

Role of Superoxide as a Signaling Molecule

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Superoxide is known to affect vascular physiology in several ways and has also been recognized to contribute significantly to vascular pathophysiology. Here we discuss the emerging role of superoxide as an essential signaling molecule in normal physiology.

Life in an oxygen-containing environment is necessarily accompanied by the production of oxygen-derived radicals. In fact, many biological processes, and probably life as we know it, are only possible because of the reactivity of oxygen and the formation of oxygen radical intermediates. The survival of organisms under these "noxious" conditions has been facilitated by the evolutionary development of suitable protection mechanisms against radical impact. In this way, organisms from bacteria to mammals contain specific enzyme systems that sense and inactivate reactive oxygen species (ROS). However, when these primary protection mechanisms are overwhelmed by excessive ROS production, one encounters a situation of oxidative stress that can result in the modification of cellular macromolecules. For these situations, most organisms have developed a secondary defense strategy that includes a variety of repair mechanisms. However, because oxygen is omnipresent and because of the evolution of life in an oxygen-containing environment, most organisms have also developed signaling mechanisms that utilize ROS. This role of ROS has only recently begun to be appreciated. Still, only little is known about where and how these radicals are produced, which radicals are used in signaling, and where and how they are sensed to exert a specific effect.

In most cases, the first reaction of O_2 is the one-electron reduction to superoxide (O_2^- , Fig. 1). Further reduction yields the nonradical ROS hydrogen peroxide (H_2O_2), which in the presence of free transition metals can give rise to the highly reactive hydroxyl radical (HO^\cdot). The major defense mechanisms that interfere with this radical flux are the superoxide dismutases (SOD) that catalyze the dismutation of O_2^- to H_2O_2 and the catalase (CAT) and glutathione peroxidases that reduce and inactivate H_2O_2 to water. Another radical of biological importance is the nitrogen monoxide (NO) radical that is at the basis of endothelial-derived relaxing factor. When NO and O_2^- are produced simultaneously, they react with each other at a diffusion-controlled rate to form the highly reactive peroxynitrite (ONOO $^-$). Formation of these primary ROS can result in the cellular formation of secondary radicals such as peroxy or alkoxy radicals. Also biologically important are the quinones, which can undergo one-electron reduction to form semiquinone radicals that then either undergo redox cycling by reducing oxygen to O_2^- or to alkylate cellular macromolecules (4).

Although many biological effects have been ascribed to H_2O_2 and have been extensively discussed elsewhere (13, 18), the insight starts to emerge that O_2^- may also be an important

mediator of cellular effects. This is partially based on the fact that most cells possess enzymatic systems that are capable of producing O_2^- , whereas to date no cellular system is known that exclusively generates H_2O_2 . In this review, we will discuss the effects of O_2^- in cellular processes with emphasis on its signaling function(s).

O_2^- and NO

The interaction of O_2^- with NO is quite extensively discussed elsewhere (18) and is not dealt with in detail here. NO is formed in neuronal cells where it serves as modulator of neurotransmission, in endothelial cells where it serves as major factor in vessel relaxation, and in neutrophils and macrophages where it serves as a mediator in blood vessel relaxation and inactivation of pathogens. Because O_2^- will react at a diffusion-controlled rate with NO, this important biological mediator will be inactivated and, for example, in blood vessels will cause vasoconstriction. In addition to that function, the resulting product ONOO $^-$ can modify cellular macromolecules, which can result in altered cellular function (Fig. 1). One of the major reactions of ONOO $^-$ appears to be nitrosylation of active site tyrosine residues in enzymes. In addition, ONOO $^-$ can result in nitrosylation of glutathione (and other cellular thiols) and thereby alter the cellular redox balance. Other effects of ONOO $^-$ are oxidation of lipids, DNA strand breaks, and nitration and deamination of DNA bases (4).

O_2^- and oxidative stress

When ROS generation overwhelms the antioxidant defense, the radicals can interact with endogenous macromolecules and alter cellular function. Although O_2^- alone is not very reactive, it has several cellular effects. It can release iron from Fe/S clusters or can abstract hydrogen atoms from target molecules such as catecholamines, DNA, RNA, fatty acids, or steroids. An excellent recent review (8) discusses many of the disease processes associated with oxidative stress and the benefit of low-molecular-weight SOD mimetics in pharmacology. ROS, and in particular O_2^- , are implicated in inflammatory diseases, ischemia-reperfusion injury, cancer, and the aging process. The fact that SOD mimetics are effective in protecting against these ROS-mediated diseases (8) suggests that it is the O_2^- that is implicated in the disease process and not H_2O_2 , the product of O_2^- dismutation. Indeed, it has recently been

described that overexpression of SOD in V79 Chinese hamster cells resulted in lower H_2O_2 levels, contrary to what was expected (16).

