**Review**

# Endothelial Dysfunction and Reactive Oxygen Species Production in Ischemia/Reperfusion and Nitrate Tolerance

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**Abstract.** Reactive oxygen species (ROS), as superoxide and its metabolites, have important roles in vascular homeostasis as they are involved in various signaling processes. In many cardiovascular disease states, however, the release of ROS is increased. Uncontrolled ROS production leads to impaired endothelial function and consequently to vascular dysfunction. This review focuses on two clinical conditions associated with elevated ROS levels: ischemia/reperfusion and nitrate tolerance. Injury caused by ischemia/reperfusion is an important limitation of transplantations, and complicates the management of stroke and myocardial infarction. Nitrates, which are used to treat transient myocardial ischemia (angina pectoris), decrease in efficacy in long-term continuous administration. There are several enzyme systems, such as xanthine oxidase, cyclooxygenase, uncoupled endothelial nitric oxide synthase, NAD(P)H oxidase, cytochrome P450 and the mitochondrial electron transport chain, which are responsible for the increased vascular production of superoxide. The contribution of particular ROS producing enzymes and the effect of antioxidant treatment are discussed in both pathological conditions.

**Key words:** Reactive oxygen species — Ischemia/reperfusion — Nitrate tolerance — NADPH oxidases — Nitric oxide synthase

**Contents**



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## Introduction

Reactive oxygen species (ROS) are recognized as important signaling molecules in the cardiovascular system. By oxidizing cysteine residues ROS alter the activity of target proteins (e.g. transcription factors, protein phosphatases) and induce redox-sensitive gene transcription, smooth muscle cell growth and motility (Chiarugi and Cirri 2003). Elevated ROS, however, inhibit cell growth and in even higher concentrations induce apoptosis (Bladier et al. 1997). Excess ROS production may injure vascular tissue by multiple mechanisms. For example, ROS can react with vasoactive substances and impair responses to vasodilators. Long-term exposure to ROS oxidizes proteins and lipids and damages DNA. These harmful actions together result in vascular injury (reviewed in Bauer and Bauer 1999). Since the endothelium is the most vulnerable component of the vessel wall, it is not surprising that the first functional abnormality common to many vascular diseases is endothelial dysfunction.

Enhanced production of ROS, in particular superoxide ($O_2^{\bullet -}$), has been implicated in the pathogenesis of a number of cardiovascular diseases including atherosclerosis, coronary artery disease, hypertension and diabetes mellitus (Cai and Harrison 2000). In addition, ROS are frequently elevated in patients with one or more cardiovascular risk factors, for example in smokers or in patients with hypercholesterolemia. Moreover, increased ROS production is associated with clinical conditions like ischemia/reperfusion (I/R). Tissue injury caused by I/R is a frequent complication of surgical procedures, transplantation, stroke, circulatory shock, coronary artery disease, etc. (Li and Jackson 2002; Carlucci et al. 2002). Myocardial ischemia (i.e. lack of oxygen due to inadequate perfusion) manifests as angina pectoris, the clinical sign of ischemic heart disease. Although nitrates are valuable drugs in treatment of recurrent ischemic heart attacks, their continuous use is complicated by the rapidly developing tolerance. Since elevated ROS concentrations are also present in nitrate tolerance, they could also play a causal role in this condition.

Involvement of ROS in the development of endothelial dysfunction is described in the first part of the review. Two pathological states in which increased ROS contribute to impaired vascular relaxation, i.e. injury caused by I/R and nitrate tolerance, are then discussed in detail. Contribution of specific ROS producing enzyme systems in these pathophysiological states will be reviewed last.

## Endothelial dysfunction as an indicator of vascular pathology

The inner layer of blood vessels, the vascular endothelium, is an active organ with paracrine, endocrine, and autocrine activities and it is crucial for the regulation of vascular tone and maintenance of vascular homeostasis. It produces the endothelium-derived relaxing factor nitric oxide ($^{\bullet}NO$) and several other vasodilating agents, including prostacyclin and the endothelium-dependent hyperpolarizing factor (EDHF). Endothelial cells also produce contracting factors, such as endothelin-1 (ET-1) and thromboxane (Vanhoutte 2000). Recently, $O_2^{\bullet-}$ has also been classified as a vasoconstrictor agent derived from endothelial cells (Vanhoutte 2000; Rey et al. 2001).

$^{\bullet}NO$ can interact whith $O_2^{\bullet-}$, forming peroxynitrite ($ONOO^-$) (Fig. 1) and also by other ROS and lipid radicals. Under physiological conditions, ROS production is low and the endogenous antioxidant systems are sufficient to maintain the balance between $O_2^{\bullet-}$ production and elimination, thus preventing the breakdown of $^{\bullet}NO$. In disease states in which the production of ROS is increased or the antioxidant capacity of the vessel is decreased, $^{\bullet}NO$ is transformed to $ONOO^-$, resulting in inhibition of endothelial-dependent relaxation (O'Donnell et al. 1997) (Fig. 1). In addition, reduced expression or activity of endothelial nitric oxide synthase (eNOS) can also decrease $^{\bullet}NO$ bioavailability.

Increased production of ROS is thought to be one of the key events in the pathogenesis of endothelial dysfunction. In 1986, before $^{\bullet}NO$ was identified as the endothelium-derived relaxing factor, Rubanyi and Vanhoutte (1986) already showed that superoxide dismutase (SOD) augmented endothelium-dependent relaxation. Their observation was confirmed by several later studies demonstrating improved vascular responses to vasodilators after SOD administration (d'Uscio et al. 2001; Steinhorn et al. 2001; Jung et al. 2003).

Impaired endothelium-dependent vasodilation is often the first sign of adverse cardiovascular events and can predict their long-term outcome (Schachinger et al. 2000; Perticone et al. 2001). Clinical evaluation of endothelial function is thus an important diagnostic tool. This is frequently done by analysis of acetylcholine (ACh)-induced relaxation (Erbs et al. 2003) or flow-dependent dilatation of the radial artery using ultrasound probes (Hambrecht et al. 2000). In endothelial dysfunction, the sensitivity and maximal relaxation in response to vasodilators which act *via* the endothelium is decreased, while vascular responses to sodium nitroprusside, which acts directly on vascular smooth muscle cells (VSMC), are unchanged. Inhibitors of nitric oxide synthase (NOS) or generators of ROS, for example pyrogallol (Haj-Yehia et al. 1999), decrease endothelium-dependent vasorelaxation to ACh. Thus, both decreased $^{\bullet}NO$ and increased ROS levels contribute to endothelial dysfunction.

