown to induce p21 gene expression by an unknown mechanism that may involve p53 (123, 369, 422). This underscores the importance of ROS scavenging mechanisms in the proliferative phase of cellular responses.

### L. Key Messages From Section v

Signal transduction from various membrane receptors is enhanced by ROS.

Membrane receptors of various growth factors, cytokines, or other ligands induce positive feedback effects on signal transduction from these receptors by triggering concomitantly the activation of NAD(P)H oxidases.

Enhancement of signal transduction from a given receptor by stimulation of ROS production through this or other receptors provides the basis for cooperativity. Because hydrogen peroxide has a relatively long half-life and can cross membranes, this cooperativity may even extend to other cells in the vicinity. In addition, the membrane receptor may function simultaneously as a sensor for extracellular signals and as a sensor for the inner metabolic state of the individual cell.

Oxidative enhancement of membrane receptor signaling and corresponding downstream signaling pathways are not well characterized at the molecular level but are likely to involve simultaneously the oxidative modification of several different redox-sensitive signaling proteins.

Certain signaling cascades involving protein tyrosine kinases can be enhanced by oxidative inhibition of protein tyrosine phosphatases. The molecular basis of this effect is relatively well-defined and involves the oxidative derivatization of a catalytically essential cysteine residue in the enzyme's active center. Therefore, tyrosine phosphatases can also be inactivated by changes in the intracellular thiol/disulfide redox state. Because most studies have been performed with relatively high concentrations of hydrogen peroxide ($\sim 1$ mM) or strong pro-oxidative changes in the intracellular thiol/disulfide redox state, the physiological relevance of this interesting mechanism needs further clarification.

Stimulation of the insulin receptor tyrosine kinase activity by insulin can be enhanced by relatively moderate (micromolar) concentrations of hydrogen peroxide or mild pro-oxidative changes in the intracellular thiol/disulfide redox state. Molecular modeling studies suggest that this redox effect may be mediated by the oxidative derivatization of any of four cysteine residues in the tyrosine kinase domain of this membrane receptor. These findings need to be confirmed by crystallographic analysis or other experimental techniques.

Signaling pathways involving JNK, p38 MAPK, and the transcription factor AP-1 are strongly responsive to redox regulation. The oxidative enhancement of Src-family tyrosine kinases and certain PKC isoforms as well as the dissociation of the JNK/GSTp and ASK1/Trx complexes may together contribute to the redox sensitivity of these pathways.

Activation of NF-$\kappa$B is another well-studied model of redox regulation. ROS or changes in the thiol/disulfide redox state are not strictly required for NF-$\kappa$B activation but induce or amplify NF-$\kappa$B activation in various cell types under various conditions. At least two different mechanisms contribute to the enhancement of NF-$\kappa$B activation. One of these mechanisms is based on the enhanced proteolytic degradation of the NF-$\kappa$B inhibitor I$\kappa$B after exposure to ROS. The second mechanism involves the increase in I$\kappa$B kinase-$\alpha$ activity after exposure to hydrogen peroxide or to pro-oxidative changes in the intracellular glutathione redox state.

The in vivo relevance of redox-sensitive signaling cascades is strongly suggested by the large number of NAD(P)H oxidase isoforms (see sect. IVA) and by the dysregulation of physiological responses in various disease-related oxidative stress conditions (see sect. VI). However, the relative contribution of individual redox-sensitive signaling proteins to redox-regulated processes in vivo is presently obscure.

The DNA-binding activity of many if not most transcription factors is sensitive to oxidative conditions. Physiological responses require, therefore, a delicate balance between the pro-oxidative conditions that are needed to strengthen the signaling cascades and the reducing conditions that are needed for the execution of these signals.

## VI. ROLE OF REACTIVE OXYGEN SPECIES IN SENESCENCE, STRESS CONDITIONS, AND DISEASE: PATHOPHYSIOLOGICAL IMPLICATIONS OF REDOX REGULATION

### A. Mediators of Excessive ROS Production

Redox-regulated physiological processes are inevitably sensitive against excessive ROS production by any

source. Such excessive levels of ROS may be generated either by excessive stimulation of the otherwise tightly regulated NAD(P)H oxidases (see sect. IV*A*) or by other mechanisms that produce ROS "accidentally" in a non-regulated fashion. These latter mechanisms include the production of ROS by the mitochondrial ETC or by xanthine oxidase.

### 1. The mitochondrial electron transport chain as a source of ROS

The mitochondrial electron transport chain (ETC) is a relatively well-investigated source of ROS. A major site for the univalent reduction of molecular oxygen to superoxide is ubisemiquinone, a component of the ETC in the mitochondrial matrix (63, 64, 86, 101, 122, 322, 360, 361, 417, 584). Practically all cells and tissues convert continuously a small proportion of oxygen into superoxide by this mechanism. If carefully deprived of contaminating superoxide dismutase, submitochondrial particles generate superoxide at a rate of $4–7$ nmol$\cdot$min$^{-1}\cdot$mg protein$^{-1}$, suggesting that the mitochondrial membrane is the quantitatively most important physiological source of superoxide in higher organisms (101). With acetone-extracted submitochondrial particles it was found that supplementation with exogenous ubiquinones led to ROS production rates that were linearly related to the amount of reducible quinone (63, 64). NADH-ubiquinone reductase and ubiquinol-cytochrome $c$ reductase, which contain ubisemiquinone as an important constitutent, were shown to generate superoxide and hydrogen peroxide (86). Mitochondrial ROS production has been implicated also in TNF-mediated oxidative stress (504, 505).

### 2. ROS production by xanthine oxidase

Xanthine oxidase generates superoxide by converting hypoxanthine into xanthine and xanthine into uric acid. The enzyme is derived from xanthine dehydrogenase by proteolytic cleavage. Under normal conditions, xanthine oxidase accounts for only a minor proportion of total ROS production (reviewed in Ref. 101). However, ROS production by xanthine oxidase has been observed in TNF-treated endothelial cells (188) and has also been implicated as a major source of oxidative stress under certain disease conditions such as ischemia and reperfusion (see sect. VI*O*).

### 3. Dopamine as a source of ROS in the central nervous system

ROS generated by oxidation of dopamine has been implicated in the aging-related destruction of dopaminergic neurons and especially in Parkinson's disease (reviewed by Luo and Roth, Ref. 365).

### 4. Other sources of ROS

In addition to the mechanisms described here, there are various other enzymic and nonenzymic mechanisms of ROS production as reviewed elsewhere (101, 122, 365, 526).

