

Review

Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure

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1. Background: the concept of ‘endothelial dysfunction’

Soon after the discovery of EDRF (endothelium derived relaxins factor) it became apparent that certain diseases are associated with an impairment of endothelium dependent vasorelaxation. In hypercholesterolemic rabbits and monkeys, vasorelaxation to acetylcholine is almost absent (Fig. 1) or changed into vasoconstriction [1,2]. Similar observations have been made in patients with coronary artery disease [3,4] or risk factors predisposing to atherosclerosis [5]. Likewise, endothelium-dependent vasorelaxation is abnormal in disease states such as heart failure, diabetes and hypertension [6]. In almost all of these disorders, there is a loss of endothelial production and/or bioavailability of NO (nitric oxide, nitrogen monoxide). This alteration of vascular function has been termed ‘endothelial dysfunction’ in the scientific literature. Although this term is widely used, it is quite imprecise. ‘Endothelial dysfunction’ may refer to impairments of important endothelial functions other than vasodilation, including anticoagulant and anti-inflammatory properties of the endothelium [7,8]. Nevertheless, ‘endothelial dysfunction’ has become widely used, and in fact, loss of nitric oxide in these conditions may contribute to alterations of other aspects of vascular function. The mechanisms underlying altered endothelium-dependent vascular relaxation in various disease states are almost certainly multifactorial, and seem to be dependent on the specific pathological condition, its duration, and the vascular bed being studied. Treatment with L-arginine or tetrahydrobiopterin has improved NO-mediated vasodilation in some instances, suggesting that there may be a

deficiency of either the substrate for the enzyme NO synthase or one of its critical co-factors. Alterations of endothelial cell signaling may impair appropriate activation of the NO synthase in response to neurohumoral or mechanical stimuli. In very advanced atherosclerosis, expression of NO synthase in the endothelium declines, almost certainly reducing endothelium-dependent vascular relaxation. Finally, there is substantial evidence that in certain disease conditions, NO production is not altered, but its bioavailability is reduced because of oxidative inactivation by excessive production of the superoxide anion (O_2^-) in the vascular wall. Evidence for this phenomenon has been found in such diverse conditions such as hypercholesterolemia, atherosclerosis, hypertension, diabetes, heart failure, and cigarette smoking. The purpose of this review is to examine the mechanisms whereby vascular cells produce excessive reactive oxygen species (and in particular superoxide), to consider cellular and molecular mechanisms underlying their reactions with NO, and to discuss the effects of reactive oxygen species on vascular diseases.

2. Vascular oxidant stress and sources of reactive oxygen species

2.1. Vascular oxidant stress

The term oxidative stress is often used to imply a condition in which cells are exposed to excessive levels of either molecular oxygen or chemical derivatives of oxygen called reactive oxygen species. In the process of normal cellular metabolism, oxygen undergoes a series of univalent reductions, leading sequentially to the production of

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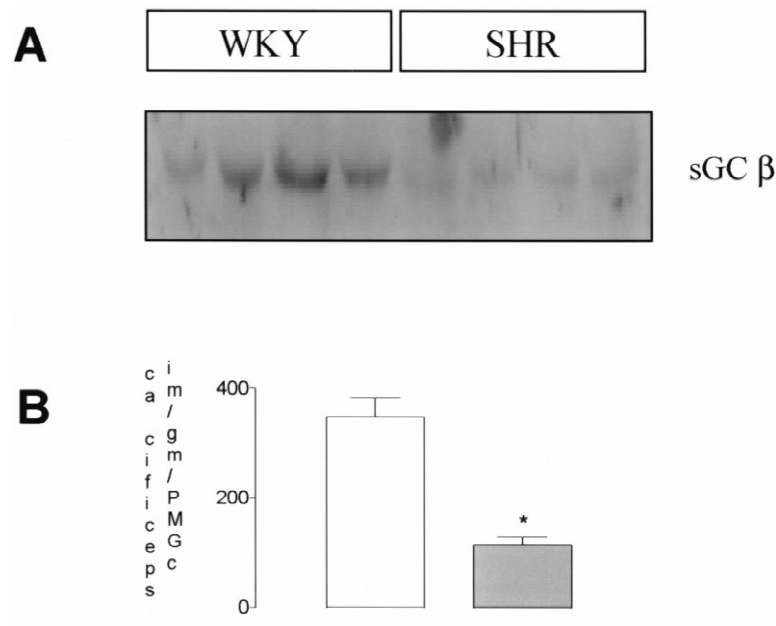