O_2^- and cellular signaling

Despite the large body of literature demonstrating cellular signaling effects of H_2O_2 (13, 18), relatively little is known of the signaling effects of O_2^- . Among the established effects of O_2^- in signaling is the activation of the ras/rac-Raf1-MAPK pathway. For some time it has been known that angiotensin II (ANG II) induces O_2^- generation in endothelial cells via the ubiquitous NAD(P)H oxidase (3). This O_2^- then activates the Raf-1-MAPK pathway, leading to altered gene expression (2). ANG II has also been shown to stimulate O_2^- production in the central nervous system to increase vasopressin secretion and sympathetic outflow (20). This was proven in mice by using adenoviral vector-mediated expression of SOD (AdSOD) where AdSOD blocked both ANG II-induced O_2^- generation and changes in blood pressure, heart rate, and drinking behavior.

In another example of the effect of O_2^- , Huang et al. (5) recently demonstrated that ERK activation induced by peroxisome proliferator-activated receptor- γ (PPAR- γ) agonists was blocked by a SOD mimetic and induced by an O_2^- -generating system. The quinone 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) has been shown to undergo redox cycling in cells without alkylating cellular proteins and was used to generate O_2^- . Treatment of C2C12 muscle cells with DMNQ, similarly to PPAR- γ agonists, induced ERK phosphorylation and activation (5).

Further effects of O_2^- on cellular signaling have been shown by Oberley et al. (19). This group showed that overexpression of the oncogenic form of the small G protein v-Ha-ras increased O_2^- generation and growth of the human keratinocyte cell line HaCaT. The fact that this effect could be blocked by lovastatin, a hydroxymethylglutaryl CoA reductase inhibitor that blocks farnesylation of the ras protein, suggests that the effect is indeed mediated by ras. However, O_2^- generation and growth could also be inhibited by the NAD(P)H oxidase inhibitor diphenylene iodonium, suggesting that the ubiquitous NAD(P)H oxidase, in addition to rac, may also interact with ras. Overexpression of virally transduced SOD blocked the growth-stimulatory effect of v-Ha-ras overexpression, suggesting that intracellular O_2^- was responsible for this effect. In addition to the keratinocyte cell line, rat kidney epithelial cells could also be stimulated to grow and form colonies in soft agar in an O_2^- -dependent manner by v-Ha-ras overexpression (19).

O_2^- and control of cell growth

From the above information, it seems that O_2^- can serve as a growth signal in different cells via a rac/ras-NAD(P)H oxidase-MAPK signaling pathway. In fact, from bacteria and yeast it is known that O_2^- serves as a growth stimulus. Bacteria such as *Escherichia coli* and *Salmonella typhimurium* or yeasts such as *Saccharomyces cerevisiae* that are deficient in SOD can

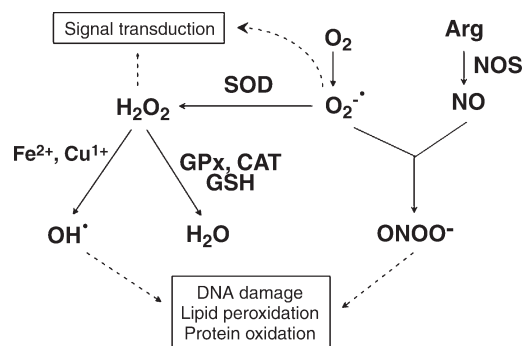


FIGURE 1. Radical flux. Oxygen is reduced to superoxide (O_2^-) by different cellular mechanisms. O_2^- is reduced to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 is reduced to water either by glutathione peroxidase (GPx) using glutathione (GSH) as substrate or by catalase (CAT). In the presence of free iron or copper, H_2O_2 undergoes the Fenton reaction to form the hydroxyl radical (OH^\cdot). When nitrogen monoxide (NO) is formed by NO synthase (NOS), O_2^- will react with NO to form peroxynitrite ($ONOO^-$). Both OH^\cdot and $ONOO^-$ are very reactive and can modify cellular macromolecules and cause toxicity. The less-reactive molecules O_2^- and H_2O_2 can serve as cellular signaling molecules.

only survive in log-phase growth and die on entering the stationary phase (14). Interestingly, when bacteria reach the stationary phase they start expressing a periplasmic SOD enzyme. Thus it seems that growth needs O_2^- , whereas growth inhibition requires a decrease in O_2^- levels that is accomplished by expression of a SOD enzyme. Similar findings have been made with eukaryotic cells, where SOD expression was found to be cell cycle dependent. Cell lines that displayed density-dependent growth inhibition demonstrated increased MnSOD expression when reaching confluence, whereas cell lines that did not display density-dependent growth inhibition failed to increase MnSOD expression at high cell density (10).