## B. The Free Radical Theory of Aging

Multicellular organisms generally undergo qualitative changes with time (aging) that are associated with progressive degeneration of biological functions, increased susceptibility to diseases, and increased probability of death within a given time period. The widely popular free radical theory of aging (234) states that the age-related degenerative process is to a large extent the consequence of free radical damage. Genetic evidence linking oxidative stress to life span has been obtained for different animal species. In *Caenorhabditis elegans*, the *daf-2* mutation causes longevity by increasing Mn-SOD expression (260). Catalase is required to extend the life span in *daf-C* and *clk-1* mutants of *C. elegans* (566), and synthetic compounds exhibiting both SOD- and catalase-like activities enhance the mean life span of wild-type worms by 44% (388). Moreover, a *mev-1 (kn1)/cyt-1* mutation, which inactivates succinate dehydrogenase cytochrome *b*, was found to render *C. elegans* susceptible to oxidative stress and, as a result, leads to premature aging (277). The *mth* mutant of *Drosophila* has a significantly extended life span and increased resistance to a free radical generator (351). An extended life span was also observed in *Drosophila* strains with extra copies of genes encoding SOD and catalase (431, 439). Last but not least, mice carrying a mutation in the p66$^{shc}$ protein were found to have an increased life span associated with increased resistance to oxidative stress (390). It is not within the scope of this review to discuss radical-mediated oxidative damage, but the following points ought to be addressed: *1*) What is the evidence that old age and/or disease conditions are associated with changes in ROS levels? *2*) What are the consequences of such changes with respect to redox-regulated processes? *3*) What are the causes for changes in ROS generation? *4*) What are the chances that therapeutic strategies can ameliorate or reverse the increase in ROS generation?

## C. Indications for an Age-Related Increase in ROS Levels

Although ROS production is difficult to measure in biological tissues, there are various indirect manifestations of oxidative stress in old age, including lipid peroxidation, DNA oxidation, protein oxidation, and a shift in the redox states of thiol/disulfide redox couples such as

glutathione, cysteine, and albumin (22, 49, 225, 343, 344, 418, 463, 537–539, 654). The finding that the skeletal muscle tissue of rhesus macaques shows massive age-related manifestations of oxidative damage (654) is of special significance, because the loss of skeletal muscle mass (sarcopenia) is one of the hallmarks of age-related wasting and a major cause of psychological stress, financial burden, and loss of social functions in elderly human subjects (72, 93, 332).

An increasing body of evidence suggests that *1*) the mitochondrial genome may be particularly susceptible to oxidative damage during aging and *2*) mitochondrial DNA deletion mutations may contribute to the fiber atrophy that causes sarcopenia. In a study on rectus phemoris muscle, fibers from 5- to 38-mo-old rats revealed that muscle fibers harboring mitochondrial deletions often display atrophy and increased steady-state levels of oxidative nucleic damage (605).

The published data do not allow us to distinguish whether these age-related changes result from an age-related accumulation of oxidative damage or from an age-related increase in ROS production per unit time. However, age-related changes corresponding to the major homeostatic control mechanisms illustrated in Figure 2 have indeed been observed and may provide an indirect indication for an age-related increase in ROS production. The analysis of gene expression profiles by oligonucleotide arrays representing 6,347 genes revealed aging-related changes indicative of a stress response in skeletal muscle and brain tissue of mice (343, 344). All three tissues tested, i.e., skeletal muscle, neocortex, and cerebellum, showed in old age an increased expression of heat shock factors and other oxidative stress-inducible transcripts. The two brain tissues showed, in addition, increased expression of immunologically relevant transcripts indicative of an inflammatory response (344). Another study examined skeletal muscle tissue from rats and revealed a significant age-related increase in the hydrogen peroxide scavenging enzymes catalase and glutathione peroxidase (284, 596). A conspicuous pro-oxidative shift in the plasma thiol/disulfide redox state has been observed in human subjects between the 3rd and the 10th decade of life (224). Nevertheless, the aging process in the elderly is remarkably slow, indicating that even the elderly have adopted a nearly stable state of redox homeostasis.

A shift in the systemic thiol/disulfide redox state may have systemic consequences because several of the redox-sensitive signal cascades respond not only to direct exposure to ROS but also to changes in the thiol redox state (see Table 2). Therefore, an age-related increase in ROS generation at a given anatomical site may cause a redox-mediated dysregulation at multiple and distant anatomical sites of the organism. The plasma thiol/disulfide ratio may be an easy target for therapeutic intervention by oral *N*-acetylcysteine or other thiol compounds (225). *N*-acetylcysteine was previously shown to protect against age-related decreases in oxidative phosphorylation in liver mitochondria (393). A shift in the redox state may cause, among other consequences, changes in the sensitivity of oxygen sensors (W. Hildebrandt and W. Dröge, unpublished observations), a decrease in the plasma albumin level (225), and, last but not least, changes in cellular functions that are under the control of redox-sensitive signaling cascades. The widely investigated phenomenon of replicative senescence in fibroblasts may be a case in point.

It is necessary, however, to add a note of caution. Although it may be a plausible and attractive paradigm to postulate that aging-related changes in the thiol/disulfide redox state and the changes in gene expression profiles are the direct consequence of an age-related increase in the rate of ROS production, this cause-and-effect relationship remains to be proven.

## D. Replicative Senescence as a Putative Consequence of Redox-Mediated Dysregulation

Somatic cells divide in cell culture only for a finite number of generations. The eventual arrest of cell division is termed "replicative senescence" (241–243, 258, 508, 625). Because the number of divisions of human dermal fibroblasts in vitro decreases with the age of the donor, cellular senescence is commonly seen as an in vitro correlate of the aging process (241). Replicative senescence of human fibroblasts in vitro is associated with morphological changes indicative of differentiation (48, 467). Studies with a histochemical marker suggested that senescent cells may accumulate with age in vivo (147). As in the case of fibroblasts, T cell lines and clones from older individuals typically show a smaller number of population doublings in vitro than observed for cells from younger persons (218, 384, 604).

More recent studies suggested, however, that the replicative life span in vitro is not an inherent property of somatic cells but determined, at least in part, by the redox state of the microenvironment. A senescence-like growth arrest is rapidly induced by hydrogen peroxide (104), and similar effects were found for primary human diploid fibroblasts after a p21$^{ras}$-mediated increase in intracellular ROS levels (342) and in fibroblast cultures exposed to 8-methoxypsoralen followed by UVA irradiation (252). Earlier studies with human diploid fibroblasts have shown that cells exhibit a prolonged life span if grown at low oxygen tension (435). Furthermore, experiments with buthionine-sulfoximine, a specific inhibitor of glutathione biosynthesis, showed that the intracellular redox state modulates the balance between self-renewal and differentiation in dividing glial precursor cells (536).

The evidence discussed above strongly suggests that the observed decrease in replicative capacity in vivo may be a result of the age-related increase in ROS levels and/or the progressive shift in the systemic thiol/disulfide redox state (see sect. VIC). This conclusion is supported by the observation that replicative senescence of human fibroblasts is associated with a dysregulation of the AP-1 transcription factor and with changes in the posttranslational modification of the Fos protein (464). Replication-incompetent cell types express new sets of genes, but the corresponding gene products do not appear to be functionally relevant. Regardless of whether these changes justify a comparison with a differentiation process or not, the observed pro-oxidative changes in elderly subjects (see sect. VIC) strongly suggest that aging of an organism may be associated with changes in redox-sensitive signaling pathways. These changes may well account for differentiation-like processes and a loss in replicative capacity. An additional complication may arise from the fact that senescent fibroblasts suffer from a marked inhibition of proteasome activity (530–532).

The conspicuous discrepancy between the cellular senescence of fibroblasts and the uncontrolled growth of malignant cells (see sect. VII) may be explained by the presence or absence of the tumor suppressor PML, which is strictly required for cellular senescence (442). Defective functioning of PML was shown to be involved in the development of malignancies (61, 143, 610).

## E. Oxidative Induction of Telomere Shortening

The discovery that telomeres get progressively shorter in aging human fibroblasts (233) has led to the popular hypothesis that telomere shortening may also be a major cause of cellular senescence. The expression of a telomerase transgene in cell lines derived from patients with Werner syndrome results in lengthened telomeres and replicative immortalization, thus indicating that the shortening of telomeres is a trigger of premature senescence in these cells (107). Several more recent reports showed that telomere shortening can be induced in fibroblasts by mild oxidative stress (533, 602, 603).

## F. Factors Contributing to Changes in ROS Production

### 1. Age-related changes in mitochondrial complex IV activity and the effect of substrate availability on mitochondrial ROS production

The probability that molecular oxygen is reduced to superoxide rather than water is increased if the proton gradient at the mitochondrial matrix is high (see Fig. 12) and the proper flux of electrons through the ETC ener-

## EFFECT OF SUBSTRATE AVAILABILITY ON ROS PRODUCTION
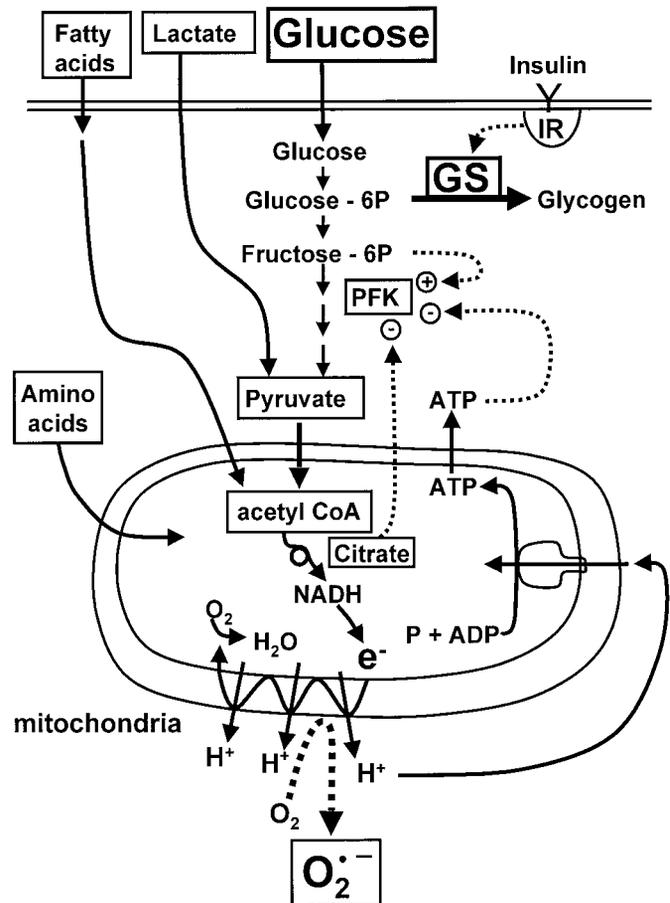


FIG. 12. Effect of substrate availability on ROS production. Substantial amounts of superoxide are produced at the semiubiquinone component of the mitochondrial electron transport chain. The probability of ROS production is increased if the influx of electrons is high and the consumption of electrons by cytochrome oxidase in the mitochondrial complex IV relatively low. Consumption of electrons is typically low if the proton gradient is high, i.e., if only a few protons are being consumed by the ATP-generating system as a result of relatively high ATP and low ADP concentrations. This condition normally inhibits the glycolytic pathway and the influx of further energy substrates into the mitochondria. At high glucose concentrations, e.g., as a result of inadequate glycogen synthase (GS) activity, this control may be overridden, resulting in increased ROS production. PFK, phosphofructokinase.

getically less favored. Because the proton gradient is coupled to the conversion of ADP into ATP (Fig. 12), mitochondrial ROS generation is particularly strong if the availability of ADP is low (64, 102, 362). Under these conditions the components of the ETC are largely in a reduced state. Because ATP consumption and APD availability are particularly low during periods of sleep when the muscular activity is low, the mitochondrial oxidative stress may be particulary high at night. Moreover, a study on the gastrocnemius muscle in mice revealed that the activity of complex IV decreases by 90% from 10 to 26 mo

of age (142). This process is likely to impair significantly the proper flux of electrons through the ETC and to enhance thereby the mitochondrial production of superoxide.

The influx of electrons into the ETC, in turn, is determined by the availability of the electron donors NADH (complex I) and succinate (complex II). Submitochondrial particles depleted of succinate dehydrogenase are still capable of ROS production (63, 360, 361), suggesting that the succinate dehydrogenase flavoprotein does not play an important role in ROS production.

The availability of NADH is determined by the availability of mitochondrial energy substrates such as acetyl CoA. An excess of substrates is normally prevented by the tight regulation of key enzymes in the glycolytic pathway (Fig. 12). Phosphofructokinase (PFK), one of the early enzymes in this pathway, is inhibited by ATP and citrate and couples thereby the generation of pyruvate and acetyl CoA to the cellular energy demand. The negative control of the glycolytic pathway can be overridden, however, by excess fructose-6-phosphate, the PFK substrate derived from glucose via glucose-6-phosphate. A disproportionate amount of the excess glucose taken up by muscle tissue in patients with non-insulin-dependent (type 2) diabetes mellitus in the postabsorptive state due to plasma hyperglycemia is converted glycolytically into lactate (reviewed in Ref. 131). In aorta endothelial cells, a pathologically relevant elevation of the extracellular glucose concentration was shown to cause a massive increase in ROS production by the mitochondrial ETC (415).