Fig. 1. Endothelium-dependent relaxation of normal and atherosclerotic arteries to acetylcholine and the calcium ionophor A23187 (adapted from Ref. [2]).

superoxide, hydrogen peroxide (H_2O_2), and H_2O . Other oxidants that have relevance to vascular biology are hypochlorous acid (HOCl), the hydroxyl radical (OH), peroxynitrite (OONO^-), reactive aldehydes, lipid peroxides, lipid radicals, and nitrogen oxides. Several of these, such as superoxide, the hydroxyl radical, and NO are radicals with an unpaired electron in their outer orbital. Other oxidants, such as hydrogen peroxide and peroxynitrate are not radicals but are biologically active.

In mammalian cells, potential enzymatic sources of reactive oxygen species include the mitochondrial electron transport chain, xanthine oxidase, cyclooxygenase, lipoxigenase, NO synthase, heme oxygenases, peroxidases, hemoproteins such as heme and hematin, and NADH oxidases. One of the best characterized sources of reactive oxygen species is the phagocytic NADPH oxidase. This enzyme system produces large, cytotoxic amounts of radicals when the phagocytic cells are activated. During the past several years, it has become apparent that a major source of reactive oxygen species in blood vessels is a membrane-associated NADH/NADPH oxidase expressed by endothelial, vascular smooth muscle cells and fibroblasts that bears some similarity to the phagocytic oxidase (discussed below).

The terms 'oxidative stress' and 'redox state' are often used interchangeably, without attention to their true meaning. In contrast to oxidative stress, defined above, the redox state or redox potential of a cell refers to the chemical environment within the cell as it relates to the number of reducing equivalents available. This can be estimated by examining ratios of so-called 'redox couples'. These include lactate/pyruvate, NADH/ NAD^+ , and the ratio of reduced and oxidized glutathione. Exposure of cells to oxidizing conditions may consume reducing equi-

valents and thus alter the redox state; however, the redox state may be altered in other ways. For example, treatment of cells with lactate can increase levels of NADH, by converting NAD^+ to NADH via the action of lactate dehydrogenase. Exposure to high concentrations of pyruvate can produce the opposite effect. Thus, the redox state may also be altered by oxidative stress; however, an altered redox state may not necessarily change the oxidative environment.

In the vascular wall, increases in oxidant stress are thought to alter several important physiological functions. Regulation of blood flow, inhibition of platelet aggregation, inhibition of leukocyte adhesion and control of cellular growth are influenced by oxidant stress. These phenomenon ultimately modulate vessel diameter, remodelling and lesion formation [8,9]. Reactive oxygen species which are thought to have relevance to vascular biology include superoxide, hydrogen peroxide, peroxynitrite, lipid hydroperoxides and hydroperoxy-radicals and probably hydroxyl-like radicals. Both hydrogen peroxide and peroxynitrite are generated as reaction products of the superoxide anion. While hydrogen peroxide mainly emerges from intra- and extracellular dismutation of superoxide by the abundantly present superoxide dismutases, peroxynitrite is formed by the rapid reaction of superoxide with NO (see below). Thus, the generation of superoxide anions most likely plays a central role as the source for many other reactive oxygen species.

2.2. Enzymatic sources of reactive oxygen species in vascular tissues

While there are a myriad of enzymes and enzyme systems which could potentially produce reactive oxygen

species in vascular cells, three have been studied rather extensively in the past few years. These include the NADH/NADPH oxidase, xanthine oxidoreductase, and NO synthase, and will be discussed separately below.

The major source of reactive oxygen species in vascular adventitia and in both endothelial and vascular smooth muscle cells are membrane bound oxidases which utilizes NADH and NADPH as substrates [10–12]. The structures of these enzyme systems have yet to be clearly elucidated. The vascular NADH/NADPH oxidases show some similarities but also striking differences to the NADPH-oxidase of neutrophils. The vascular enzymes have a lower output and do not show the ‘burst activity’ typical for the neutrophil enzyme. These enzymes predominantly prefer NADH as a substrate [13]. The endothelial and vascular smooth muscle cell NADH/NADPH oxidases are also probably not all similar. All components of the functional NADPH-oxidase enzyme have been found in endothelial cells [14]. In contrast, only p22fox could be identified in smooth muscle cells and has been shown to participate in the increased superoxide production upon stimulation with angiotensin II and TNFalpha [11,15,16].