A further example of O_2^- requirement in growth and development is seen in the multicellular development of *Dicystostelium discoideum*. In this organism, O_2^- is generated during the transition to the multicellular phase of development and scavenging of O_2^- with diethyldithiocarbamate or overexpression of SOD inhibited the formation of multicellular aggregates (1).

A role of O_2^- in growth regulation has also been demonstrated in other systems. Overexpression of SOD was shown to inhibit cell proliferation (11). In contrast, inhibition of SOD by pharmacological means increased O_2^- levels and stimulated cell proliferation. On the other hand, at least in these studies, apoptosis, the counterregulator of cell proliferation, seemed to be inversely regulated to cell growth. Increased, nontoxic O_2^- levels achieved by downregulation of SOD with antisense oligonucleotides inhibited apoptosis, whereas overexpression of SOD or inhibition of NAD(P)H oxidase led to a decrease in O_2^- and increased susceptibility of cells to undergo apoptosis. The growth-stimulatory effect of low levels of O_2^- has been confirmed by the group of Sawyer and coworkers (12), who used modulation of SOD in cardiomyocytes to manipulate O_2^- levels. However, in contrast to Pervaiz et al. (11), this group found that high levels of O_2^- were necessary to induce cells to undergo apoptosis (12). In these

studies moderate inhibition of SOD or application of moderate stretch stress to cardiomyocytes resulted in cell growth and differentiation via phosphorylation and activation of ERK and increased protein synthesis. On the other hand, complete inhibition of SOD or application of excessive stretch stress led to apoptosis and was mediated by both ERK and JNK activation and increased expression of the proapoptotic protein Bax. All of these effects could be blocked with SOD mimetics. The need for increased O_2^- production necessary to induce apoptosis has also been confirmed by other authors (15). Together, these data suggest that a graded increase in O_2^- levels can decide between life (growth and differentiation) and death, as depicted in Fig. 2.

O_2^- and cyclosporin A-mediated vasoconstriction

We have recently added another aspect of O_2^- -mediated cellular regulation to this growing list of effects. In our investigations of the side effects of the immunosuppressive drug cyclosporin A (CsA), we and others (6) have found that CsA was able to generate O_2^- . Because our earlier studies have shown that CsA increased the cell surface expression of the type 1A vasopressin receptor (V_{1A} receptor) at the protein and mRNA levels, probably via a posttranscriptional effect on mRNA stabilization, we investigated whether this effect was mediated by O_2^- . Indeed, we found that the ROS produced by CsA were responsible for the observed increase in V_{1A} receptor expression in rat aortic smooth muscle cells (RASMC) as shown in Fig. 3 (6). Short-term treatment (30–60 min) of RASMC with several pro-oxidants was sufficient to increase V_{1A} receptor expression at both the protein and mRNA levels. This effect could be blocked by antioxidants such as *N*-acetylcysteine or α -tocopherol. Further experiments were undertaken to establish that O_2^- was the mediator of this effect. For this we used cell-permeable forms of SOD and CAT [polyethylene glycol (PEG)-coupled SOD and CAT]. As shown in Fig. 4, PEG-SOD decreased specific binding of [3 H]Arg⁸-vaso-

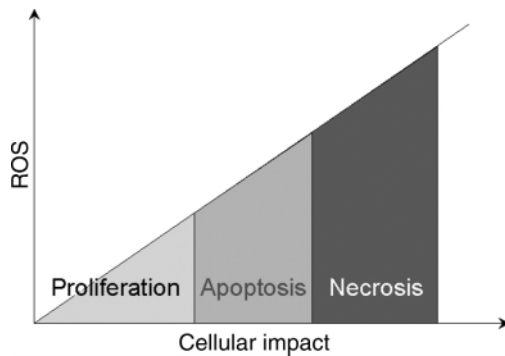


FIGURE 2. Schematic illustration of the cellular impact of reactive oxygen species (ROS). At low concentrations, ROS appear to exert a growth-stimulatory effect on a wide variety of cells and organisms. However, when ROS levels increase, other signaling pathways may be activated that lead to apoptosis. When ROS levels rise even higher, a cell will probably die a sudden necrotic death. Only the latter 2 modes of ROS action are currently being considered to be due to “oxidative stress.” The signaling function of ROS is now starting to be appreciated.