TNF-mediated wasting may be mediated, at least to some extent, by a TNF-induced increase in glycolytic activity and lactate production (46). Under certain conditions, NO and peroxynitrite may contribute to mitochondrial superoxide production by enhancing the build-up of semi-ubiquinone (87).

### 2. Effect of caloric restriction on ROS production, age-related diseases, and life span

Because the rate of mitochondrial ROS production is significantly influenced by the availability of mitochondrial energy substrates, it is not surprising that dietary restriction is today the best investigated and most promising experimental strategy to increase life span and to improve the quality of life in old age (181, 343, 344, 537, 539, 613, 614, 654). Studies in several animal species have shown that caloric restriction ameliorates certain manifestations of oxidative stress and causes a substantial increase in life span. Dietary restriction in rodents also delays the onset of age-associated diseases, decreases the incidence of malignancies, and ameliorates the decline in mitochondrial complex IV activity (142, 613). The life-extending benefits of caloric restriction depended in all the cited studies on the prevention of malnutrition and a reduction in total caloric intake rather than in any particular nutrient (613).

### 3. Can endurance exercise substitute for caloric restriction?

Whereas rigorous caloric restriction may be an unattractive regimen for human subjects, endurance exercise may yield similar effects with lower risk of malnutrition. About 80% of glucose deposition is mediated by skeletal muscle tissues (131, 292). A young, healthy organism can rapidly convert excess glucose from dietary carbohydrates into high-molecular-weight glycogen, thereby decreasing the plasma glucose concentration to the normal level required by the central nervous system. Abnormally high plasma glucose concentrations are typically seen in elderly subjects and diabetic patients and are indicative of inadequate muscular glycogen synthase activity (131, 256, 598, 650). This enzyme is regulated by physical activity and strongly enhanced by endurance exercise (40, 58, 597, 598), suggesting that the sedentary life-style of the elderly may contribute to an age-related increase in systemic mean glucose level.

### G. ROS Production in Skeletal Muscle Tissue During Physical Immobilization and Intensive Physical Exercise

ROS production was reported to increase in skeletal muscle tissue after immobilization (314, 315). In view of the massive loss of skeletal muscle mass and body cell mass in immobilized subjects (110, 202, 259, 262, 540, 583), this finding certainly deserves more intensive investigation and underscores the importance of muscular activity.

However, intensive muscular activity was also reported to enhance ROS production (129, 382, 383, 534). With the use of electron spin resonance, it was found that free radical concentrations were increased more than twofold in rat skeletal muscle and liver tissues after exhaustive exercise (129, 280). Repetitive muscular contraction was reported to increase ROS levels and thereby promote the low-frequency fatigue of muscle fibers (459). Direct evidence for increased rates of ROS production during intensive physical exercise is still scarce but is supported by other putative manifestations of oxidative stress, such as changes in the thiol/disulfide redox state in blood plasma and erythrocytes, in the context of intensive physical exercise. A decrease in intracellular GSH/GSSG ratios was found in skeletal muscle of rats after intense muscular exercise (348, 511); a similar decrease in GSH/GSSG ratios was detected in the blood of human volunteers after strenuous exercise (164, 201, 485, 513). The thiol compound *N*-acetylcysteine was found to ameliorate muscle fatigue in humans (460).

The rate of ROS production is generally believed to increase during intensive physical exercise as a consequence of the increased oxygen consumption of the exercising muscle. However, xanthine oxidase may also contribute to the production of superoxide in the context of intensive physical exercise. After exhaustive physical exercise, patients with chronic obstructive pulmonary disease exhibit a significant increase in the GSSG/GSH ratio in the arterial blood and a significant increase in malondialdehyde, a product of lipid peroxidation (253). These symptoms are ameliorated by treatment with allopurinol, a potent inhibitor of xanthine oxidase. A marked increase in xanthine oxidase activity was also found in the plasma of rats after exhaustive exercise (599). Treatment with allopurinol ameliorated the oxidative shift in the glutathione redox state of the blood and the appearence of the cytosolic enzymes creatine kinase and aspartate aminotransferase in the plasma of human volunteers and rats after exhaustive physical exercise (599). Under normal conditions, xanthine oxidase accounts for only a minor proportion of total ROS production (reviewed in Ref. 101).

## H. Oxidative Stress as a Frequent Complication in Disease Conditions

There is a growing awareness that oxidative stress plays a role in various clinical conditions. Malignant diseases, diabetes, atherosclerosis, chronic inflammation, human immunodeficiency virus (HIV) infection, ischemia-reperfusion injury, and sleep apnea are important examples. These diseases fall into two major categories. In the first category, diabetes mellitus and cancer show commonly a pro-oxidative shift in the systemic thiol/disulfide redox state and impaired glucose clearance, suggesting that skeletal muscle mitochondria may be the major site of elevated ROS production (see sect. vIF). These conditions may be referred to as "mitochondrial oxidative stress." Without therapeutic intervention these conditions lead to massive skeletal muscle wasting, reminiscent of aging-related wasting. The second category may be referred to as "inflammatory oxidative conditions" because it is typically associated with an excessive stimulation of NAD(P)H oxidase activity by cytokines or other agents. In this case increased ROS levels or changes in intracellular glutathione levels are often associated with pathological changes indicative of a dysregulation of signal cascades and/or gene expression, exemplified by altered expression of cell adhesion molecules (120, 152, 325, 405, 438, 574).

## I. Malignant Diseases

ROS are potential carcinogens because they facilitate mutagenesis, tumor promotion, and progression (155, 223, 269, 410, 483). The growth-promoting effects of ROS are related to redox-responsive signaling cascades, some of which have been discussed in section v. A placebo-controlled clinical study of patients with previous adenomatous colonic polyps, i.e., a group with an increased risk for colon cancer and increased proliferative index of colonic crypts, revealed a significant decrease in the proliferative index after treatment with N-acetylcysteine (170). Even normal cells often show increased proliferation and expression of growth-related genes if exposed to hydrogen peroxide or superoxide (81, 118, 123, 419). In addition, certain types of cancer cells produce substantial amounts of ROS (36, 223, 387, 553, 557, 562, 653). ROS production is induced after the expression of several genes associated with a transformed phenotype including H-Ras$^{v12}$ or $mox1$ (276, 553). Ras transformation of fibroblasts and the induction of ROS production were shown to involve Rac1 and may thus be similar to the pathway by which extracellular growth factors induce ROS production (see sects. ivA3 and vA). The apparent inconsistency between the uncontrolled cell growth in ROS-producing malignant cells and the ROS-induced senescence in normal cells suggests, however, that ROS production may be necessary but not sufficient to induce malignant cell growth (see sect. vID).