Altered function of the vascular endothelium is associated with arterial hypertension (Taddei and Salvetti 2002), preeclampsia (Page 2002), diabetes (Makimattila and Yki-Jarvinen 2002), atherosclerosis (Anderson 2003), heart failure (Linke et al. 2003), coronary artery disease (Schachinger et al. 2000), nitrate tolerance (Mun-

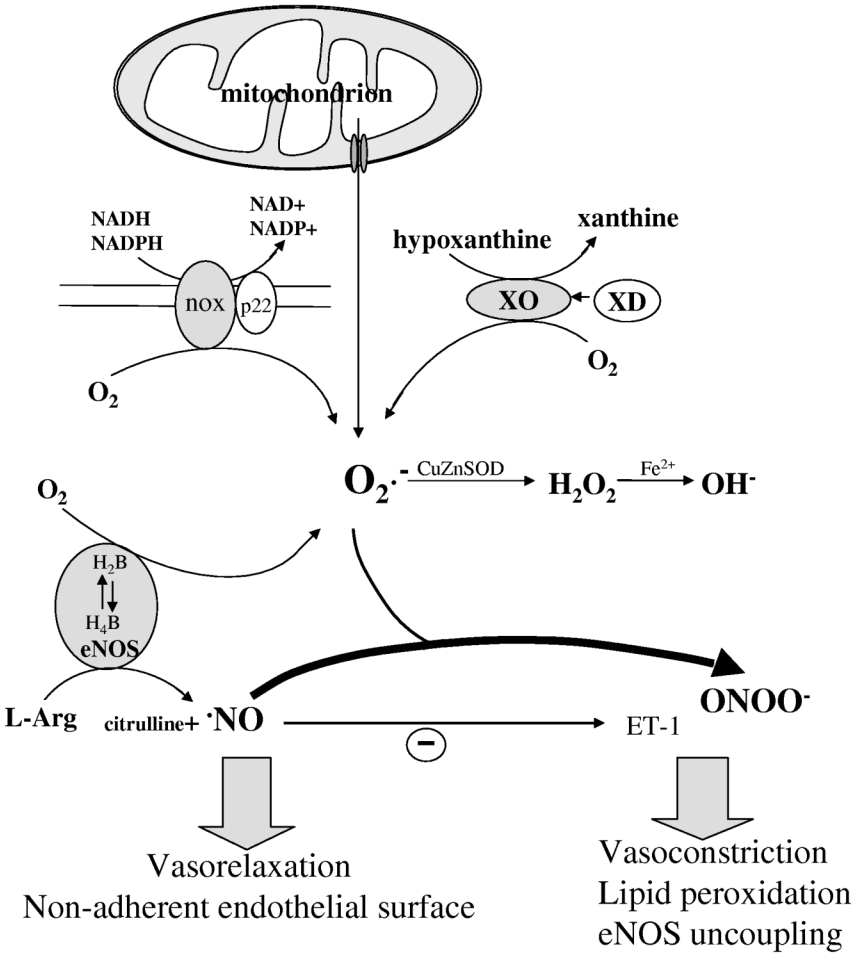


**Figure 1.** ROS production and its consequences during I/R and nitrate-tolerance in vessel wall. Under physiological conditions, eNOS forms ·NO and citrulline from L-arginine. ·NO, directly or by inhibiting ET-1 release, promotes vasorelaxation. In I/R and nitrate-tolerance there is an imbalance between ·NO and $O_2^{\cdot-}$ formation. Enzymes contributing to increased $O_2^{\cdot-}$ production include the mitochondrial electron transport chain, NAD(P)H oxidase, xanthine oxidase (XO), eNOS. Excess of $O_2^{\cdot-}$ results in ·NO inactivation through peroxinitrite ($ONOO^-$) formation and consequently to decreased endothelium-dependent vasorelaxation, and eventually to vasoconstriction. $ONOO^-$ is also a potent oxidant, it mediates lipid peroxidation and eNOS uncoupling. $O_2^{\cdot-}$ can be dismutated to $H_2O_2$ (spontaneously or by CuZnSOD). In the presence of metal ions, $H_2O_2$ can be further metabolized to hydroxyl radical. L-Arg, L-arginine; $H_2B$, dihydrobiopterin; $H_4B$, tetrahydrobiopterin; XD, xanthine dehydrogenase; SOD, superoxide dismutase.

zel et al. 1995) and clinical conditions linked to ischemia followed by reperfusion (Sotnikova et al. 1998; Pagliaro et al. 2003). In these disease states, the production of ROS is increased and the beneficial effect of antioxidants suggests that ROS contribute to the development of endothelial dysfunction (Cai and Harrison 2000). Administration of probucol to cholesterol fed rabbits, for example, decreased vascular $O_2^{\bullet -}$ production and improved endothelium-dependent relaxations (Inoue et al. 1998). The membrane-permeable polyethylene glycol SOD (Mügge et al. 1991), glutathione (GSH) (Prasad et al. 1999), vitamin C (Ting et al. 1995; Gokce et al. 1999) and vitamin E (Heitzer et al. 1999) increase $^{\bullet}$NO bioavailability. Estradiol, which also has indirect antioxidant properties, was found to increase coronary blood flow in postmenopausal women with coronary artery disease (Blumel et al. 2003). Antioxidants reverse also endothelial vasomotor dysfunction in patients with risk factors for coronary artery disease (smokers, hypercholesterolemic patients) and improve vasodilation of atherosclerotic vessels. Recent clinical data showed that acute administration of vitamin C to patients with coronary artery disease restored peripheral endothelial function by reducing elevated levels of ROS (Erbs et al. 2003).

In summary, endothelial dysfunction is present in many vascular diseases and pathological conditions. The protective role of antioxidants indicates that increased ROS production, and consequently low $^{\bullet}$NO bioavailability, are responsible for the impaired vasorelaxation. Vascular injury, as caused by I/R, and tolerance to nitrates, drugs used in the treatment of myocardial ischemia and its prevention, are both associated with elevated ROS levels. The following sections are focusing on the involvement of ROS in the pathogenesis of these two conditions.

*Ischemia/reperfusion injury*

One of the earliest events following I/R injury is vascular dysfunction linked to altered function of endothelial cells. Both ischemia (hypoxia) and reperfusion (reoxygenation) are important in human pathophysiology as they occur in various clinical conditions, for example during circulatory shock, stroke, organ transplantations (Li and Jackson 2002) or coronary artery bypass surgery (Carlucci et al. 2002). These events are associated with increased oxidative stress and endothelial damage.

Increased ROS levels have been classically attributed to exposure of ischemic tissues to molecular oxygen during reperfusion (reoxygenation) (Zweier et al. 1994; Kim et al. 1998, Bauer et al. 2002). Indeed, the largest increase in ROS production is usually observed within the first 15 min of reperfusion (Kim et al. 1998; Buttemeyer et al. 2002; Bertuglia and Giusti 2003; Bertuglia et al. 2004). Furthermore, increased ROS production was detected by luminol or lucigenin in rat superior mesenteric arteries one hour after reperfusion following a 30-min ischemia (Haklar et al. 1998). This rapid burst of oxygen-derived free radicals during reperfusion coincides with the time course of endothelial dysfunction. While 90 or 120 min of ischemia alone did not alter vascular responses, the reactivity of the endothelium to ACh was attenuated as soon as 2.5 min following initiation of reperfusion (Tsao et al. 1990;

Hayward and Lefer 1998). Neither did partial occlusion of the abdominal aorta of rats for 18 h change vascular responses significantly. However, if prolonged ischemia was followed by a 30-min reperfusion, endothelium-dependent vasorelaxation was impaired (Sotníková et al. 1998). Reperfusion lasting up to 120 min further reduced vascular responses to endothelium-dependent vasodilators, which remained altered even more than 4 h after blood flow renewal (Tsao et al. 1990; Hayward and Lefer 1998). Similarly, in superior mesenteric arteries of mice and rats, I/R for either 45/45 min or 30/60 min decreased vasodilatory responses to ACh by approximately 40 %, compared to sham-operated animals (Banda et al. 1997; Chen et al. 2000).

Although initial studies suggested that ROS were predominantly produced during reperfusion, recent discoveries showed that hypoxia itself could also increase ROS production in various tissues (Vanden Hoek et al. 1997; Li and Jackson 2002; Pearlstein et al. 2002). For example, increased superoxide production was detected *in vivo* within 10 min of femoral artery occlusion (Buttemeyer et al. 2002). In arteries injured by ischemia, ROS production returned to basal levels when oxygen tension recovered completely, which may take 30 min after clamp removal (Bertuglia and Giusti 2003).

Endothelial dysfunction induced by I/R is manifested with a delay of a few minutes following increased vascular ROS production and is maintained for a longer period (hours) than elevated ROS levels. For example, in hamster cheek pouch arterioles, while elevated ROS returned to normal as soon as 30 min after ischemia, the arteriolar diameter kept decreasing (Bertuglia and Giusti 2003). Coronary blood flow was also found significantly decreased after 30 min of reperfusion (Pernow and Wang 1999). Similarly, impaired vasodilatory responses to ACh were observed in superior mesenteric arteries 120 min after reperfusion (Hayward and Lefer 1998). However, 60/120 min I/R did not affect sensitivity to ACh in rabbit major conduit arteries (Koksoy et al. 2000). This may be due to the higher resistance of some large rather than small arteries to I/R. Thus the small vessels seem to be more sensitive to hypoxia than the large ones. In arterioles and capillaries, hypoxic conditions are maintained even after restoration of blood flow in adjacent arteries. Hypoxic injury, manifested by endothelial cell swelling, leads to the "no reflow" phenomenon within the first minutes of reperfusion (Menger et al. 1992). If the ischemic period lasts for several hours, hypoxia-induced continuous ROS release impairs the endothelial barrier, increases vascular permeability and thus damages nearby tissues (Plateel et al. 1995). Electronmicroscopical examination of 18 h/30 min I/R revealed structural changes, such as edematous mitochondria in both endothelial and vascular smooth muscle cells, microvilli formation on the surface of endothelial cells, increased pinocytic activity and disturbed tight junctions between endothelial cells (Sotníková et al. 1998). 20 min after reperfusion, polymorphonuclear cells adhered to the endothelium (Hayward and Lefer 1998). Adhesion of polymorphonuclear cells to the vessel wall further impairs vascular function. Recent data indicate that leukocyte adhesion is due to endothelial dysfunction, as a consequence of oxidative stress. Both decomposition of $^{\bullet}$NO during reperfusion and inhibition of $^{\bullet}$NO synthesis increased leukocyte adherence to the venular wall (Bertuglia and Giusti

2003), suggesting a protective role of $^{\bullet}NO$ in maintaining a non-adherent endothelial surface. These phagocytic cells continue to produce ROS and injure endothelial cells, which contributes to later loss of vasomotor tone control. This could explain the prolonged endothelial dysfunction and the protective action of SOD long after ROS production in the vessel wall (within endothelial cells and VSMC) has returned to normal.

Decreased $^{\bullet}NO$ availability, at least in part, has been attributed to the activity of $O_2^{\bullet-}$ formed during reperfusion in endothelial cells (Zweier et al. 1994) and neutrophils (Fabian and Kent 1999). Studies performed *in vivo* suggest the involvement of at least two ROS in I/R injury. As demonstrated with dihydroethidium (DHE) and dichlorofluorescein (DCF) fluorescence, both $O_2^{\bullet-}$ and hydrogen peroxide ($H_2O_2$) were elevated in endothelial cells during hypoxia (Kim et al. 1998; Li and Jackson 2002; Pearlstein et al. 2002). Another mechanism by which ROS injure cells and impair endothelial function involves generation of hydroxyl radical ($^{\bullet}OH$) by surface-associated iron, which reacts with $H_2O_2$. This was demonstrated by the protective effect of iron chelators and $^{\bullet}OH$ scavengers on reoxygenated endothelial cell permeability (Terada 1996). Pretreatment with stobadine, a pyridoindole derivative, which is known to scavenge $^{\bullet}OH$, exerted protective effect on I/R induced injury (Sotníková et al. 1998).

MnSOD overexpression or exogenous SOD administration protects against I/R injury (SOD mimetics were reviewed in Salvemini and Cuzzocrea (2003). For example, pretreatment with SOD before reperfusion maintained ROS at normal levels, prevented lipid peroxidation, increased arteriolar diameter and decreased leukocyte adhesion in hamster microcirculation (Bertuglia and Giusti 2003; Nakae et al. 2003). Similarly catalase, if delivered directly to the endothelium using specific antibodies, protected against oxidative stress during lung transplantation in the rat. This antioxidant enzyme restores the barrier function of the endothelium, as shown by decreased lung graft edema and reduced number of intracapillary, interstitial and intraalveolar neutrophils (Christofidou-Solomidou et al. 2003; Kozower et al. 2003).

Upregulation of endogenous antioxidant enzymes is likely to contribute to the beneficial effect of ischemic or hyperthermic preconditioning (Hoshida et al. 2002; Pagliaro et al. 2003). These techniques, involving application of a short ischemia or hyperthermia for several minutes to hours before I/R, improved the ability of the tissue to withstand subsequent ischemia. For example, a brief ischemia applied before prolonged occlusion of the left anterior descending coronary artery attenuated reperfusion injury in dogs. In this experiment both pre- and postconditioning (a short reperfusion period during ischemia) decreased oxidative stress and increased maximal vasodilatory response to ACh (Zhao et al. 2003). Similarly, a short-term body temperature elevation prior to I/R preserved endothelium dependent vasodilation (Chen et al. 2000). Exercise is another physical stress that induces protection against ischemia. In patients with coronary artery disease, long-term aerobic exercise improves endothelium-dependent vasodilation (Hambrecht et al. 2000). Following exercise, vascular CuZnSOD activity is induced and NAD(P)H-oxidase

expression is decreased. These changes are expected to decrease oxidative stress and thereby to contribute to improved endothelial function (Rush et al. 2003).

•NO, besides acting directly as a vasodilator, inhibited the release of ET-1 (Vanhoutte 2000). This potent vasoconstrictor is produced in arteries by endothelial and vascular smooth muscle cells. Similarly, production of ET-1 was enhanced by I/R injury in the myocardium (Gourine et al. 2001). ET-1 levels were increased also in blood samples collected from the coronary sinus 30 min after reperfusion, and they most likely contribute to the vasoconstrictive response (Carlucci et al. 2002).

Thus, I/R is linked to increased ROS production both during the ischemic and early reperfusion period, leading to decreased •NO availability, vasoconstriction and leukocyte adhesion. Upregulation of antioxidant enzymes by preconditioning techniques or physical exercise improves vascular responses to vasodilators. In addition to non-pharmacological management of coronary artery disease, organic nitrates are generally used to treat transient ischemic attacks. This medication, however, is not straightforward, since it is accompanied by undesirable vascular reaction, i.e. nitrate tolerance, as seen in the next section.

*Nitrate tolerance*

Nitrates are frequently used in the management of coronary artery disease. They cause vasorelaxation by releasing •NO from the parent molecule in vascular smooth muscle (Wong and Fukuto 1999). Due to general vasodilation, ventricular volume and arterial pressure are lowered, thus myocardial oxygen requirement decreases, which manifests as relief from angina. Although acute application of nitrates is a highly efficient vasodilator and anti-ischemic therapeutic, prolonged continuous administration for more than 12 to 24 h leads to desensitization, an effect referred to as nitrate tolerance (Munzel et al. 1995). In healthy volunteers, a continuous 6-day treatment with nitroglycerine patches led to decreases in ACh-induced vasodilation and consequently to a decrease in forearm blood flow (Gori et al. 2001). In order to minimize the tolerance and restore useful responses to the drug, patients are advised to keep nitrate-free periods daily, at least for 8 h. This approach, however, has two drawbacks. The first is the lack of tissue protection during the nitrate-free period, and the second is rebound ischemia upon nitrate withdrawal (Thadani 1997).

Tolerance has most frequently been reported in the case of nitroglycerine (NTG), historically the first therapeutic organic nitrate (Munzel et al. 1995; Leopold and Loscalzo 2003). However, all other members of this pharmacological class, such as amyl nitrite and isosorbide dinitrate, induce tolerance and cross-tolerance (Munzel 2001). This means that the vascular response is decreased not only to NTG but also to other endothelium-dependent and -independent nitrovasodilators.

Several mechanisms may account for the phenomenon of nitrate tolerance, or tachyphylaxis. The first one is oxidative changes following nitrate administration. Intracellular thiols, such as GSH and cysteine, are involved in the conversion of nitrates to their bioactive metabolite (•NO). For example, in rat aortic cytosol,

glutathione S-transferases, which catalyze the conversion of NTG, were shown to require glutathione as a cosubstrate (Nigam et al. 1996). The activity of the mitochondrial aldehyde reductase, recently identified as another site of NTG bioactivation, seems to depend also on sulfhydryl groups (Chen et al. 2002).

The second mechanism explaining the beneficial effects of thiol donors and other antioxidants on vascular relaxation is based on their $O_2^{\bullet -}$ lowering property. Thus, exogenous administration of the thiol compound N-acetylcysteine augmented the hypotensive effect of NTG even if intracellular arterial and venous thiol levels were similar in nitrate-tolerant and control animals (Boesgaard 1995). Other antioxidants improving vascular responses to NTG (assessed by forearm plethysmography) in long-term nitrate-treated patients include vitamins C and E (Watanabe et al. 1997; Watanabe et al. 1998). Consistent with this effect of antioxidants, increased $O_2^{\bullet -}$ generation was observed in nitrate-tolerant rabbit arteries (Munzel et al. 1995). In this model, the main site of its production appears to be the vascular endothelium, since endothelial denudation decreased ROS levels. Platelets of healthy volunteers also enhanced $O_2^{\bullet -}$ generation upon 3-day exposure to NTG (McVeigh et al. 2002). Both tolerance to nitrates and cross-tolerance to other vasodilators, ACh for example, are specific to long-term treatment, and can be reversed by SOD administration. Treatment with liposomal SOD, which can act intracellularly, significantly enhanced relaxations to NTG in NTG-tolerant rabbit aortic segments with endothelium (Munzel et al. 1995). However, as expected, administration of conventional SOD at lower concentration did not significantly improve vasodilatory responses of aortic strips in the same model (Nakae et al. 2003).

A third possible mechanism of nitrate tolerance involves downstream targets of $^{\bullet}NO$ (Fig. 2) since impaired endothelium-dependent vasodilation to ACh was also observed following overexpression of eNOS in mice leading to elevated basal $^{\bullet}NO$ and cyclic guanosyl monophosphate (cGMP) (Ohashi et al. 1998; Yamashita et al. 2000). The expression of two downstream $^{\bullet}NO$ targets, soluble guanylyl cyclase (GC) and the cGMP-dependent protein kinase (cGK), remained unaltered upon long-term nitrate intake (Schulz et al. 2002). GC activity was found increased if NTG was administered in combination with cysteine (Artz et al. 2002). Therefore in the presence of thiol groups nitrate tolerance may result from impaired cGK activity. This enzyme catalyzes vasodilator-stimulated phosphoprotein (VASP) phosphorylation and mediates vasorelaxation (Lincoln et al. 2001) (Fig. 2). A recent study showed decreased phospho-VASP (P-VASP) staining, a marker of cGK activity, in arteries of patients previously treated with nitroglycerine. Their arteries also exhibited increased superoxide production (Schulz et al. 2002). Treatment with the antioxidant vitamin C partially reversed tolerance, correlating with reduced vascular $O_2^{\bullet -}$ and elevated P-VASP levels (Mulsch et al. 2001). The peroxynitrite scavenger, ebselen, showed similar effects. In nitrate-tolerant aortic rings ebselen enhanced P-VASP formation, decreased ROS levels and improved vascular responses to NTG (Hink et al. 2003).

Other factors contributing to nitrate tolerance include neurohormonal counter-

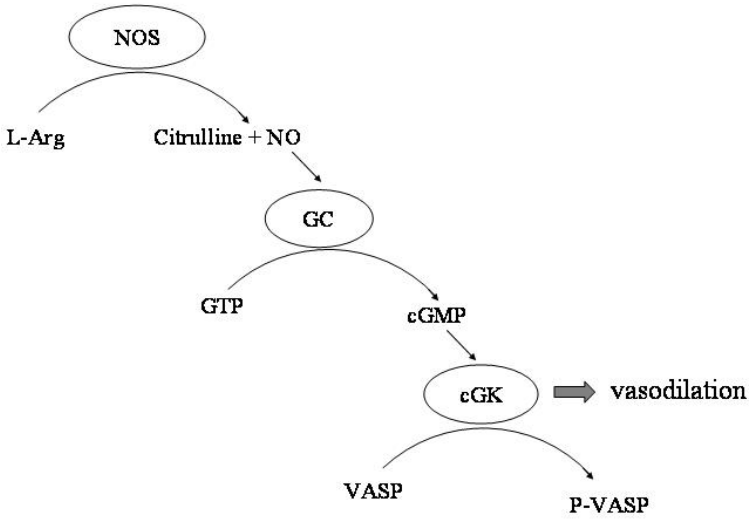


**Figure 2.** Downstream targets of $^{\bullet}$NO. $^{\bullet}$NO, produced by NOS, activates the target enzyme – soluble guanylyl cyclase – and increases tissue levels of cGMP. cGMP activates a cGMP-dependent protein kinase that mediates vasorelaxation. NOS, nitric oxide synthase; GC, soluble guanylyl cyclase; cGK, cGMP-dependent protein kinase; VASP, vasodilator-stimulated phosphoproteinf.

regulatory mechanisms activated by the fall in blood pressure upon $^{\bullet}$NO intake. One of these is the renin-angiotensin system (RAS). Its inhibition by angiotensin converting enzyme inhibitors or $AT_1$ receptor blockers ameliorated nitrate tolerance (Berkenboom et al. 1999; Kurz et al. 1999).

Thus, several mechanisms may contribute to nitrate tolerance (reviewed in detail by Leopold and Loscalzo 2003). These mechanisms are not mutually exclusive, while increased ROS production is considered the main factor. Neurohormonal activation leads to stimulation of the renin angiotensin system, thus increasing ROS formation, which in turn deplete intracellular sulfhydryl groups. Increased oxidative stress is associated with decreased cGK activity, which manifests as endothelial dysfunction.

## Sources of ROS in blood vessels

Multiple ROS are produced in the vascular wall, such as $O_2^{\bullet -}$, $H_2O_2$, and $^{\bullet}$OH (Fig. 1). These molecules, if present in physiological concentrations, have important functions in homeostasis. The primary oxygen-derived free radical produced in the vasculature appears to be $O_2^{\bullet -}$, which is formed by univalent reduction of molecular oxygen ($^3O_2$). This radical is involved in signal transduction pathways leading for example to vascular smooth muscle proliferation, migration and hypertrophy (Cai

and Harrison 2000). These effects are mediated either directly by $O_2^{\bullet -}$ or by its metabolites.

Dismutation of $O_2^{\bullet -}$ by SOD or nonenzymatic univalent reduction of this radical generates $H_2O_2$, which acts as a second messenger (Griendling et al. 2000a) and also regulates vascular tone. Depending on the concentration and the type of artery, $H_2O_2$ can induce either contraction (Sotnikova 1998; Nowicki et al. 2001) or relaxation (Matoba et al. 2000). $H_2O_2$ may be inactivated by conversion to water and molecular oxygen in a reaction catalyzed by glutathione peroxidase or catalase.

In the presence of metal ions, $H_2O_2$ can give rise to $^{\bullet}OH$ (Fenton reaction). This very reactive radical participates in lipid hydroperoxidation and leads to injury of endothelial cells, for example upon reperfusion (Kontos 2001). On the other hand, $^{\bullet}OH$ may also induce relaxation of uninjured, norepinephrine-precontracted aortic strips (Prasad and Bharadwaj 1996). Since this effect of the $^{\bullet}OH$ generating system is concentration-dependent, it is possible that certain physiological amounts of this radical do not damage the endothelium.

There are many potential sources of cellular $O_2^{\bullet -}$, including membrane and mitochondrial NAD(P)H oxidases, cyclooxygenase, cytochrome P450, xanthine oxidase (XO) and NOS (Fig. 1). In unstimulated systems, non-mitochondrial NAD(P)H oxidases are a major source of $O_2^{\bullet -}$ in the vessel wall (Griendling et al. 2000b). In contrast to the neutrophil enzyme, vascular NAD(P)H oxidases are activated more slowly and produce superoxide at a lower rate. However, both the neutrophil and vascular oxidases are flavoproteins and most likely have similar subunit composition (Griendling et al. 2000b; Lassegue and Clempus 2003). The phagocytic oxidase consists of 5 major subunits: gp91phox (which is also referred to as nox2) and p22phox are the membrane subunits; p47phox, p67phox and rac1 are located in the cytoplasm (Babior et al. 2002) (Fig. 3). Most of these subunits and some of their homologues were recently detected in vascular tissue. Homologues of the catalytic subunit gp91phox which are expressed in the vasculature include nox1, nox4, nox5 and duox1 (Lassegue et al. 2001; Lassegue and Clempus 2003). Homologues of the cytosolic subunits were also recently described (Banfi et al. 2003; Geiszt et al. 2003) but their expression in the vascular wall has not been reported.

eNOS is another important source of $O_2^{\bullet -}$ in the vessel wall. Besides being the source of $^{\bullet}NO$, this enzyme can produce $O_2^{\bullet -}$ and $H_2O_2$ in the absence of either L-arginine (L-Arg) or tetrahydrobiopterin ($H_4B$) (Parker et al. 2002). In this dysfunctional state, known as NOS uncoupling, reductive activation of molecular oxygen to form $O_2^{\bullet -}$ is not followed by oxidation of L-Arg and $^{\bullet}NO$ synthesis (Landmesser and Harrison 2001).

Arachidonic acid metabolism is another potential source of ROS. Cyclooxygenase, which converts arachidonic acid into prostaglandin, as well as lipoxygenase, responsible for leukotriene synthesis, can both contribute to ROS production (Kukreja et al. 1986). However, in the vessel wall these enzymes usually seem to be insignificant sources of ROS, since their inhibitors, indomethacin and eicosatetraenoic acid, do not decrease superoxide production (Mohazzab and Wolin 1994).

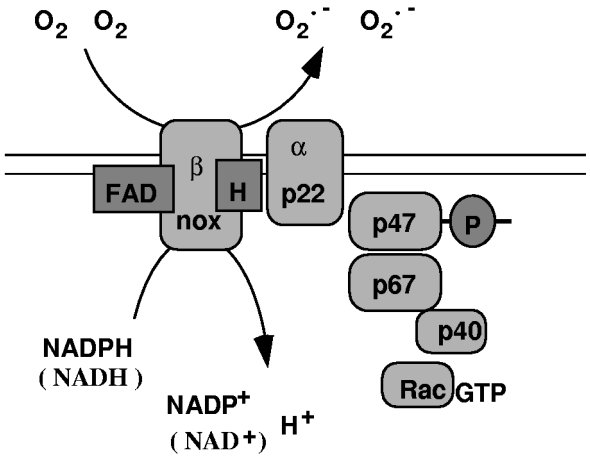


**Figure 3.** Structure of the NAD(P)H oxidases. The phagocytic NAD(P)H oxidase is composed of 2 membrane subunits, nox2 (a. k. a. gp91phox, the catalytic component) and p22phox, and of 3 cytosolic subunits, p47phox, p67phox, p40phox, and of a small molecular weight G protein, rac. These components are also expressed in vascular cells, but the interaction between them remains to be determined. In addition, novel members of the nox family of proteins, such as nox1, nox4 and nox5, seem to serve as catalytic components of the vascular oxidase. The catalytic component contains flavine adenine dinucleotide (FAD) and heme (H) groups, which participate in electron transfer. P, phosphate group; GTP, guanosyl triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate. (Scheme adapted from Lassegue et al. 2001).

Microsomal cytochrome P450 enzymes (CYP), which convert arachidonic acid to epoxyeicosatrienoic acids (EET), can also generate varying amounts of oxygen-derived free radicals (Puntarulo and Cederbaum 1998). One of the CYP isoforms, CYP 2C9, identified as an EDHF synthase, is another source of ROS in the vasculature. Expression of this enzyme was detected in porcine coronary endothelial cells, where it most likely contributes to basal ROS production (Fleming et al. 2001).

The xanthine oxidoreductase enzyme, which is involved in purine metabolism, is present in vascular tissue in two forms: xanthine dehydrogenase (XD) and xanthine oxidase (XO) (Fig. 4). The constitutively expressed XD can be converted in certain conditions to XO (reviewed in Meneshian and Bulkley 2002), which is capable of generating $O_2^{\bullet -}$ and $H_2O_2$ by reducing molecular oxygen.

Finally, the mitochondrial electron transport chain is another possible source of vascular ROS (Fig. 5). During oxidative phosphorylation, electrons are transferred through complexes I–III to cytochrome oxidase (complex IV), which in normoxia reduces molecular oxygen to water. Under physiological conditions only a small fraction of the oxygen consumed by mitochondria is converted to superoxide,

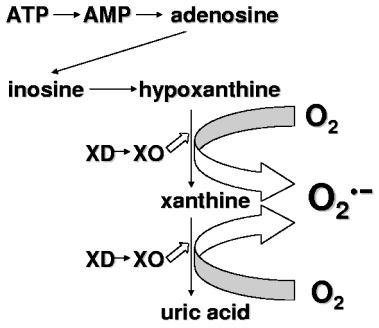


**Figure 4.** ROS formation by xanthine oxidase. During purine metabolism, ATP is degraded to ADP, AMP and adenosine, which is further metabolized to inosine and hypoxanthine. XO catalyzes the final step of this metabolic pathway, i.e. the conversion of hypoxanthine to xanthine and xanthine to uric acid. In these reactions $O_2$ serves as an electron acceptor. By its respective univalent or divalent reduction, $O_2^{\bullet -}$ and $H_2O_2$ are formed. (Scheme adapted from Bauer et al. 2002). XD, xanthine dehydrogenase; XO, xanthine oxidase.

which is rapidly dismutated by MnSOD (Raha et al. 2000). In contrast in hypoxia, mitochondria seem to be an important source of ROS in endothelial cells (Pearlstein et al. 2002). There are multiple possible sites for ROS generation within the mitochondrial respiratory chain. For example, complexes I and III were identified as main sites of $H_2O_2$ generation (Kwong and Sohal 1998).

The enzymes just mentioned have been associated with vascular $O_2^{\bullet -}$ overproduction. In addition, decreased activity of antioxidant enzymes also contributes

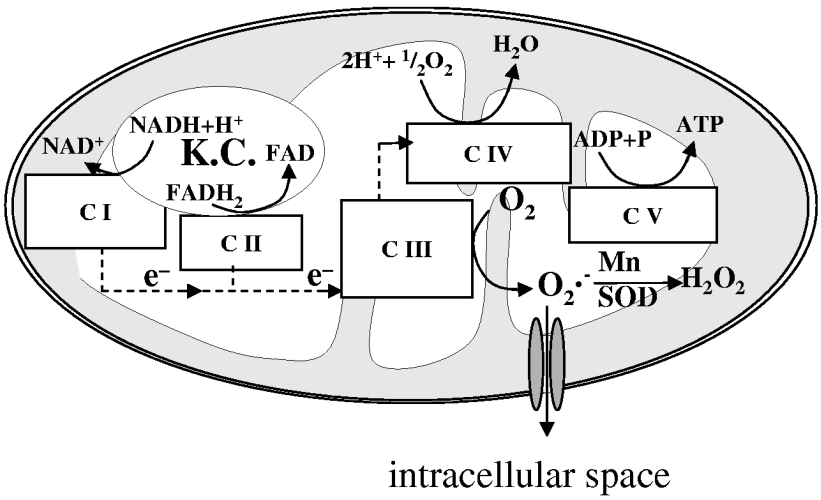


**Figure 5.** ROS generation in mitochondria. Under physiological conditions, the free energy of electron transfer from NADH and $FADH_2$ to $O_2$ is coupled to ATP synthesis. Due to leakage from the mitochondrial electron transport chain, only a small amount of $O_2^{\bullet -}$ is produced, which is converted by MnSOD to $H_2O_2$. In ischemia, the inhibition of complex IV increases ROS production mainly by complex III. K.C., Krebs cycle; C I–V, mitochondrial complexes.

to elevated ROS levels in pathological conditions. Both prooxidant and antioxidant enzyme systems have an important role in the development of endothelial dysfunction in I/R injury and nitrate tolerance.

## ROS sources in ischemia/reperfusion

During ischemia and reperfusion, increases in various ROS, such as $O_2^{\bullet -}$ and $^{\bullet}OH$, as well as reactive nitrogen species, e.g. $ONOO^-$ can be detected in tissues (Li and Jackson 2002). These reactive oxygen and nitrogen species are derived from diverse cellular and molecular sources in vessels. Both endothelial cells and circulating phagocytes produce ROS during I/R (Al-Mehdi et al. 1998; Kaminski et al. 2002). While endothelial cells produce $O_2^{\bullet -}$ continuously, neutrophils become more important with longer periods of reperfusion as they adhere to the endothelium and become activated (Fabian and Kent 1999). The most important source of ROS in phagocytic cells is the NADPH oxidase, which catalyzes the reduction of molecular oxygen to $O_2^{\bullet -}$. This radical in turn gives rise to other reactive oxygen metabolites, which can diffuse into endothelial cells and injure them. Endothelial cells themselves also produce ROS. For example, in cerebral arteries $O_2^{\bullet -}$ production is increased 1–4 h after reperfusion. Subcellular microscopical examination shows that $O_2^{\bullet -}$ is localized in cytosolic vesicles (Kim et al. 2002). Various enzymes are potential sources of ROS in the vessel wall. The involvement of each of these oxidases to I/R injury will be presented in more detail in the following sections.

### *Xanthine oxidase*

The reaction of XO with hypoxanthine and molecular oxygen to produce a burst of oxygen radicals was implicated in reperfusion models more than 20 years ago (Parks et al. 1983). Xanthine dehydrogenase, which uses $NAD^+$ as a substrate, is converted under ischemic conditions to XO, which uses oxygen as an electron acceptor (substrate). Due to ATP consumption during hypoxia, hypoxanthine and xanthine are accumulated in the tissue, which upon reoxygenation are metabolized by XO and yield $O_2^{\bullet -}$ and $H_2O_2$ (Granger 1988) (Fig. 4). In the vessel wall, this conversion and increased activity of XO occurs in the endothelial layer during I/R. Some of this enzyme also circulates in the blood and can bind to endothelial cells.

The activity of XO is increased for example in reoxygenated human umbilical veins 2 h after the hypoxic period (termed late reoxygenation) (Sohn et al. 2003). Inhibition of this enzyme has protective effect in some models of I/R. Oxypurinol similarly to SOD decreases brain cortical endothelial cell damage caused by 10-h anoxia and 4-h reoxygenation, as measured by lactate dehydrogenase efflux into the culture medium (Beetsch et al. 1998). Another XO inhibitor, allopurinol, also restores venodilation responses to ACh in rats (Flynn et al. 1999).

However, I/R injury also occurs when XO activity is low or absent (Muxfeldt and Schaper 1987). This is the case in patients undergoing coronary bypass surgery. At the end of the ischemic period, and also 30 min following clamp removal, the ratio of reduced/oxidized glutathione (GSH/GSSG) in the plasma is decreased,

indicating increased oxidative stress. Levels of hypoxanthine were increased in the plasma collected from the coronary sinus. Nevertheless, xanthine and uric acid levels were found to be low, suggesting low XO activity (Carlucci et al. 2002).

Thus, the role of XO in I/R injury is variable. This oxidase seems to be important for example in human umbilical vein reperfusion (Sohn et al. 2003), in maintaining optimal mesenteric artery microcirculation upon resuscitation from hemorrhagic shock (Flynn et al. 1999) and in the *in vitro* model of cerebral endothelial cell reoxygenation (Beetsch et al. 1998). On the other hand, there are several reports, where the contribution of XO is not critical for ROS generation in I/R. This suggests involvement of other enzyme systems, as discussed in the following sections.

*Mitochondrial oxidase*

Absence of the final electron acceptor increases ROS production in the mitochondria by complexes I–III (Fig. 5), of which complex III seems to be the most important (Pearlstein et al. 2002; Waypa and Schumacker 2002). Increased ROS levels observed in endothelial cells during hypoxia could be due to inhibition of cytochrome oxidase (complex IV) activity and increased $O_2^{\bullet -}$ production by the ubisemiquinone at complex III (Pearlstein et al. 2002). $O_2^{\bullet -}$ and $H_2O_2$ released to the cytoplasm through anion channels and by diffusion, respectively, can act as intracellular signaling messengers linking tissue hypoxia to the subsequent responses. Mitochondria, by means of ROS production have been suggested to function as oxygen sensors in pulmonary artery endothelial cells and myocytes. Although the exact mechanism is still being debated, ROS derived from the mitochondria are thought to mediate vasoconstriction in hypoxic pulmonary arteries, thereby decreasing blood flow through hypoventilated alveoli (Waypa and Schumacker 2002).

In contrast to these signaling roles, ROS produced by the mitochondria during hypoxia/reoxygenation have harmful effects. Increased endothelial cell permeability is related to hypoxia-induced oxidative stress (Pearlstein et al. 2002). Exposure to hypoxia for 6 h followed by a 45-min reoxygenation period increased $O_2^{\bullet -}$ generation in bovine brain endothelial cells. Excess $O_2^{\bullet -}$ production was prevented by blocking the first two complexes of the mitochondrial electron transport chain, suggesting that mitochondria are the main sources of ROS formed during reoxygenation (Kimura et al. 2000). A negative correlation between the number of perfused postischemic sinusoids and the mitochondrial redox state also indicates an important role of mitochondria in hepatic microcirculation (Glanemann et al. 2003).

Decreased activity of antioxidant mitochondrial enzymes, such as MnSOD, further increases ROS release during hypoxia. Cardiac I/R significantly reduced mitochondrial SOD and glutathione peroxidase activities, so that enzymatic degradation of $O_2^{\bullet -}$ and $H_2O_2$ was compromised (Shlafer et al. 1987). There are thus two mechanisms, inhibited electron transfer through cytochromes and decreased antioxidant enzymatic activity, leading to excessive mitochondrial ROS production during I/R.

*Nitric oxide synthase*

In the absence of its substrate and/or cofactor NOS was shown to produce $O_2^{\bullet -}$ and $H_2O_2$ (Vasquez-Vivar et al. 1998). Administration of the substrate L-Arg decreased coronary vascular resistance, increased blood flow and thereby reduced the extent of myocardial I/R injury (Agullo et al. 1999; Pernow and Wang 1999). Infusion of $^{\bullet}NO$ donors had a similar effect on the ischemic myocardium, indicating that maintenance of $^{\bullet}NO$ release is an important factor in protecting tissues from reperfusion injury. In an *in vitro* model of vascular hypoxia and reoxygenation, incubation of hypoxic endothelial cells with L-Arg decreased ferryl hemoglobin formation in the medium, which is a measure of oxidative stress (D'Agnillo et al. 2000). A recent study showed that L-Arg may provide protection against I/R injury in transplantations as well as in routine cardiac surgery. This was demonstrated in a model of rat heart transplantation, where polymers of L-Arg improved coronary flow and reduced myocardial oxidative stress (Kown et al. 2003).

$^{\bullet}NO$ synthesis also depends on the availability of $H_4B$, a NOS cofactor modulated by the redox state of the cell. $H_4B$ is decreased in several pathophysiological conditions, including coronary artery disease and I/R. Deficiency of $H_4B$ seems to accelerate endothelial dysfunction and myocardial I/R injury, while pretreatment with this cofactor reduced functional and metabolic abnormalities (Yamashiro et al. 2003).

In contrast to short ischemia, eNOS upregulation and increased $^{\bullet}NO$ formation was detected in endothelial cells exposed for 8 h to mild hypoxia (Sohn et al. 2003). Long-term hypoxic conditions thus may lead to increased $^{\bullet}NO$ levels by activation of antioxidant enzymes and NOS upregulation.

Hypoxia alone or followed by reoxygenation also increased $O_2^{\bullet -}$ production in endothelial cells (D'Agnillo et al. 2000; Kimura et al. 2000). As soon as it is produced, $O_2^{\bullet -}$ acts as a scavenger of $^{\bullet}NO$ to which it combines to form $ONOO^-$. This radical is more potent in oxidizing $H_4B$ than $O_2^{\bullet -}$ and uncouples NOS (Laursen et al. 2001). Therefore, $O_2^{\bullet -}$ production by eNOS appears to be a self-induced mechanism triggered by interaction with a primary $O_2^{\bullet -}$ source. An important candidate for such interaction is the non-phagocytic NAD(P)H oxidase.

*NAD(P)H oxidase*

The role of the vascular NAD(P)H oxidase in I/R injury can be studied with the help of inhibitors, such as diphenyleneiodonium (DPI), apocynin, as well as a natural proline- and arginine-rich antibactetrial peptide (PR-39). While DPI inhibits all flavoproteins, the latter two are more specific since they prevent oxidase assembly by binding to the cytosolic subunit p47phox. For example, apocynin concentration-dependently inhibited NADH-stimulated $O_2^{\bullet -}$ production in rat aorta, thus increasing $^{\bullet}NO$ bioavailability and vasorelaxation (Hamilton et al. 2002).

Endothelial cells exposed to 24-h hypoxia showed an almost 3-fold increase in $H_2O_2$ production immediately upon reperfusion, which was not reversed by inhibitors of XO, cyclooxygenase, NOS, CYP450 or mitochondrial electron transport

(Zulueta et al. 1995). In contrast, DPI, and/or the protein kinase C (PKC) inhibitor calphostin, reduced $H_2O_2$ release in whole endothelial cells as well as in a membrane fraction (Zulueta et al. 1995). These results suggest that a PKC-dependent flavoprotein, such as NAD(P)H oxidase, is involved in hypoxia-induced $O_2^{\bullet -}$ production.

In isolated sheep lungs, 30 min of ischemia followed by 180 min of reperfusion increased vascular permeability, which was prevented by preincubation with apocynin (Dodd and Pearse 2000). High concentrations (0.3 and 3 mmol/l) of this NAD(P)H oxidase inhibitor increased pulmonary artery pressure observed in I/R specimens after 30-min reperfusion. In the same model, apocynin or DPI administration decreased $O_2^{\bullet -}$ production in the blood (Dodd and Pearse 2000), possibly by inhibiting both the neutrophil and vascular NAD(P)H oxidase.

In rat liver, I/R obtained by hepatic artery and portal vein clamping induced a biphasic increase in ROS production (Ozaki et al. 2000). The early oxidative burst in this tissue occurred within 5 min of reperfusion in the absence of neutrophils, suggesting that ROS were produced by the ischemic tissue itself. This increase in superoxide production was inhibited by SOD and dominant-negative rac1, while gp91phox deficiency had no effect on $O_2^{\bullet -}$ production. Therefore a rac1 containing oxidase, which is different from the neutrophil oxidase, such as one of the vascular homologues, appears to be responsible for the early increase in $O_2^{\bullet -}$ production in hepatic tissue during I/R (Ozaki et al. 2000).

The production of ROS in vascular smooth muscle cells also seems to require the small GTP-binding protein rac1. Primary cultures of rat vascular smooth muscle cells showed a 10-fold increase in ROS production after 16 h/5 min hypoxia/reoxygenation, which was reversed by rac1 inhibition using dominant-negative adenovirus transduction (Kim et al. 1998). Similarly, ROS production, detected by DCF, was inhibited when the cells were preincubated with N-acetylcysteine, catalase or DPI (Kim et al. 1998). Although this method of ROS measurement detects predominantly $H_2O_2$, results from electron spin resonance measurements showed that rac1 increased $O_2^{\bullet -}$ generation, which is subsequently dismutated to $H_2O_2$ (Irani et al. 1997). Thus, rac1 as a component of the NAD(P)H oxidase seems to contribute to increased $O_2^{\bullet -}$ production in vascular smooth muscle during hypoxia/reoxygenation.

ROS production is similarly increased in a model of lung ischemia, called – because of maintained ventilation – "oxygenated ischemia". Blocking lung perfusion for 1 h led to a 7-fold increase in DCF fluorescence indicating $H_2O_2$ generation (Al-Mehdi et al. 1998). Tissue infiltration by phagocytes was low in this model, and fluorescence was localized predominantly to endothelial cells. PR-39 dose dependently inhibited ROS generation and tissue lipid peroxidation. Thus a non-phagocytic NAD(P)H oxidase appears to be responsible for ROS production.

Multiple cellular sources of ROS may account for their increased generation during I/R. XO was identified as the first enzyme producing excess $O_2^{\bullet -}$, later studies demonstrated the important contribution of the mitochondrial respiratory chain, NAD(P)H oxidase and eNOS to ROS-induced reperfusion injury. A low

activity of antioxidant enzymes, such as GSH peroxidase, GSH reductase, SOD, and catalase was also observed after prolonged (24–48 h) endothelial cell hypoxia (Plateel et al. 1995). Overall, increased ROS production by oxidases and decreased antioxidant capacity lead to injury in I/R.

Maintaining the balance between $^{\bullet}$NO and ROS is important to protect blood vessels from I/R-induced injury. $^{\bullet}$NO prevents neutrophil infiltration, has anti-platelet activating factor properties, preserves vascular permeability and endothelial function. Treatments increasing $^{\bullet}$NO bioavailability, for example L-Arg, $H_4B$, or simply $^{\bullet}$NO donors, have great therapeutic potential in conditions associated with I/R. However, long-term nitrate administration can also lead to increased ROS formation, as further elaborated.

## ROS sources in nitrate tolerance

In nitrate tolerant arteries, the production of $O_2^{\bullet-}$ and its metabolite $ONOO^-$ are increased. The main source of $O_2^{\bullet-}$ appears to be the endothelium (Munzel and Harrison 1997), although, as shown by dihydroethidium staining, other components of the vessel wall also produce $O_2^{\bullet-}$ (Schulz et al. 2002). Positive staining for nitrotyrosine indicates that $ONOO^-$ formation is also increased throughout the vessel wall (Mihm et al. 1999). Moreover, platelets are other potential sources of $O_2^{\bullet-}$ in nitrate tolerance (McVeigh et al. 2002).

Two enzymes are important sources of increased ROS production in nitrate tolerance. Firstly, uncoupled endothelial NOS and secondly, the vascular NAD(P)H oxidase, which releases low concentrations of $O_2^{\bullet-}$ into the cytoplasm and further alters the physiological function of NOS.

### *Nitric oxide synthase*

Recent studies suggest that prolonged NTG treatment induces a dysfunctional state of NOS (Munzel et al. 2000). In healthy vessels, ACh induces vasodilation mediated by $^{\bullet}$NO release, which is blocked by NOS inhibitors. For example, the L-Arg antagonists N-monomethyl-L-Arg (L-NMMA), $N^{\omega}$-nitro-L-arginine (L-NNA) and L-nitroarginine methyl ester (L-NAME) decrease vasorelaxation and increase $O_2^{\bullet-}$ production in healthy large arteries (for references see Table 1). In contrast, when applied to nitrate-tolerant vessels NOS inhibitors improved responses to ACh, suggesting that NOS was uncoupled, thus producing the vasoconstrictor $O_2^{\bullet-}$ rather than $^{\bullet}$NO (Gori et al. 2001). Similar results were obtained in cultured endothelial cells exposed to NTG (Kaesemeyer et al. 2000). Vascular responses to L-NAME were, however, restored when the animals were pretreated with low doses of pravastatin or atorvastatin, most likely due to their $O_2^{\bullet-}$ lowering effect (Fontaine et al. 2003).

Both $O_2^{\bullet-}$ and $ONOO^-$ can oxidize $H_4B$ to $H_2B$, and thereby uncouple eNOS (Landmesser et al. 2003). SOD administration decreases tissue superoxide levels and improves relaxation (Munzel et al. 1995). Similarly, increased ROS production in nitrate-tolerant vessels can be reversed by supplementation with L-Arg,

**Table 1.** Effect of nitric oxide synthase (NOS) inhibitors on $O_2^{\bullet -}$ production and endothelium-dependent vasorelaxation

| NOS | Untreated vessels | | Nitrate-tolerant vessels | |
|---|---|---|---|---|
| inhibitor | $O_2^{\bullet -}$ production | Vasorelaxation | $O_2^{\bullet -}$ production | Vasorelaxation |
| L-NMMA | ↑(Guzik et al. 2002) | ↓(Ishibashi et al. 2001) | | ↑(Gori et al. 2001) |
| | ↑(Boger et al. 2000) | ↓(Gori et al. 2001) | | |
| | | ↓(Heitzer et al. 2000) | | |
| L-NNA | ↑(Oelze et al. 2000) | ↓(Yada et al. 2003 | ↓(Munzel et al. 2000) | ↑(Gruhn et al. 2001) |
| | ↑(Munzel et al. 2000) | ↓(Matoba et al. 2000) | | |
| | ↑(Mollnau et al. 2002) | ↓(Sotnikova 1998) | | |
| L-NAME | ↑(Laursen et al. 2001) | ↓(Guzik et al. 2002) | ↓(Kaesemeyer et al. 2000) EC | ↑(Nakae et al. 2003) |
| | ↑(Muzaffar et al. 2003) | ↓(Ruiz et al. 1997) | ↑(Fontaine et al. 2003) statins | ↓(Yamashita et al. 2000) |
| | | ↓(Imaoka et al. 1999) | | ↓(Fontaine et al. 2003) statins |

EC, endothelial cells.

$H_4B$, or antioxidant folates. Depleted intracellular stores of L-Arg are thought to contribute to abnormal NOS activity in nitrate-tolerant patients, as suggested by the fact that supplementation with L-Arg improves NOS dysfunction caused by nitrates (Parker et al. 2002). *In vivo* administration of $H_4B$ also reduced $O_2^{\bullet-}$ production (Vasquez-Vivar et al. 1998) and improved $^{\bullet}NO$ availability (Tiefenbacher 2001). Similarly, $H_4B$ or folic acid can prevent endothelial dysfunction induced by continuous nitroglycerin treatment in healthy volunteers (Gori et al. 2001; