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Endothelial Function and Oxidant Stress

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Summary: Both endothelial cells and vascular smooth muscle cells are capable of producing reactive oxygen species from a variety of enzymatic sources. In disease states such as atherosclerosis and hypertension, vascular production of these reactive oxygen metabolites can increase substantially. Increases in the production of superoxide anion can lead to decreases in ambient levels of nitric oxide via a facile radical-radical reaction that occurs more rapidly than the reaction of superoxide anion with superoxide dismutase. This phenomenon alters endothelial regulation of vasomotion in a variety of disease conditions. Recent evidence suggests that the major source of vascular superoxide ion and hydrogen peroxide is a membrane-bound, reduced nicotinamide-adenine dinucleotide (NADH)-dependent oxidase. The activity of this enzyme system is regulated by angiotensin II and is elevated following prolonged exposure to nitroglycerin. Alterations of vascular oxidant state caused by angiotensin II may contribute substantially to vascular pathology and may also provide a link between hypertension and atherosclerosis.

Key words: atherosclerosis, angiotensin II, endothelial function, hypertension, nitric oxide, superoxide anion, superoxide dismutase

Introduction

During the past decade, it has become apparent that numerous disease states are associated with abnormalities of endothelium-dependent vascular relaxation in both large and arteriolar vessels. While the mechanisms whereby these various disease processes alter endothelium-dependent vascular relaxation are likely multifactorial, several studies from our laboratory and others have suggested that oxidative inactivation of nitric oxide (NO) may be important in some circumstances. These studies indicate that a tenuous balance exists in the vessel wall between the steady-state levels of NO and superoxide anion (·O₂⁻). This review will discuss factors that may modulate vascular levels of ·O₂⁻ and NO and the evidence that imbalances between these two can predispose to alterations of vascular regulation in several important disease conditions.

Interactions between Nitric Oxide and Superoxide: A Potential Mechanism for Modulating Vasomotor Tone?

Even before the endothelium-derived relaxing factor (EDRF) was shown to be NO or a closely related compound, it was known that its half-life could be shortened by exposure to artificially generated superoxide and prolonged by superoxide dismutase (SOD). Shortly after the identification of EDRF with NO, Eric Feigl, in a commentary to the journal Science, suggested that an important role of the EDRF might be to scavenge ·O₂⁻. This suggestion was based on knowledge of chemical reactions in which superoxide and NO reacted with one another in a facile radical-radical reaction. Since NO could be inactivated by superoxide, it was reasoned that superoxide itself might be inactivated by NO.

At first glance, a role for NO in scavenging superoxide seemed unlikely. Both prokaryotic and eukaryotic cells contain large amounts of various SOD enzymes with a high affinity for superoxide. One might suspect that these SODs would scavenge all of the superoxide made in the vessel wall, preventing the radical from reacting with NO. Recent evidence suggests, however, that in the vessel wall there exists a tenuous balance between superoxide, NO, and cellular antioxidant defense mechanisms (particularly SODs) and that reactions between superoxide and NO may be quite important. For example, it has been shown that the reaction rate between superoxide and NO is extremely rapid (6.7 × 10⁸ M⁻¹ s⁻¹). This rate is approximately three times faster than the reaction rate of superoxide with either the manganese or the copper-zinc form of SOD. In a compartment containing both NO and SOD, there
may be a propensity for superoxide to react preferentially with NO rather than with SOD, depending on the relative concentrations of each.

In addition, there may exist compartments in the cellular and extracellular space in which the scavenging of superoxide by SOD is limited. For example, both SOD and vascular cell membranes carry a negative charge, leading to an electrostatic repulsion between the two. Therefore, interactions between NO and superoxide may occur preferentially over reactions between superoxide and SODs in or near cellular membranes. Such reactions might also occur in interstitial spaces.

Regulation of Vascular Superoxide Production

Studies in Hypercholesterolemia: Possible Role of Xanthine Oxidase

Some of the earliest evidence that vascular levels of superoxide could modulate NO bioactivity came from studies of vessels from cholesterol-fed rabbits. Several conditions, including hypercholesterolemia and atherosclerosis, are associated with altered bioactivity of NO as reflected by abnormal endothelium-dependent vascular relaxation. The responsible mechanisms may vary depending on the stage of the disease, the vascular bed examined, and the animal model studied. Nevertheless, we found that aortas from cholesterol-fed rabbits produce even larger amounts of NO than aortas from control rabbits, but that the NO seemed to be oxidatively degraded. Treatment of the rabbits with polyethylene-glycolated SOD dramatically increased endothelium-dependent vascular relaxation, further suggesting a role for superoxide. Finally, direct measurements of superoxide production using lucigenin chemiluminescence have shown that aortic segments from cholesterol-fed rabbits (4 weeks on diet) produce approximately threefold more superoxide than the aortas of control rabbits. The superoxide production seemed to come from xanthine oxidase, as it was inhibited by oxypurinol. In more recent studies, White et al. have shown that levels of xanthine oxidase are increased in the plasma of cholesterol-fed rabbits and that this circulating xanthine oxidase binds to heparin-binding sites on the vessel wall, where it acts to produce excess superoxide. Inhibition of xanthine oxidase with oxypurinol improves endothelium-dependent vascular relaxation, further suggesting a role for superoxide.

The role of xanthine oxidase may be limited to the early stages of hypercholesterolemia. As atherosclerosis develops and more complicated plaques form, other oxidases become predominant, and oxypurinol or similar agents become less effective in either decreasing superoxide or improving endothelium-dependent vascular relaxation. Preliminary studies in apolipoprotein E-deficient mice indicate a substantial increase in reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase in atherosclerotic vessels, perhaps due to accumulation of macrophages containing the NADPH dependent enzyme.

Studies of Other Oxidase Systems: Role of NADH/NADPH-Dependent Oxidases

Oxidases are present in virtually all mammalian cells and are often named based on their substrate preference, as with xanthine oxidase. One of the best-characterized oxidase systems is neutrophil oxidase, which is composed of at least five components: two cytosolic components (p47phox and p67phox), a small-molecular-weight G protein similar to rac-2, and two membrane-bound components. The last two, p22phox and gp91phox, comprise the cytochrome b558. When activated, the cytosolic components translocate to the membrane components and, upon assembly, create an active oxidase. Neutrophil oxidase is activated by phorbol esters, f-met-leu-phe, and opsonized zymozan. When activated in neutrophils, the enzyme transfers electrons from NADPH to flavins, subsequently to a heme group, and eventually to molecular oxygen. The neutrophil enzyme prefers NADPH over NADH in a ratio of 3:1. Mutations occurring in the various protein subunits of the neutrophil oxidases are associated with chronic granulomatous disease.

What does the neutrophil oxidase have to do with vascular biology? During the past 2 to 3 years, it has become apparent that both the endothelium and vascular smooth muscle contain membrane-bound oxidases that utilize NADH and NADPH as substrates for electron transfer to molecular oxygen. These oxidases have similarities to neutrophil NADPH oxidase in that they possess flavin-binding and heme-binding regions, which are likely important in the transfer of electrons. A common component of neutrophil oxidase and vascular smooth muscle oxidase is p22phox. Dr. Kathy Griendling has recently cloned the p22phox of vascular smooth muscle and has shown that it exhibits a high homology to the neutrophil analog. Inhibition of p22phox expression in vascular smooth muscle using antisense techniques results in a loss of NADH oxidase activity.

Despite these similarities, there are important differences between the vascular and neutrophil oxidases. First, the output of vascular oxidase is much lower than that of neutrophil oxidase (nmol vs. pmol/min/mg protein). Second, vascular oxidase does not exhibit “bursts” of activity as does neutrophil oxidase. This property of low output does not lessen the importance of the vascular oxidase system. The neutrophil oxidase system serves a bactericidal role, while vascular oxidase may have other functions, such as modulation of NO activity. Third, unlike neutrophil oxidase, smooth muscle oxidase uses NADH for electron transfer in preference to NADPH.

Regulation of Oxidase Activity by Angiotensin II: Studies in Cultured Cells and Intact Animals

A particularly important aspect of the vascular NADH/NADPH oxidase systems is that their activity is regulated by angiotensin II and certain cytokines. Studies by Dr. Griendling have shown that a 4-h treatment of cultured vascular smooth muscle cells with nanomolar levels of angiotensin II...
markedly increases NADH and NADPH oxidase activity (Fig. 1).12

Recently, we extended these findings to an in vivo model of angiotensin II-induced hypertension.19 We used osmotic minipumps to infuse angiotensin II subcutaneously (0.6 mg/kg/day) in Sprague-Dawley rats. To study a model of hypertension associated with low levels of angiotensin II, we also treated rats with a subcutaneous infusion of norepinephrine for a similar period of time. After 5 days the animals were sacrificed and their aortas removed for studies (by lucigenin chemiluminescence) of \( \cdot \mathrm{O}_2^\cdot \) production. Systolic blood pressure and \( \cdot \mathrm{O}_2^\cdot \) production are shown in Figure 2. The difference illustrated in \( \cdot \mathrm{O}_2^\cdot \) production between angiotensin II-treated and sham-operated animals persisted in experiments in which we intentionally removed the endothelium, suggesting the vascular smooth muscle as a likely source for the increase in \( \cdot \mathrm{O}_2^\cdot \).

Characterization of the Source of \( \cdot \mathrm{O}_2^\cdot \) in Angiotensin II-Treated Rats

In additional experiments, we sought to characterize the enzyme systems involved in this increase in \( \cdot \mathrm{O}_2^\cdot \) production. Diphenylene iodonium, an inhibitor of flavin-containing enzymes, normalized \( \cdot \mathrm{O}_2^\cdot \) production in vessels removed from angiotensin II-infused animals. In contrast, \( \cdot \mathrm{O}_2^\cdot \) production was not altered by rotenone, oxyurinol, indomethacin, or L-nitroarginine, suggesting that the source of the \( \cdot \mathrm{O}_2^\cdot \) was not mitochondrial electron transport, xanthine oxidase, cyclooxygenase, or NO synthase. We made homogenates of tissue and examined the ability of various agents to serve as substrates for \( \cdot \mathrm{O}_2^\cdot \) production. Arachidonic acid, xanthine, and succinate (in the presence of antimycin) had only a minimal effect on \( \cdot \mathrm{O}_2^\cdot \) production. In contrast, both NADH and, to a lesser extent, NADPH served as substrates for \( \cdot \mathrm{O}_2^\cdot \) production. In both cases, more than 98% of the activity occurred in the membrane and was greater in the vessels from angiotensin II-treated animals (Fig. 3).19

Role of Increased \( \cdot \mathrm{O}_2^\cdot \) Production in Angiotensin II-Induced Hypertension and Changes in Vascular Reactivity

The increase in vascular smooth muscle production of \( \cdot \mathrm{O}_2^\cdot \) caused by angiotensin II treatment was associated with a marked impairment in endothelium-dependent vascular relaxation. This impairment was not observed in vessels from rats treated with norepinephrine (Fig. 4).19

Because loss of NO can contribute to hypertension, we reasoned that an increase in \( \cdot \mathrm{O}_2^\cdot \) in the resistance circulation might contribute to the hypertension caused by infusion of angiotensin II. In a recent study,20 we lowered endogenous steady-state levels of vascular \( \cdot \mathrm{O}_2^\cdot \) by treating rats with daily injections of pH-sensitive, liposome-entrapped SOD. This

![Graph showing the effect of angiotensin II and norepinephrine on systolic blood pressure](image)

**Fig. 2** (A) Effect of either angiotensin II (A II) or norepinephrine (NE) infusion on systolic blood pressures in rats (measured by tail cuff sphygmomanometry). Although blood pressure rose more rapidly in norepinephrine-treated animals, pressures after 5 days in both groups had plateaued at similar values. (B) Lucigenin counts, reflecting \( \cdot \mathrm{O}_2^\cdot \) production from intact aortic segments from norepinephrine-infused and angiotensin II-treated animals. Sham-operated animals served as controls. Also shown is the effect of concomitant treatment with the angiotensin receptor antagonist, losartan. Losartan treatment completely prevented (and, in fact, reduced below normal levels) the effect of angiotensin II on \( \cdot \mathrm{O}_2^\cdot \) production. *p<0.01 versus sham. Reproduced from Ref. No. 19 by copyright permission of the American Society for Clinical Investigation.