A pro-oxidative shift in the plasma thiol/disulfide redox state has been observed in patients with various types of advanced malignancies (225). This shift is reminiscent of similar changes in diabetes mellitus, old age, and intensive physical exercise. Because cancer patients commonly have decreased glucose clearance capacity (371, 414, 447, 469, 568) and, in addition, abnormally high glycolytic activity and lactate production (517, 518, 568, 611), it is reasonable to assume that the observed pro-oxidative shift is mediated by an increased and basically uncontrolled availability of mitochondrial energy substrate.

## J. Diabetes Mellitus

Elevated ROS levels have also been implicated in diabetes mellitus (47, 629). In this case oxidative stress is associated with a pro-oxidative shift of the glutathione redox state in the blood (137). Hyperglycemia is a hallmark of both non-insulin-dependent (type 2) and insulin-dependent diabetes mellitus (type 1). Elevated glucose levels are associated with increased production of ROS by several different mechanisms (47, 415, 420, 482, 593). In cultured bovine aortic endothelial cells, hyperglycemia was shown to cause increased ROS production at the mitochondrial complex II (415). Several independent strategies that ameliorate mitochondrial ROS production were shown to prevent some of the typical secondary complications of the disease, including the activation of protein kinase C or NF-$\kappa$B and the formation of advanced glycation end products (415).

In addition, superoxide is generated by the process of glucose auto-oxidation that is associated with the formation of glycated proteins in the plasma of diabetic patients (47, 420, 482, 593, 628, 630). The interaction of advanced glycation end products with corresponding cell surface receptors stimulates ROS production and decreases intracellular glutathione levels (645). The increase in ROS production contributes to the development of diabetic complications such as atherosclerosis and other vascular complications (25, 47). In addition, hyperglycemia enhances cell-mediated low-density lipoprotein (LDL) peroxidation in endothelial cells (381). Treatment with antioxidants ameliorates diabetic complications including the dysfunction of endothelial cells or increased platelet aggregation (90, 99, 271, 298, 364, 528, 626).

## K. Atherosclerosis

Atherosclerosis is a multifactorial disease characterized by hardening and thickening of the arterial wall. The vascular areas affected by this disease contain mononuclear cells, proliferating smooth muscle cells, and extracellular matrix components. Atherosclerosis is commonly viewed as a chronic inflammatory disease and is associated with certain risk factors such as hyperlipidemia, diabetes (see sect. VI*J*), and hypertension. Excessive ROS production has been implicated in the pathogenesis of atherosclerosis and hypertension (19, 29, 98, 210, 227, 328, 547, 550). Oxidative stress induces the expression of protein kinases such as focal adhesion kinase and intercellular adhesion molecules such as ICAM-1 (106). The invasion of the artery wall by monocytes and T lymphocytes is one of the earliest events in the development of atherosclerotic lesions. Monocytes, macrophages, and smooth muscle cells possess the so-called scavenger receptor for oxidized LDL. Binding of oxidized LDL leads to the activation of monocytes and macrophages and stimulates the expression of Mn-SOD which, in turn, increases the concentration of hydrogen peroxide by perturbing the steady-state levels of ROS (305, 306, 308). This process is associated with massive macrophage apoptosis and contributes thereby to the formation of the atherosclerotic lesions (309, 461). The process may be further enhanced by cytokines and other factors such as TNF, interleukin-1$\beta$, angiotensin II, and interferon-$\gamma$, which induce superoxide production by the membrane-bound NADPH oxidase in endothelial cells (136, 210, 396, 424).

Lipid peroxidation and atherogenesis may be ameliorated by vitamin E. A study of atherosclerosis-susceptible APO-lipoprotein E knock-out mice revealed that induction of vitamin E deficiency by disruption of the $\alpha$-tocopherol transfer protein gene (Ttpa) increased the severity of atherosclerotic lesions in the proximal aorta (570). A randomized placebo-controlled study of patients with an-

giographically proven coronary artery disease revealed that NO-dependent, flow-mediated dilation was significantly improved by treatment with the cysteine pro-drug and glutathione precursor L-2-oxo-4-thiazolidine carboxylate (600).

In view of the enhancement of T-cell signaling by oxidative conditions (see sect. IV*F*), it is not surprising that the atherosclerotic process also shows signs of an autoimmune component (618, 619). T cells isolated from atherosclerotic lesions in rabbits receiving a cholesterol-rich diet were found to express a preferential reactivity toward the mycobacterial heat shock protein hsp65, which shows >50% sequence homology with human hsp60 on the DNA and protein levels (641). A role of hsp65/60 in association with oxidative stress has also been implicated in other autoimmune diseases such as rheumatoid arthritis (303, 462, 594) (see also sect. VI*M*).

## L. Neurodegenerative Diseases

Down's syndrome or trisomy 21 is the most frequent genetic cause of mental retardation and is commonly associated with the development of Alzheimer's disease (AD) in adult life. Cultured cortical neurons from fetal Down's syndrome cases exhibit a three- to fourfold higher intracellular ROS level than age-matched normal brain cells. Treatment with free radical scavengers or catalase prevents the degeneration of Down's syndrome neurons in culture (84). The gene of the antioxidant enzyme Cu/Zn-SOD is localized at 21q22.1 (529) and, through increased gene dosage, is thought to play a prominent role in some of the clinical features of Down's syndrome. Cu/Zn-SOD was found to be elevated in a variety of cell types and organs including erythrocytes, platelets, fibroblasts, lymphocytes, and fetal brain (24, 73, 133, 135, 180; reviewed in Ref. 55). This increase in SOD is expected to result in increased generation of hydrogen peroxide and a displaced equilibrium in the steady-state levels of ROS.

Stably transfected cells overexpressing Cu/Zn-SOD show a higher level of lipid peroxidation than control cells (168). Cells exhibiting an increase in the ratio of Cu/Zn-SOD to glutathione peroxidase and in catalase activity also have a higher intracellular level of hydrogen peroxide and show typical features of cellular senescence (see sect. VI*D*), as indicated by a slower growth rate and altered morphology (134). A similar senescent phenotype is observed in cells from children with Down's syndrome and in cells exposed directly to hydrogen peroxide (56, 134). Cu/Zn-SOD transgenic mice also exhibit signs of premature aging and neuromuscular dysfunction (30–32, 648). The neuromuscular junctions of the leg muscles from these mice were found to exhibit pathological changes that are similar to those observed in the skeletal muscle tissue of aging rats and mice (95, 172) and in the

tongue muscles of individuals with Down's syndrome (646, 647).

AD is a neurodegenerative disorder characterized by a progressive decline in cognitive function and extensive neuronal loss. The brains of affected patients show numerous amyloid plaques and neurofibrillary tangles. The production of ROS in the brains of AD patients and its implication in AD pathogenesis are implicated by the significant amount of lipid peroxidation detected in the brain as well as by the increased levels of 4-hydroxynonenal found in postmortem cerebrospinal fluid of AD patients (363, 398, 404, 450, 487). Furthermore, ROS were found to mediate amyloid $\beta$-protein damage (51, 293, 404).

