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Vascular oxidant stress: Molecular mechanisms and pathophysiological implications

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The term oxidative stress refers to a situation in which cells are exposed to excessive levels of either molecular oxygen or chemical derivatives of oxygen (ie, reactive oxygen species). Three enzyme systems produce reactive oxygen species in the vascular wall: NADH/NADPH oxidase, xanthine oxidoreductase, and endothelial nitric oxide synthase. Among vascular reactive oxygen species superoxide anion plays a critical role in vascular biology because it is the source for many other reactive oxygen species and various vascular cell functions. It is currently thought that increases in oxidant stress, namely excessive production of superoxide anion, are involved in the pathophysiology of endothelial dysfunction that accompanies a number of cardiovascular risk factors including hypercholesterolemia, hypertension and cigarette smoking. On the other hand, vascular oxidant stress plays a pivotal role in the evolution of clinical conditions such as atherosclerosis, diabetes and heart failure.

Key words: Nitric oxide, Oxidative stress, Peroxynitrite, Superoxide anion, Vascular diseases.

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 particulary interactions between nitric oxide or nitrogen monoxide (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 purpose of this review is to examine the mechanisms whereby vascular cells produce excessive

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reactive oxygen species (and in particular superoxide anion, O_2^-), to consider cellular and molecular mechanisms underlying their reactions with NO, and to discuss the effects of reactive oxygen species on vascular diseases.

Vascular oxidant stress

The term oxidative stress is often use 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 productions of O_2^-, 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 O_2^-, OH^-, and NO are radicals with an unpaired electron in their outer orbital. Other oxidants, such as H_2O_2 and $OONO^-$ 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, lipoxygenase, 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 oxidant 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 equivalents 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, namely excessive generation of O_2^-, are thought to alter several important physiological functions (fig. 1). 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 (1, 14). Reactive oxygen species which are thought to have relevance to vascular biology include O_2^-, OH^-, $OONO^-$, lipid hydroperoxydes and hydroperoxy-radicals and probably

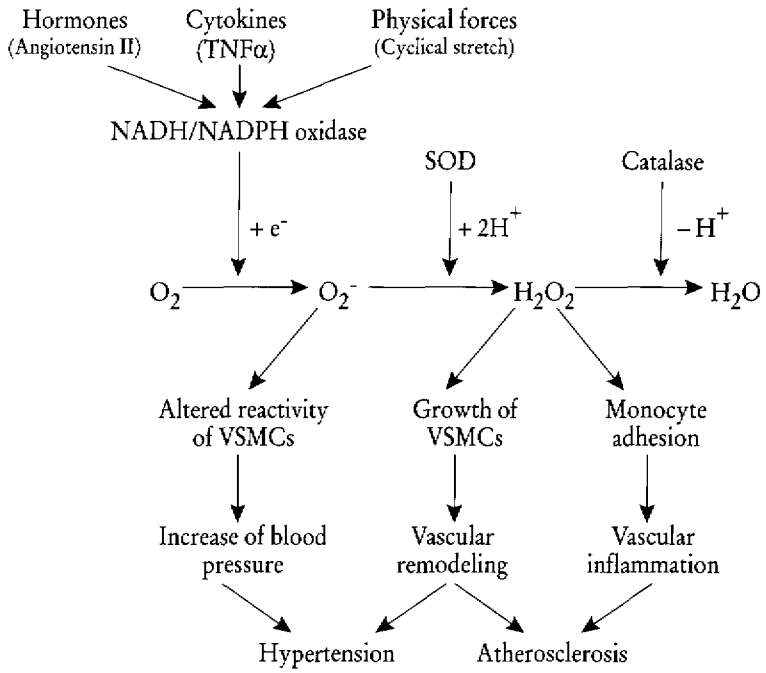

Fig. 1. *Formation and potential actions of superoxide anion (O_2^-) in the vascular wall.*
SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; VSMCs, vascular smooth muscle cells.

hydroxyl-like radicals. Both H_2O_2 and $OONO^-$ are generated as reaction products of the O_2^-. While H_2O_2 mainly emerges from intra- and extracellular dismutation of O_2^- by the abundantly present superoxide dismutases, $OONO^-$ is formed by the rapid reaction of O_2^- with NO (see below). Thus, the generation of O_2^- most likely plays a central role as the source for many other reactive oxygen species.

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 (NOS), and will be discussed separately below.

NADH/NADPH oxidase.– 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 (5, 12, 39). 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 (21). In contrast, only p22phox could be identified in smooth muscle cells and has been shown to participate in the increased O_2^- production upon stimulation with angiotensin II and TNFα (5, 8, 36).

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 (fig. 1). Exposure of cultured vascular smooth muscle cells to angiotensin II and TNFα increased the activity of NADH/NADPH oxidases and subsequent formation of reactive oxygen species was observed (5, 12). Accordingly, treatment of rats with angiotensin II increased vascular O_2^- production independent of the concomitant hypertension as evidenced by parallel experiments using permanent infusions of norepinephrine (31). The O_2^- generation induced by angiotensin II was NADH/NADPH dependent and most likely occurred in the smooth muscle (7). Cyclical strech has also been shown to increase the production of both O_2^- and H_2O_2 by endothelial and vascular smooth muscle cells (16, 17, 19).

Xanthine oxidoreductase.– 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 oxygen, leading to the production of both O_2^- and H_2O_2 (34). It has been shown that early stages of hypercholesterolemia are associated with increased O_2^- production derived from endothelial xanthine oxidase (27, 41). 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 (33). 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 (34), and thus could masquerade as an NADH oxidase, similar to the enzyme system discussed above. Methods to separate the function of the two enzyme systems are not yet universally available.

Endothelial NOS.– Another potential source of vascular O_2^- production is the endothelial isoform of NOS (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 (15, 28). Interestingly, the NOS co-factor tetrahydrobiopterin has also been shown to non-enzymatically generate O_2^-, and this limits the ability of the NOS to produce free NO in the absence of superoxide dismutase (18). Recently, such studies have been extended to the eNOS. XIA *et al.* have shown that in the absence of tetrahydrobiopterin, eNOS can generate

O_2^-, likely via its heme center. In this study, O_2^- production by eNOS was not affected by L-arginine (42). VASQUEZ-VIVAR *et al.* have reported that eNOS can produce considerable amounts of O_2^- by two differents mechanisms (37). In the absence of sufficient cofactors, the oxygenase domain of eNOS can generate O_2^- from the dissociation of the heme ferrous-dioxygen complex. These investigators also showed that O_2^- can be produced by flavins in the reductase domain of eNOS. While eNOS generation of O_2^- 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* so as to be able to serve as a source of O_2^-. PRITCHARD and coworkers have provided evidence that treatment of endothelial cells in culture with native low density lipoprotein (LDL) may increase their production of O_2^- in a fashion which seems to be dependent on eNOS, perhaps due to the dissociation of L-arginine from eNOS (29). The mechanism whereby LDL could affect eNOS function in this manner has not been defined, however such a mechanism could have substantial pathological consequences.

Reactions of O_2^- with NO in the vascular wall

Both O_2^- and NO are highly reactive and unstable radicals. Thus, it is not surprising that they react very rapidly at a rate estimated to be 6.7×10^9 mol s^{-1} to form the major product $OONO^-$ (fig. 2) (9). This reaction is approximately three times faster than the dismutation of O_2^- by superoxide dismutases, implying that increased generation of O_2^- in the vascular wall may very well inhibit the physiological functions of NO. In addition, $OONO^-$ is a strong oxidant and is more stable than either NO or O_2^- (3). At neutral pH, $OONO^-$ can undergo protonation to form peroxynitrous acid which upon homolytic cleavage can yield hydroxyl-like and nitrogen dioxide radicals which are also strong oxidants (2, 43).

Although $OONO^-$ can produce vasodilation, this effect occurs at concentrations far in excess of the effective vasorelaxant concentrations of NO (23, 35, 38). Oxidation reactions induced by $OONO^-$ such as modifications of iron-sulfur clusters, zinc-fingers, protein thiols and membrane lipids are likely to be involved in numerous pathophysiological processes (2, 40, 42). Effects probably related to vascular disease are ilustrated in figure 2.

Besides its exaggerated production from NO and O_2^-, excessive $OONO^-$ may result also from a deficient metabolism of this compound by "detoxifying" agents present in the vascular environment. Up to now, two pathways of

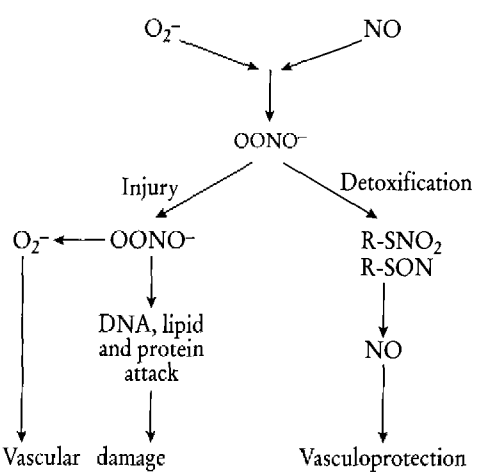

Fig. 2. *Formation and potential actions of peroxynitrite ($OONO^-$) in the vascular wall.*
O_2^-, superoxide anion; NO, nitric oxide; $R\text{-}SNO_{(2)}$, nitrosothiol.

$OONO^-$ metabolism have been described: one involving the generation of nitrosothiol components (25) and the other implicating a thiol-independent mechanism not yet identified (11).

Pathophysiological relevance

Soon after the discovery of EDRF (endothelium derived relaxing 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 or changed into vasoconstriction (6, 20). Similar observations have been made in patients with coronary artery disease (10, 24) or risk factors predisposing to atherosclerosis (45). Likewise, endothelium-dependent vasorelaxation is abnormal in disease states such as heart failure, diabetes and hypertension (26). In almost all of these disorders, there is a loss of endothelial production and/or bioavailability of NO. 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 endothelium (4, 14). Nevertheless, endothelial dysfunction has become widely used, and in fact, loss of NO 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 eNOS or one of its critical co-factors. Alterations of endothelial cell signaling may impair appropriate activation of eNOS in response to neurohumoral or mechanical stimuli. In very advanced atherosclerosis, expression of eNOS 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 O_2^- in the vascular wall. Evidence for this phenomenon has been found in such diverse conditions such as hypercholesterolemia, hypertension, cigarette smoking, atherosclerosis, diabetes and heart failure (22). Furthermore, some of the vascular alterations present in these conditions can be attributed to the deleterious effects of an excess of $OONO^-$ (32).

Summary and Perspectives

There is a growing body of evidence from animal experiments and clinical investigations indicating that a variety of cardiovascular diseases are indeed associated with increased vascular O_2^- production impairing the important functions of endothelial NO production and providing enhanced availability of the harmful compound $OONO^-$. The mechanisms whereby vascular cells produce increased O_2^- are only presently coming to light, and almost certainly will prove to be a focus for future therapies. For instance, recent data from our laboratory suggest that chronic administration of the angiotensin

II type 1 (AT_1) receptor antagonist irbesartan to rats with spontaneous hypertension corrects enhanced NADH/NADPH oxidase-dependent O_2^- production by the vascular wall (44). Interestingly, this effect was associated with restoration of normal endothelial function in treated animals. Further work is required to investigate whether chronic blockade of AT_1 receptors will also correct endothelial dysfunction via suppression of vascular oxidant stress in hypertensive patients.

G. ZALBA, J. BEAUMONT, G. SAN JOSÉ, A. FORTUÑO, M. A. FORTUÑO y J. DÍEZ. *Estrés oxidativo vascular: mecanismos moleculares e implicaciones patofisiológicas* (minirrevisión). J. Physiol. Biochem., **56** (1), 57-64, 2000.

El término estrés oxidativo hace referencia a una situación en la que las células están expuestas a elevadas concentraciones de oxígeno o de sus derivados (especies reactivas del oxígeno). Tres son los sistemas enzimáticos principales que producen especies reactivas del oxígeno en la pared vascular: La NADH/NADPH oxidasa, la xantino óxido-reductasa y la sintasa endotelial del óxido nítrico. La especie reactiva del oxígeno fundamental de la pared vascular es el anión superóxido, pues es la fuente de otras especies reactivas y modifica múltiples funciones de las células vasculares. Actualmente se piensa que un aumento del estrés oxidativo, relacionado sobre todo con una excesiva producción de anión superóxido, interviene en la fisiopatología de la disfunción endotelial asociada a factores de riesgo cardiovascular como la hipercolesterolemia, la hipertensión y el tabaquismo. Además, el estrés oxidativo vascular puede ser determinante de la evolución de enfermedades como la aterosclerosis y la diabetes y de cardiopatías con insuficiencia cardiaca.

Palabras clave: Anión superóxido, Enfermedades vasculares, Estrés oxidativo, Óxido nítrico, Peroxinitrito.

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