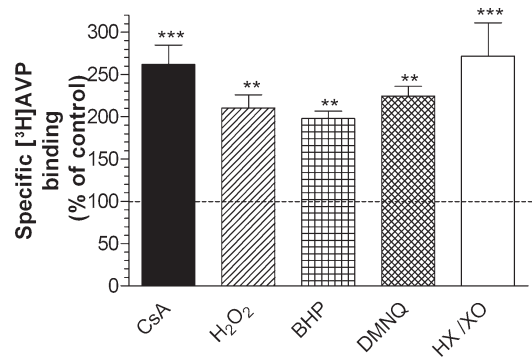


FIGURE 3. Effect of pro-oxidants on V_{1A} receptor protein levels. Rat aortic smooth muscle cells (RASMC) were treated with cyclosporin A (CsA; 3 μ M) or the pro-oxidants H_2O_2 (30 μ M), tert-butylhydroperoxide (BHP; 3 μ M), dimethylnaphthoquinone (DMNQ; 10 nM), or hypoxanthine (HX; 10 μ M)/xanthine oxidase (XO; 1 mU/ml) for 6 h at 37°C. Analysis of [3 H]Arg⁸-vasopressin (AVP) binding was performed after 20 h of treatment. Results are means \pm SE of at least 3 independent experiments done in triplicate or quadruplicate. Asterisks indicate values significantly different from control at $P < 0.01$ (**) or at $P < 0.001$ (***). Reproduced from Ref. 6, with permission.

pressin (AVP) to its receptor, consistent with an effect of O_2^- as mediator. In contrast, PEG-CAT had no effect on [3 H]AVP binding, demonstrating that H_2O_2 was not able to serve as a mediator in V_{1A} receptor increase.

This is consistent with the finding that higher concentrations of H_2O_2 were needed to increase V_{1A} receptor expression compared with the O_2^- donors. Indeed, the concentrations of the O_2^- donors (DMNQ and the hypoxanthine/xanthine oxidase system) needed to mimic the effect observed with CsA were extremely low. On the basis of published values for O_2^- generation by DMNQ (17), we calculated that the 10 nM DMNQ used in our study generated ~ 2 pmol $O_2^- \cdot$ mg protein⁻¹·min⁻¹. This is in the range of endogenous O_2^- generation that was shown to be ~ 4 pmol $O_2^- \cdot$ mg protein⁻¹·min⁻¹ in

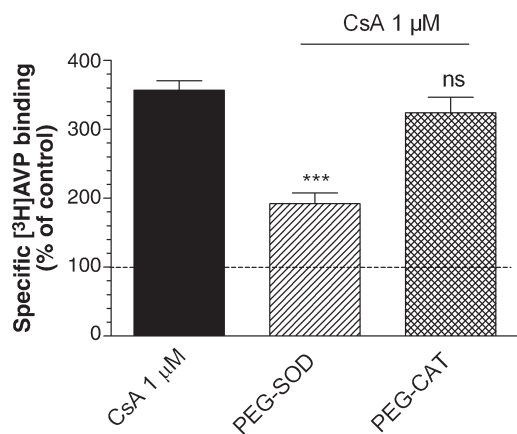


FIGURE 4. Effect of polyethylene glycol (PEG)-SOD and PEG-CAT on CsA-mediated increase in [3 H]AVP binding. RASMC were loaded with PEG-SOD (100 U/ml) or PEG-CAT (50 U/ml) for 6 h at 37°C in culture medium without serum. The medium was changed with medium containing CsA (1 μ M) but no PEG-SOD or PEG-CAT, and [3 H]AVP binding was measured 20 h later. Data are means \pm SE of at least 4 independent experiments performed in triplicate. Asterisks indicate values significantly different from CsA at $P < 0.001$ (***); ns, statistically not significant. Reproduced from Ref. 6, with permission.

aortic rings in the presence of NADH or NADPH. Thus increasing endogenous O_2^- production by 50% can have a physiological effect, in this case an increase in V_{1A} receptor expression. The consequence of this is a higher responsiveness of the tissue to AVP, resulting in increased vasoconstriction and perhaps also growth and differentiation.

Perspectives

Our findings further support the importance of O_2^- as physiological, local signaling molecule. In growing or differentiating cells or tissues, O_2^- may serve to increase cell or tissue responsiveness to growth- and/or differentiation-enhancing factors. In adult tissues, an exposure to O_2^- could additionally result in tissue hypertrophy. Similar to ANG II (20), AVP can also serve as a growth factor for vascular smooth muscle cells (9), and increased expression of the V_{1A} receptor can render these cells more responsive to this growth-stimulatory effect. In line with this argument, it has been shown that CsA can result in intimal thickening and enhanced growth factor-dependent growth of SMC (7). Thus O_2^- can alter cellular response(s) to growth factors and vasoconstrictor hormones and thereby alter cellular function.

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