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects primarily motor neurons in the spinal cord and brain stem. Approximately 10% of the cases are inherited in an autosomal dominant manner. One-fifth of these familial ALS patients carry mutations in the Cu/Zn-SOD gene, suggesting the involvement of ROS in this neurodegenerative disease (470). Several lines of transgenic mice carrying mutant SOD transgenes have been shown to develop a pathology and clinical phenotype similar to that of familial ALS patients (582). The mutation in the Cu/Zn-SOD gene causes neuronal death by apoptosis through the sequential activation of caspase-1 and caspase-3 (440).

Transmissible spongiform encephalopathies (TSEs) are characterized by the conversion of the cellular form of the prion protein (PrP$^C$) into a conformationally modified protease-resistant isoform called PrP$^{Sc}$ (451). A prominent example of such a prion disease is the bovine spongiform encephalopathy (BSE). The function of PrP$^c$ and particularly its role in the neurodegenerative processes, which are typically found in prion diseases, are still unknown. It has been proposed, however, that PrP$^C$ may play a role in the control of the oxidative state of the cell through a regulation of the copper transport (441) and/or through a modification of Cu/Zn-SOD activity (74). More recently, Milhavet et al. (392) demonstrated that prion-infected neuronal cells displayed a higher sensitivity to oxidative stress over noninfected cells. Infected cells also showed increased lipid peroxidation and a dramatic decrease in various redox-related enzymes such as glutathione peroxidase, glutathione reductase, and Mn-SOD (392). Collectively, these findings suggest that prion infection compromises the cellular resistance to ROS.

## M. Rheumatoid Arthritis

While the enhancement of immune reactivity by pro-oxidative conditions may be critically important for the immune system to control and defeat rapidly multiplying pathogens (see sect. IV$F$), such enhancement also bears the risk of inducing autoimmune processes. Rheumatoid arthritis is a systemic autoimmune disease characterized by chronic joint inflammation with infiltration of macrophages and activated T cells. Several lines of evidence suggest that production of free radicals at the site of inflammation may contribute decisively to the pathogenesis of this disease (26, 370, 379). T cells isolated from the synovial fluid of patients with rheumatoid arthritis are characterized by a decreased intracellular GSH level and the "primed" CD45RO phenotype (378). These T cells exhibit severely impaired phosphorylation of the adaptor protein linker for T-cell activation (LAT). Changes in intracellular GSH level were shown to alter the subcellular localization of LAT (214). The migration of monocytes and lymphocytes into the rheumatoid arthritis synovium is mediated by the abnormal expression of several adhesion molecules including ELAM-1, VCAM-1, ICAM-1, and ICAM-2 (119, 120, 595), an effect which may be explained by the abnormal induction of redox-sensitive signaling pathways (see sect. IV$E$). Oxidative conditions in synovial tissue are also associated with a higher incidence of p53 mutations (179). Although malignant tumors of the synovium are rare, it has been hypothesized that the presence of transformed cells in the synovium of rheumatoid arthritis patients may lead to progressive joint destruction without malignant degeneration (563). The heat shock protein hsp65/60, which has been implicated in the pathogenesis of atherosclerosis (see sect. VI$K$), is also a candidate (auto)antigen in the pathogenesis of rheumatoid arthritis (303, 462, 594).

## N. HIV Infection

HIV infection is associated with progressive deterioration of the immune system, leading eventually to lethal opportunistic infections. Relatively early in the course of HIV infection, there is a decrease in various functional activities of lymphoid cells followed by a conspicuous decrease in CD4$^+$ T-lymphocyte numbers. In the late stages of the disease, the patients often suffer from massive skeletal muscle wasting.

HIV infection is also associated with massive catabolism of cysteine into sulfate. This process can be detected even in the early asymptomatic state of the disease and accounts for a mean net loss of more than 4 g cysteine/day (70, 156). Excessive cysteine catabolism can be detected most easily as urinary sulfate excretion or as muscular sulfate excretion determined from arterial-venous differences in the lower extremities (70). The ratio of urinary sulfate to urea (i.e., sulfur/nitrogen) indicates that the excessive cysteine catabolism proceeds largely at the expense of glutathione rather than protein. This is in agreement with studies of SIV-infected rhesus macaques which exhibit a progressive decrease in the glutathione level of skeletal muscle tissue followed by skeletal muscle

wasting (216). The mechanism responsible for excessive cysteine catabolism is unclear. Old age and intensive physical exercise, i.e., two conditions believed to be associated with increased muscular ROS production, show typically increased rather than decreased plasma cystine (cysteine disulfide) levels. In contrast, HIV-infected patients and SIV-infected rhesus macaques show, on the average, abnormally low plasma cystine levels (157, 158, 166, 225, 261) and low intracellular glutathione levels in peripheral blood lymphocytes (165, 468).

The limiting role of glutathione and its precursor cysteine in the execution of the immune response was discussed in section v*J*. Numerous lymphocyte functions are exquisitely sensitive to a decrease in intracellular glutathione levels (reviewed in Ref. 160). That the virus-induced cyst(e)ine deficiency is indeed a causative factor for the progressive impairment of the immune system is suggested by two independent placebo-controlled double-blind studies, which demonstrated that treatment of HIV-infected patients with *N*-acetylcysteine leads to a significant improvement of various proliferative T-cell responses and to a reconstitution of NK cell activity to almost normal levels (71). In view of the importance of glutathione levels in several redox-regulated systems (see sect. v*J*), it is believed that the HIV-induced decrease in intracellular glutathione levels facilitates the induction of signaling pathways leading to lymphocyte activation but renders the cells more sensitive to oxidative stress. Ex vivo labeling studies have shown that, compared with healthy controls, HIV-infected patients have indeed significant increases in the number and faction of dividing $CD4^+$ and $CD8^+$ T cells. The fact that $CD4^+$ T-cell counts decline during the course of HIV infection suggests, however, that the increase in $CD4^+$ T cell destruction is greater than the increase in T-cell production (345). Taken together, the available evidence suggests that depletion of the systemic cysteine pool may be one of several ways by which a virus can prevent its elimination by the immune system. Because immune reconstitution is a widely accepted aim of HIV therapy, cysteine supplementation may be considered as a standard therapy for these patients.

### O. Ischemia and Reperfusion Injury

Ischemia and reperfusion can lead to tissue injury and are serious complications in organ transplantation, myocardial infarction, and stroke (194, 196, 203). Massive ROS production was identified as an important causative factor (100, 116, 153, 282, 375, 376, 385, 432, 433, 579). Xanthine dehydrogenase, which normally utilizes $NAD^+$ as electron acceptor, is converted under the conditions of ischemia/reperfusion into xanthine oxidase which uses oxygen as substrate. During the ischemic period, excessive ATP consumption leads to the accumulation of the purine catabolites hypoxanthine and xanthine, which upon subsequent reperfusion and influx of oxygen are metabolized by xanthine oxidase to yield massive amounts of superoxide and hydrogen peroxide (208). More recently, a Rac1-regulated NAD(P)H oxidase distinct from the phagocytic NAD(P)H oxidase was shown to be critically involved in ROS production in a mouse model of hepatic ischemia/reperfusion injury (434).

Neutrophils are the principal effector cells of reperfusion injury, and the inhibition of neutrophil adhesion to the endothelium attenuates the process (573). Antioxidant treatment ameliorates both leukocyte adhesion and leukocyte-mediated heart injury in the postischemic period (514). Also, treatment with a synthetic SOD mimetic was shown to ameliorate tissue damage in a rat model of ischemia/reperfusion injury (484).

Experimental induction of ischemia and reperfusion in the rat heart was found to be associated with the activation of the redox-responsive trancription factors NF-$\kappa$B and AP-1 and the MAPKs JNK and p38 in the presence of minimal activation of ERK (112, 341, 377). This activation may account for inflammatory responses and apoptotic cell death in the affected tissue (296, 502).

### P. Obstructive Sleep Apnea

A substantial proportion of the adult population in Western countries suffers from a breathing disorder characterized by repeated episodes of apnea or hypopnea during sleep (652). This condition is associated with the development of hypertension (444). In its more severe manifestations, obstructive sleep apnea is associated with increased mortality due to cardiovascular morbidity, including arterial hypertension, coronary artery disease, and cerebrovascular disease (244, 443, 519). It is one of the most important cardiovascular risk factors.

The resulting repetitive hypoxia/reoxygenation stress is reminiscent of the ischemia/reperfusion condition. An involvement of ROS in the pathogenesis of cardiovascular complications is suggested by the finding that polymorphonuclear neutrophils from the blood of patients with obstructive sleep apnea show significantly increased superoxide production after exposure to different stimuli (503). In addition, obstructive sleep apnea is associated with increased expression of several cell adhesion molecules such as ICAM-1 and VCAM-1, suggesting that the pathogenesis of the cardiovascular complications are mechanistically similar to the development of atherosclerosis (see sect. vi*K*) (425).

### Q. Key Messages From Section vi

The important role of ROS in the regulation of physiological responses is underscored by the apparent dys-

regulation of physiological responses in various disease-related oxidative stress conditions.

Excessive levels of ROS may be generated either by excessive stimulation of otherwise tightly regulated NAD(P)H oxidases or by other mechanisms that produce ROS "accidentally" in a nonregulated fashion. The latter situation includes the production of ROS by the mitochondrial ETC, the quantitatively most important source of ROS in higher organisms.

ROS production in the mitochondrial ETC is determined in part by substrate availability. In endothelial cells elevation of the extracellular glucose concentration was shown to cause a massive increase in ROS production by the mitochondrial ETC and a corresponding activation of NF-$\kappa$B.

Caloric restriction is an effective experimental strategy to decrease oxidative stress in vivo and to increase the life span of experimental animals. Endurance exercise can serve to some extent as a substitute for caloric restriction in humans.

Intensive physical exercise induces various manifestations of oxidative stress.

Diabetes mellitus and cancer commonly show a pro-oxidative shift in the systemic thiol/disulfide redox state, similar to the shift seen in old age. These "mitochondrial oxidative stress" conditions are typically associated with skeletal muscle wasting. In diabetes mellitus ROS may also be generated by glucose auto-oxidation, which is associated with the formation of glycated proteins in the plasma. ROS are potential carcinogens because they facilitate mutagenesis, tumor promotion, and progression. The growth-promoting effects of ROS are related to redox-responsive signaling cascades.

Another group of diseases designated as "inflammatory oxidative conditions" includes atherosclerosis and chronic inflammation and is typically associated with excessive stimulation of NAD(P)H oxidase activity. Some of the pathological complications are indicative of a redox-mediated dysregulation of signaling cascades and/or gene expression, e.g., the overproduction of cell adhesion molecules.

ROS production by xanthine oxidase has been implicated in the pathology of ischemia and reperfusion injury. Similar mechanisms may also operate in obstructive sleep apnea.

The free radical theory of aging (234) states that age-related degenerative processes are to a large extent the consequence of damage induced by free radicals. A growing body of evidence now suggests that aging involves, in addition, progressive changes in free radical-mediated regulatory processes, resulting in altered gene expression.

There are various indirect manifestations of oxidative stress in old age, including lipid peroxidation, DNA oxidation, protein oxidation, and a shift in the redox states of thiol/disulfide redox couples such as glutathione, cysteine, and albumin. All of these manifestations suggest that the rate of ROS production per time unit increases with age. However, this conclusion needs to be tested experimentally. ROS production is difficult to measure in biological tissues.

Regardless of whether the documented age-related shift in the thiol/disulfide redox state is caused by increasing ROS levels or not, this shift itself may cause a progressive redox-mediated dysregulation of signaling cascades (see sect. III$A3$).

The term *replicative senescence* describes the fact that somatic cells divide in cell culture only for a finite number of generations. Recent evidence suggests that the decrease in replicative capacity may be related to an increase in ROS levels and/or a pro-oxidative shift in the thiol/disulfide redox state. Mild oxidative stress was also shown to cause telomere shortening in fibroblasts.

# VII. CONCLUSIONS

## A. Physiological Aspects of Redox Regulation

The radicals NO (NO·) and superoxide anion ($O_2^-$·) play an important role in biological regulation. Superoxide gives rise to other forms of ROS that serve as mediators in many regulatory processes. Most redox-responsive regulatory mechanisms in bacteria and mammalian cells serve to protect the cells against oxidative stress and to reestablish redox homeostasis. The oxidative induction of protective enzymes by the redox-sensitive bacterial OxyR and SoxR proteins or the inhibition of NOS by NO are prominent examples. Redox regulation of other physiological responses in higher organisms is embedded in these basic mechanisms of redox homeostasis.

The relatively large number of NAD(P)H oxidase isoforms and NOS indicates that nature has "learned" to use free radicals to her advantage in processes not directly related to protection against oxidative stress. The production of superoxide and NO, respectively, by these enzymes is strictly regulated by hormones, cytokines, or other inducing mechanisms. The resulting oxidative species, in turn, act as secondary messengers to control a variety of physiological responses. The regulation of vascular smooth muscle relaxation, the monitoring of the oxygen concentration in the regulation of respiratory ventilation and erythropoietin production, and the enhancement of signaling cascades from various membrane receptors are prominent examples.