Interestingly, the activity of these oxidases appears to be

regulated by cytokines, physical forces, and tissue hormones which are critically involved in the pathogenesis of oxidant stress-related vascular diseases. Exposure of cultured vascular smooth muscle cells to angiotensin II and tumor necrosis factor alpha increased the activity of NADH/NADPH oxidases and subsequent formation of reactive oxygen species was observed [10,11]. In accordance, treatment of rats with angiotensin II increased vascular superoxide production independent of the concomitant hypertension as evidenced by parallel experiments using permanent infusions of norepinephrine (Fig. 2) [17]. The superoxide generation induced by angiotensin II was NADH/NADPH dependent and most likely occurred in the smooth muscle [18]. Cyclical stretch has also been shown to increase the production of both superoxide and hydrogen peroxide by endothelial and vascular smooth muscle cells [19–21].

The xanthine oxidoreductase is a molybdoenzyme capable of catalyzing the oxidation of hypoxanthine and xanthine in the process of purine metabolism. Xanthine oxidoreductase can exist in two interconvertible forms, either as xanthine dehydrogenase or xanthine oxidase. The former reduces NAD^+ , while the latter prefers molecular

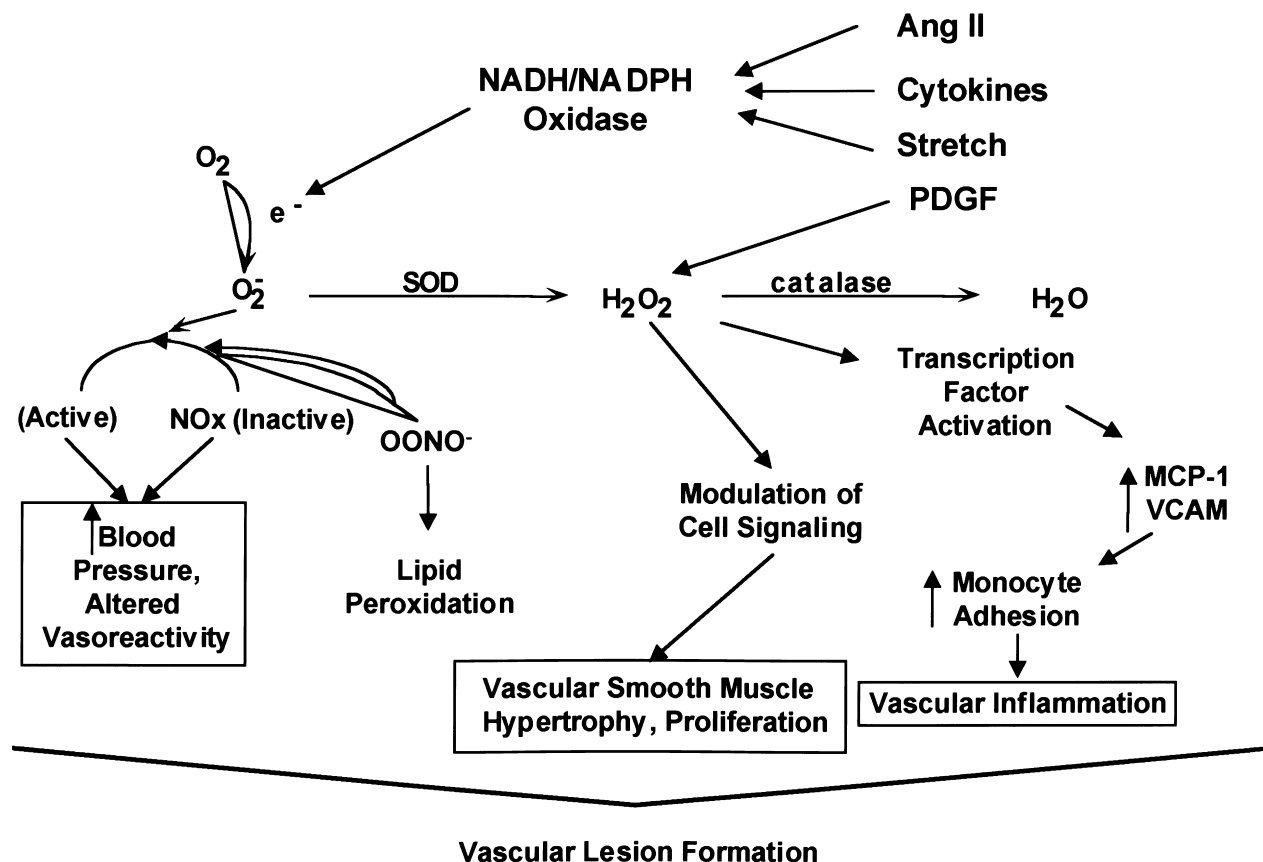


Fig. 2. Impairment of endothelium-dependent vasorelaxation to acetylcholine and the calcium ionophor A23187 after experimental hypertension in rats. Both infusion of angiotensin II and of norepinephrine resulted in a comparable degree of hypertension but only treatment with angiotensin II induced an impairment of endothelium-dependent vasorelaxation. While empty liposomes had no effect on blood pressure, infusion of liposomes containing superoxide dismutase (SOD) selectively diminished hypertension in angiotensin II-treated rats suggesting a contribution of vascular superoxide production to the effects of angiotensin II on blood pressure and endothelium-dependent vasorelaxation (adapted from Ref. [60]).

oxygen, leading to the production of both superoxide and hydrogen peroxide [22]. It has been shown that early stages of hypercholesterolemia are associated with increased superoxide production derived from endothelial xanthine oxidase (Fig. 3) [23,24]. Both inhibition of xanthine oxidase with oxypurinol and its displacement from the heparin binding site by infusion of heparin improved the impairment of endothelium-dependent vasorelaxation. Recently, it has been shown that xanthine oxidoreductase is asymmetrically localized on the outer surface of human endothelial cells in culture [25]. The role of xanthine oxidoreductase in vascular production of reactive oxygen species remains poorly defined, in part because in the oxidase form, the enzyme is not inhibited by oxypurinol and can use NADH as a substrate for reduction of oxygen [22], and thus could masquerade as an NADH oxidase, similar to the enzyme system discussed above. Methods to separate the function of two enzyme systems are not yet universally available.