![Graph showing superoxide production in response to NADH and NADPH](image)

**Fig. 3** Superoxide production in response to either NADH or NADPH in particulate (A) and cytosolic (B) fractions of aortic homogenates from sham-operated and angiotensin II-treated rats. The data indicate an activation of a membrane-bound NADH-dependent oxidase in aortic tissue from the angiotensin II-treated rats. *p<0.01 versus sham. Reproduced from Ref. No. 19 by copyright permission of The American Society for Clinical Investigation.
treatment had no effect on blood pressure in either control or norepinephrine-infused rats, but lowered blood pressure by 60 mmHg in rats with angiotensin II-induced hypertension (Fig. 5). These data suggest that a portion of the hypertension in conditions of elevated angiotensin II is associated with an increase in vascular superoxide production.

These findings may provide some insight into why forms of hypertension associated with elevated plasma renin activity (and presumably elevated effects of angiotensin II) are associated with increased rates of cardiovascular events. It is of interest that hypertension induced by norepinephrine infusion was not associated with an increase in vascular •O₂⁻ production and did not alter endothelial regulation of vasomotion. We also found that infusion of lower doses of angiotensin II, which had minimal effects on blood pressure, also increased NADH oxidase activity by about twofold. This result suggested that hypertension per se is not a stimulus for increased •O₂⁻ production, but that conditions in which circulating or local levels of angiotensin II are increased may have unique effects on the vessel wall independent of elevating blood pressure. Further, hypertension not associated with increases in angiotensin II and activation of vascular oxidases may be less prone to produce vascular disease.

The effect of hypertension on endothelium-dependent vascular relaxation is somewhat controversial (for a review, see Ref. No. 22). Furthermore, the cause of altered endothelial regulation of vasomotion may vary in different forms of hypertension. Based on our current findings, we may speculate that animal models or human subjects with hypertension associated with elevated levels of angiotensin II might exhibit greater alterations of endothelium-dependent vascular relaxation than do hypertensive conditions associated with low-renin, low-angiotensin II states. Future studies of endothelium-dependent vascular relaxation in humans should take into account the renin-angiotensin II profiles of the subjects enrolled. Such studies may provide insight into why treatment with angiotensin-converting enzyme inhibitors or angiotensin II-receptor antagonists may have beneficial effects not seen with other drugs.

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Role of NADH/NADPH Oxidase in Nitrate Tolerance

Angiotensin II-induced hypertension is not the only situation in which vascular oxidases are activated. In other studies, we found that nitrate tolerance is in part due to an increase in vascular superoxide production. Rabbits treated for 3 days with nitroglycerin demonstrated decreased vascular relaxations to nitroglycerin and cross-tolerance to NO endogenously released by acetylcholine. Tolerance to nitroglycerin was greatest when the endothelium was present. In these studies, we found that superoxide production by aortic segments from nitrate-tolerant animals was increased twofold. It is of interest
that, as with tolerance, this increase in superoxide production was greatest in vessels with intact endothelium, suggesting that a major source of the superoxide was the endothelium itself. Subsequent studies of homogenates of these vessels showed an increase in NADH-dependent oxidase activity, identical to that previously observed in angiotensin II-treated rats. Because nitrate therapy is associated with an increase in plasma renin activity, we hypothesized that the increase in oxidase activity might be secondary to angiotensin II. In preliminary studies, we found that treatment with the angiotensin-receptor antagonist, losartan, completely normalized vasodilation to nitroglycerin and reduced vascular superoxide production to normal.

A related finding in these studies was that treatment with hydralazine markedly inhibited superoxide production and reduced vascular NADH oxidase activity tremendously. Since hydralazine prevents nitrate tolerance, its inhibition of NADH oxidase may play a role in this phenomenon. These various studies have provided some insight into how vascular production of superoxide can play a role in regulating the bioactivity of NO, produced either endogenously or administered exogenously. Given that the levels of production of both NO and superoxide are subject to modest degrees of control, the interplay between the two may be quite dynamic. A final manner in which the levels of superoxide and NO may be modulated relates to scavenging of superoxide in the vessel wall.

Regulation of Vascular Antioxidant Defense Mechanisms

While a substantial amount has been learned about control of vascular NO and superoxide levels, less is known about what may control endogenous antioxidant defense mechanisms. The most important of these mechanisms in terms of superoxide are the SODs. There are three types of SOD—mitochondrial manganese-containing SOD, cytosolic copper-zinc SOD, and an extracellular SOD—and their regulation varies substantially. Copper-zinc SOD plays an important role in modulating the release of bioactive NO. Pharmacologic inhibition of copper-zinc SOD results in release of NO from the endothelium in an oxidatively inactive form, likely as nitrite and nitrate. Thus, copper-zinc SOD plays an important role in protecting NO in the endothelium.

Based on this observation, we recently hypothesized that shear stress might modulate the expression of copper-zinc SOD. This hypothesis was based on our earlier finding that endothelial NO synthase is regulated in response to shear stress. We reasoned that an increase in copper-zinc SOD might assure that any additional NO made by vessels previously exposed to high shear might be released more efficiently. We exposed human aortic endothelial cells to various levels of shear and examined the expression of copper-zinc SOD at the mRNA, protein, and activity level. The results are shown in Figures 6 and 7.

It has been observed that vessels exposed to elevated shear stresses exhibit enhanced endothelium-dependent vascular relaxations. Shear stress also increases the expression of endothelial cell NO synthase mRNA and protein (both approximately threefold for shears of 15 dynes/cm² compared with static conditions), and increases the capacity of endothelial cells to release NO (approximately twofold at 15 dynes/cm² for 24 h compared with static conditions). These findings of shear regulation of copper-zinc SOD expression suggest that augmented endothelium-dependent relaxations in vessels exposed to high shear stress may be mediated by increases in the expression of endothelial cell NO synthase and that increased expression of copper-zinc SOD might synergistically potentiate the vasorelaxant capacity of endothelium-derived NO. Although the measured increase in SOD activity was modest in these studies, even a small increase in SOD activity will markedly decrease the half-life of $\cdot O_2^-$. The distribution of hemodynamic forces is thought to influence the development of atherosclerosis substantially. In humans, regions of low shear stress are more prone to develop atherosclerosis than regions exposed to high shear stress. In experimental animals, plaque formation is greater in regions with low shear stresses, while elevated shear stresses tend to protect against plaque formation and intimal thickening.

Differences in expression of copper-zinc SOD may in part ex-
plain these observations. It is evident now that the reaction of NO and superoxide leads to the formation of peroxynitrite anion, which is protonated to form peroxynitrous acid. The latter can yield the hydroxyl radical and nitrogen dioxide. Peroxynitrite has been shown to produce endothelial cell injury and to oxidize sulfhydryl groups. Both superoxide and the hydroxyl radical may contribute to oxidation of low-density lipoproteins. Recently, it has become evident that reactive oxygen species contribute to cell activation and intracellular signal transduction via redox-sensitive genes, such as vascular cell adhesion molecule-1, tissue factor, monocyte chemotactic protein-1, and others. Preservation of the half-life of NO may also have other antiatherogenic properties, such as inhibition of platelet and neutrophil adhesion and inhibition of vascular smooth muscle growth. These lines of evidence suggest that induction of copper-zinc SOD by shear stress might have antiatherogenic properties by reducing superoxide levels and subsequent formation of peroxynitrite.

Conclusion

The data presented in this review suggest levels of regulation not only of NO but also of superoxide and SOD in cells in the vessel wall. While none of these is dramatically altered, the effect of concomitant regulation of all three may greatly affect several aspects of vascular biology, including vasomotor tone, redox state, predisposition for disease, and gene regulation. Clearly this work is in its infancy, and a great deal more remains to be learned regarding these interactions. It is likely that specific interventions in these processes may lead to therapeutic advances in the future.

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