The enhancement of signal transduction from a given receptor by stimulation of ROS production through this or other receptors may serve two physiological purposes. First, it provides a basis for cooperativity, and second, the membrane receptor may function simultaneously as a

sensor for the extracellular ligand and as a sensor for the inner metabolic state of the individual cell. The cooperativity between the angiotensin II receptor and the EGF receptor is a well-studied example, but other examples will likely be found. Because hydrogen peroxide has a relatively long half-life and crosses membranes, the cooperativity principle may even extend to other cells in the vicinity. By enhancing the intracellular signaling pathways of lymphocytes, ROS from activated macrophages and neutrophils may contribute decisively to the activation of the antigen-specific immune response and may allow the immune system to respond to minute amounts of invading pathogens. Signaling pathways involving JNK, p38 MAPK, and the transcription factors AP-1 and NF-$\kappa$B are particularly responsive to redox regulation.

## B. Molecular Aspects of Redox Regulation: Gain of Function, Loss of Function, or Outright Destruction

The capacity of ROS to damage proteins and to hasten their proteolytic degradation has been employed as a regulatory mechanism in several cases, e.g., in the degradation of the transcription factor subunit HIF-1$\alpha$ and the NF-$\kappa$B inhibitor I$\kappa$B. The inhibition of protein tyrosine phosphatases is well-defined on a molecular basis and provides an example of redox regulation by loss of function. In other cases, NO or ROS induce a gain of function in a signaling protein. This mechanism is involved in the regulation of vascular tone and the functional activation of the bacterial OxyR and SoxR proteins. The oxidative enhancement of membrane receptor signaling and the corresponding downstream signaling pathways are not well-characterized at the molecular level but are likely to involve the simultaneous induction of several different redox-sensitive signaling proteins. This redundancy does not preclude selective effects. The in vivo relevance of redox-sensitive signaling cascades is strongly suggested by the mere existence of the many NAD(P)H oxidase isoforms (see sect. IV*A*) and by the apparent dysregulation of physiological responses in various disease-related oxidative stress conditions (see sect. VI). However, the relative contributions of individual redox-sensitive signaling proteins to redox-regulated processes in vivo are presently obscure.

## C. Regulated Versus Uncontrolled Free Radical Production: Increased ROS Levels in Old Age and Disease

There is evidence that ROS production may be substantially elevated in old age and certain disease conditions. Excessive stimulation of NAD(P)H oxidase by cytokines or other mediators is implicated in various disease conditions. Other sources of superoxide such as the mitochondrial ETC and xanthine oxidase are not tightly regulated and may become increasingly relevant in old age, diabetes mellitus, and malignant diseases. With respect to the free radical theory of aging, we are seeing a shift in paradigm. Changes in redox-responsive signaling cascades and in the expression of corresponding target genes may have a similar or even greater impact on senescence as the direct radical-inflicted damage of cellular constituents.

Receptor signaling was also found to be strongly influenced by the intracellular glutathione level in all

## OXIDATIVE STRESS AND SENESCENCE: observed age-related changes and putative mechanisms
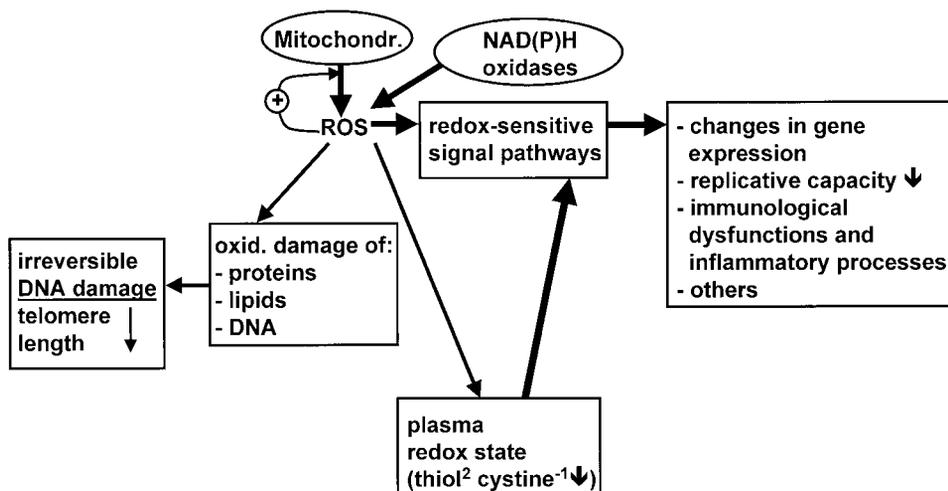


FIG. 13. Oxidative stress and senescence: observed age-related changes and putative mechanisms. A persistent increase in ROS production either by mitochondria in skeletal muscle tissue or by chronic stimulation of NAD(P)H oxidase activity in leukocytes may cause a dysregulation of redox-sensitive signaling pathways in addition to direct oxidative damage. Progressive changes in the systemic plasma thiol/disulfide redox state may affect changes in redox-sensitive signaling pathways at any anatomic site.

cases where appropriate experiments were performed. Many redox-sensitive signaling cascades respond equally well to ROS or to changes in the intracellular thiol/disulfide redox state. The massive oxidative shift in the human plasma thiol/disulfide redox state between the 3rd and the 10th decade of life may, therefore, alter the set point of redox-sensitive signaling pathways in various somatic cells. The pro-oxidative shift may account for age-related immunological dysfunctions and inflammatory processes as well as for the loss of the replicative capacity of fibroblasts, as illustrated schematically in Figure 13.

## D. Chances for Therapeutic Intervention and Perspectives

The development of procedures to ameliorate undesirable ROS production may be one of the central issues in research on aging and oxidative stress-related diseases in the near future. The available evidence suggests that the age-related increase in ROS production may be due, at least in part, to the age-related increase in systemic glucose levels. Rigorous caloric restriction was found to ameliorate various manifestations of oxidative stress in experimental animals but may not be feasible for humans. Flooding the system with antioxidants or the overexpression of antioxidative enzymes may be just as detrimental as excessive exposure to free radicals. To ensure ordered redox-mediated signaling, life requires a delicately balanced intermediate level of free radicals and radical-derived ROS. There are some encouraging recent reports about the use of SOD/catalase mimetics in certain experimental systems. Dietary antioxidants are widely used to ameliorate excessive oxidative stress, but scientific proof of their efficacy is scarce. There is, nevertheless, a strong possibility that the process of senescence and disease-related wasting results, at least to some extent, from a progressive shift in biochemical conditions that may not be irreversible in principle.

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