Another potential source of vascular superoxide production is eNOS. Early studies with neuronal NOS showed that this enzyme type is capable of producing reactive oxygen species if either L-arginine or tetrahydrobiopterin is absent [26,27]. Interestingly, the NOS co-factor tetrahydrobiopterin has also been shown to non-enzymatically generate superoxide, and this limits the ability of the NOS to produce free NO in the absence of superoxide dismutase [28]. Recently, such studies have been extended to the endothelial isoform of NOS. Xia et al. have shown that in the absence of tetrahydrobiopterin, eNOS can generate superoxide, likely via its heme center. In this study, superoxide production by eNOS was not affected by L-arginine [29]. Vasquez-Vivar et al. have reported that eNOS can produce considerable amounts of superoxide by two different mechanisms [30]. In the absence of sufficient cofactors, the oxygenase domain of eNOS can generate superoxide from the dissociation of the heme ferrous-

dioxygen complex. These investigators also showed that superoxide can be produced by flavins in the reductase domain of eNOS. While eNOS generation of superoxide can be demonstrated in in-vitro biochemical preparations, it is less clear that the NOS enzymes are ever sufficiently depleted of co-factors in vivo to serve as a source of superoxide. Pritchard and co-workers have provided evidence that treatment of endothelial cells in culture with native low density lipoprotein (LDL) may increase their production of superoxide in a fashion which seems to be dependent on eNOS, perhaps due to dissociation of L-arginine from eNOS [31]. The mechanism whereby LDL could affect eNOS function in this manner has not been defined, however such a mechanism could have substantial pathological consequences.

3. Reactions of superoxide with NO in the vascular wall.

3.1. Reaction products

Both superoxide and NO are highly reactive and unstable radicals. Thus, it is not surprising that they react very rapidly at a rate estimated to be $6.7 \times 10^9 \cdot \text{M} \cdot \text{s}^{-1}$ to form the major product peroxynitrite [32]. This reaction is approximately three times faster than the dismutation of superoxide by superoxide dismutases, implying that increased generation of superoxide in the vascular wall may very well inhibit the physiological functions of NO. In addition, peroxynitrite is a strong oxidant and is more stable than either NO or superoxide [33]. At neutral pH, peroxynitrite can undergo protonation to form peroxynitrous acid which upon homolytic cleavage can yield hydroxyl-like and nitrogen dioxide radicals which are also strong oxidants [34,35].

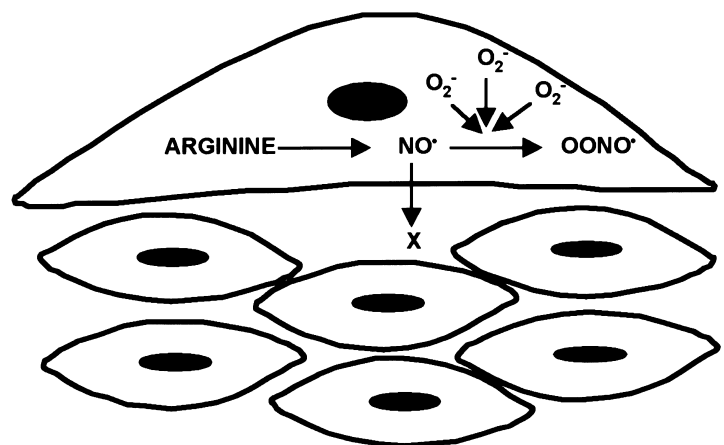
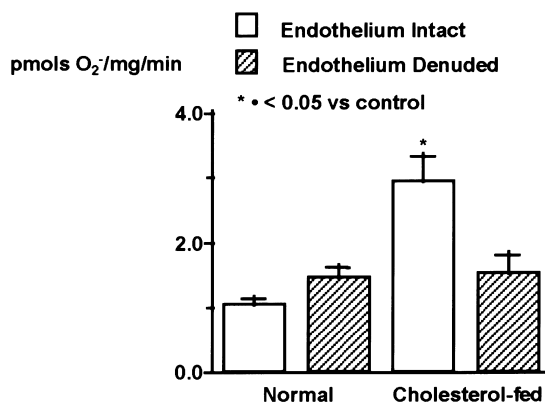


Fig. 3. Vascular superoxide production in normal and atherosclerotic arteries before and after endothelial denudation. The majority of superoxide in early atherosclerosis is produced in the endothelium. Superoxide induces an impairment of vascular functions by both the reduction of the bioavailability of NO and the generation of the toxic oxidant peroxynitrate (adapted from Ref. [23]).

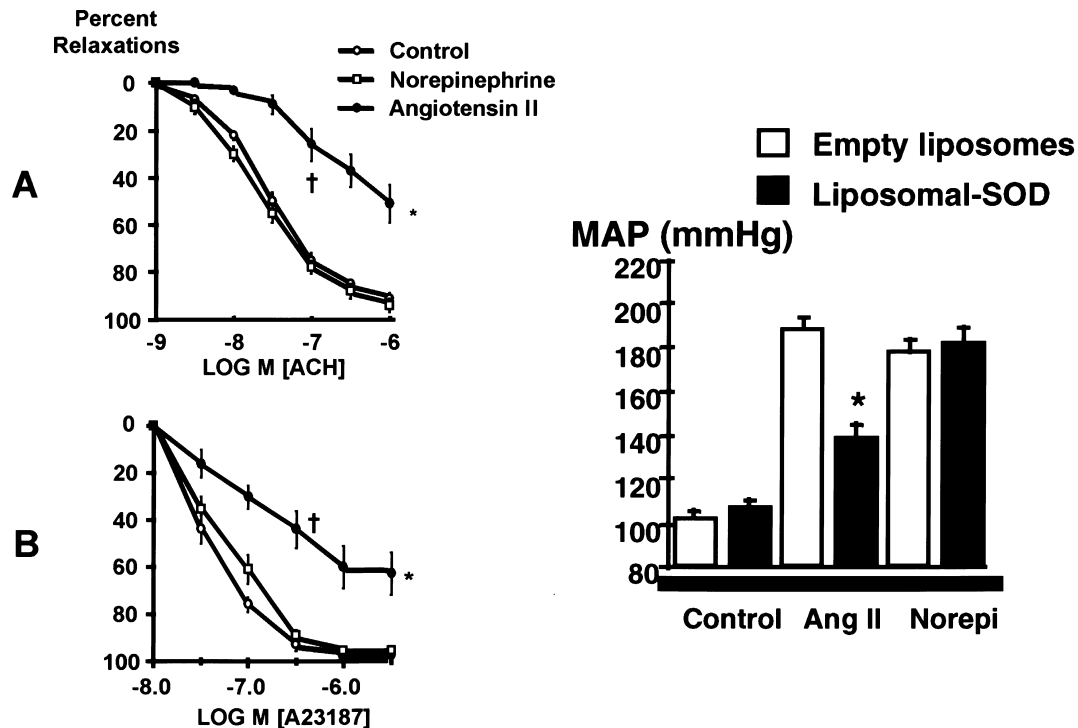


Fig. 4. Mechanisms of vascular oxidant stress. Stimulation of vascular oxidases results in overproduction of superoxide, which reduces the bioavailability of NO and increases the formation of the toxic oxidant peroxynitrate. Dismutation of superoxide results in increased cellular levels of hydrogen peroxide promoting inflammation, smooth muscle hypertrophy and smooth muscle proliferation.

3.2. Effects in the vascular wall

Although peroxynitrite can produce vasodilation, this effect occurs at concentrations far in excess of the effective vasorelaxant concentrations of NO [36–38]. Oxidation reactions induced by peroxynitrite such as modifications of iron–sulfur clusters, zinc-fingers, protein thiols and membrane lipids are likely involved in numerous pathophysiological processes [34,39,40]. Effects likely relevant to vascular disease are illustrated in Fig. 4. There is a growing body of evidence from animal experiments and clinical investigations indicating that a variety of diseases are indeed associated with increased vascular superoxide production impairing the important functions of endothelial NO production. Of these, hypercholesterolemia, hypertension, heart failure and diabetes are briefly reviewed below.

4. Hypercholesterolemia and coronary artery disease

Hypercholesterolemia was the first pathological condition shown to be associated with an impaired endothelium-dependent vasorelaxation both in animals and man [1–4]. Interestingly, hypercholesterolemia impairs endothelium-dependent vasodilation not only in large conductance vessels. Several investigations demonstrated a similar

effect in the microcirculation of the rabbit cremaster muscle [41], in primate and pig coronary microvessels [42,43] and in human coronary and peripheral circulation [44,45]. A major mechanism underlying impaired endothelium-dependent vasodilation in hypercholesterolemia is thought to be the destruction of readily formed endothelium-derived NO by excess ambient levels of superoxide. Chemiluminescence measurements of NO detected a two- to three-fold increase in the release of total nitrogen oxides from atherosclerotic versus normal rabbit aortas, despite the fact that the bioactivity of the nitrogen oxides was markedly diminished in the atherosclerotic vessels. These findings strongly suggested that the NO produced by the endothelium of atherosclerotic vessels had undergone oxidative destruction [46]. Subsequent studies have shown that treatment with polyethylene-glycolated superoxide dismutase partially restores impaired endothelium-dependent relaxation of rabbit aorta [47]. Excessive production of vascular superoxide was subsequently confirmed by direct demonstration of a three-fold increase of aortic superoxide production in the hypercholesterolemic rabbit [23]. This effect was completely abolished by endothelial denudation identifying the endothelial cell as the major source of abnormal vascular superoxide production in early atherosclerosis (Fig. 3). In later stages of the atherosclerotic process, other sources of superoxide are likely to be involved, such as activated macrophages in the intima

and smooth muscle cells [48]. Components of oxidized low density lipoprotein such as lysophosphatidylcholine, which accumulate in advanced atherosclerotic lesions, have been shown to stimulate protein kinase C and superoxide production in the entire vascular wall [49,50]. More recently, preliminary data from our laboratories have shown that endothelium-dependent relaxations to acetylcholine and A23187 can be corrected to normal in aortas of Apo(E)-deficient mice by acute treatment of the vessels with liposome-entrapped SOD (unpublished data). These findings in experimental animals have recently been extended to humans with hypercholesterolemia. Treatment with intra-arterial infusions of vitamin C improves endothelium-dependent vasodilation in the forearm of human subjects with hypercholesterolemia [51,52]. Taken together, these results strongly indicate that hypercholesterolemia and coronary artery disease is associated with a shift in the balance of vascular production of NO and superoxide that favors the generation of toxic radicals or radical products such as peroxynitrite or hydroxyl radicals, which may in turn promote the disease process.

5. Hypertension

Early evidence for superoxide production in hypertension was obtained by indirect measurements such as reduction of nitroblue tetrazolium in animals made acutely hypertensive by infusion of vasoconstrictors [53,54]. Interestingly, endothelium-dependent vasorelaxation was found to be markedly impaired at the same time. These observations led to the suggestion that superoxide may play a key role in pathologic changes of vascular reactivity induced

by hypertension [54,55]. In accordance, conventional copper/zinc-superoxide dismutase prevents vascular damage and increases survival in rats made hypertensive by infusion of either angiotensin II or norepinephrine [56,57]. Nakazano et al. showed that a form of superoxide dismutase modified to bind to heparan sulfates in the vessel extracellular matrix acutely lowered blood pressure in spontaneously hypertensive rats, while having no effect on blood pressure in normal rats [58]. In further studies, superoxide production was directly measured in both endothelial cells cultured from spontaneously hypertensive rats and aortic segments of rats made hypertensive by infusion of angiotensin II [17,59]. In the latter model treatment with liposomencapsulated superoxide dismutase normalized not only aortic superoxide production but also blood pressure (Fig. 2) [18,60]. More recently Schnackenberg et al. have shown that the membrane permeable superoxide dismutase mimetic tempol normalizes blood pressure in spontaneously hypertensive rats [61]. The significance of the results of these animal experiments was underlined by clinical data indicating the occurrence of increased superoxide production in man having essential hypertension [62,63].

The increase in superoxide generation in hypertension was shown to impact on the production and activity of endogenous vascular NO. Endothelium-dependent relaxation is strongly impaired following experimental elevation of blood pressure [17,53,59,60] and a similar situation was observed in man having essential hypertension [5,64]. Direct NO-measurement in endothelial cells and aortic rings of hypertensive rats have shown a decreased release of NO upon stimulation with the calcium ionophor A23187, and that this can be corrected by addition of

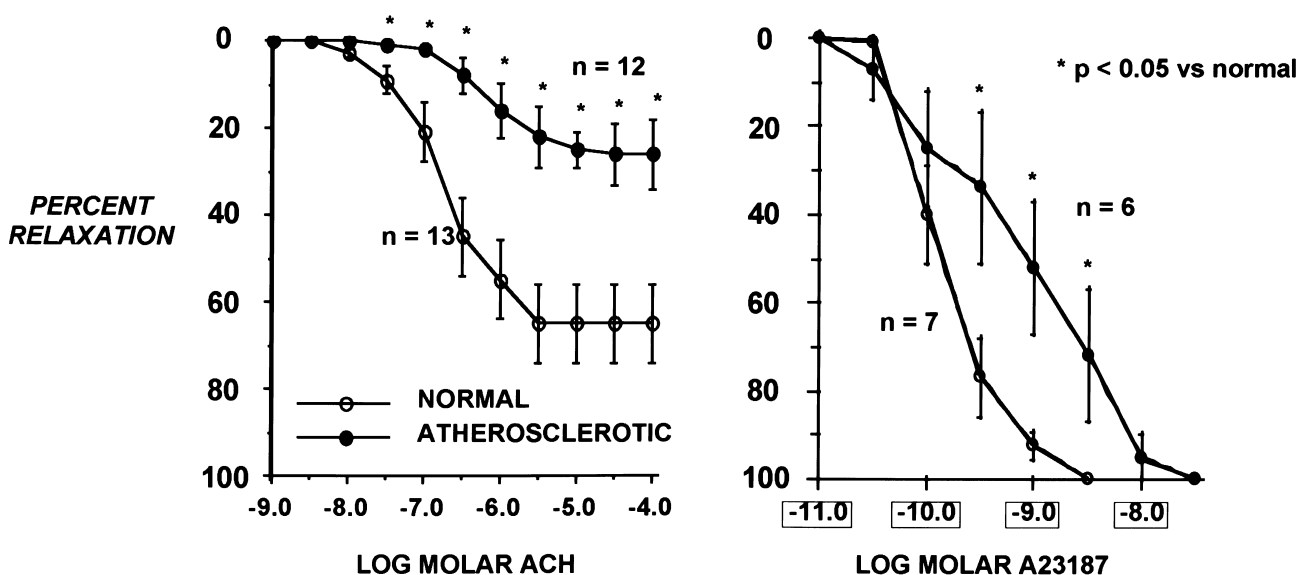


Fig. 5. Impairment of the NO/cGMP pathway in a genetic model of hypertension. In the vascular smooth muscle of spontaneously hypertensive rats (SHR) both expression and specific activity of soluble guanylate cyclase is strongly reduced suggesting that alterations of the key enzyme of the NO/cGMP pathway may contribute to the pathophysiology of hypertension (adapted from [66,67]).

superoxide dismutase [59,65]. Thus, reactions between NO and superoxide (see Chapter 3) are most likely a major cause of impaired endothelium dependent vasorelaxation in hypertension. In addition, recent reports have presented evidence for an impairment of the NO signal transduction pathway in animal models of hypertension (Fig. 5). It was shown that both the expression and the activity of vascular soluble guanylate cyclase in spontaneously hypertensive rats is markedly reduced [66,67]. To date, the precise alterations of the NO signal transduction pathway in hypertension remains unknown and further studies are needed to determine if oxidative stress contributes to this process.

6. Heart failure

It is well known that heart failure is accompanied by changes of reactivity of the coronary and systemic vessels. Treasure et al. [68], found impairment of endothelium-dependent vasorelaxation in the coronary circulation of patients with dilated cardiomyopathy. A similar effect was seen in conductive blood vessels in experimental heart failure [69] and in the peripheral circulation of patients with heart [70–72]. Oxygen-derived radicals have been implicated as a cause of endothelial dysfunction in heart failure; either as a result of enhanced radical formation or by a reduction of the antioxidant tissue reserve [73,74]. Oxidative stress in experimental and clinical heart failure has been assessed indirectly by measuring superoxide dismutase activity, glutathione peroxidase activity, catalase activity and plasma levels of lipid peroxides, thiobarbituric acid reactive substances, malondialdehyde or reduced thiols. Several clinical studies have reported enhanced oxidative stress in heart failure and have suggested a major contribution of superoxide [75–78]. Similar changes have been observed in experimental heart failure following myocardial infarction in rats [79].

Reactive oxygen species such as superoxide and peroxynitrite (see above) have been shown to impair myocardial function and thus are of interest in heart failure. Superoxide is able to rapidly and irreversibly inhibit calcium-induced force development in isolated rat myocardial muscle [80]. Peroxynitrite can inhibit mitochondrial electron transport, enhance the production of hydrogen peroxide and inhibit the citric acid cycle enzyme aconitase [81,82]. Application of peroxynitrite to isolated cardiomyocytes induces a pronounced cell injury leading to uncontrolled calcium influx and an impairment of the contractile apparatus [83]. Thus, cardiac work is markedly depressed after exposure of working rat hearts to exogenous peroxynitrite [84]. Further, formation of endogenous peroxynitrite likely contributes to myocardial depression during posthypoxic reoxygenation of isolated rat left ventricular papillary muscle preparations [85]. A recent *in vivo* study indicated that peroxynitrite is a likely candidate

for promoting myocardial dysfunction induced by cytokines in dogs [86].

7. Diabetes

Endothelium-dependent vasodilation is substantially impaired in diabetic animals [87] and humans [88,89]. Interestingly, endothelial dysfunction not only occurs in overt diabetes but is also inducible by simple pretreatment of isolated vessels with high glucose *in vitro* [90]. There is evidence that generation of increased vascular production of superoxide contributes to this impairment of endothelium-dependent vasorelaxation. Pretreatment of diabetic rat aorta with superoxide dismutase produces significantly greater relaxations to acetylcholine than the untreated controls [91] and feeding rabbits the antioxidant probucol prevented the impairment of endothelium-dependent relaxations in aortic rings incubated in high glucose [90]. Likewise, pretreatment with either superoxide dismutase plus catalase or an inhibitor of metal-facilitated hydroxyl radical formation (DETAPAC) has been shown to improve endothelial dysfunction in aortic rings of streptozotocin-induced diabetic rats suggesting that vascular production of both superoxide and hydroxyl radicals may contribute to endothelial dysfunction in this model [92]. Superoxide also seems to be involved in the oxidative modification of low density lipoprotein induced by glucose concentrations occurring in the diabetic state [93]. Another relevant mechanism involves direct inactivation of EDRF by advanced glycosylation products and increased adhesion of leucocytes to the endothelium [94,95].

The source of vascular oxygen-free radicals in diabetes is not clear. A recent study showed that NADH oxidase activity was increased in the retina of diabetic rats [96]. Interestingly, treatment of patients having insulin-dependent diabetes with an angiotensin converting inhibitor improves endothelium-dependent vasorelaxation as measured by changes in forearm bloodflow upon intrabrachial infusion of acetylcholine [97]. This result raises the possibility that the induction of vascular oxidases by angiotensin II (see Chapter 2) and subsequent production of superoxide and peroxynitrite (see Chapter 3) might also play a role in diabetes. Importantly, there is evidence that superoxide and peroxynitrite are important mediators of pancreatic cell death and thus might serve as pathogenic factors precipitating the disease itself [98–100].

8. Summary

There is a growing body of evidence suggesting that numerous pathological conditions are associated with increased vascular production of reactive oxygen species. This form of vascular oxidant stress and particularly interactions between NO and oxygen-derived radicals

represent a common pathological mechanism present in many so-called risk factors for atherosclerosis. Furthermore, reactive oxygen species seem to serve important cellular signalling mechanisms responsible for many of the features of vascular lesion formation. The mechanisms whereby vascular cells produce reactive oxygen species are only presently coming to light, and almost certainly will prove to be a focus for future therapies.

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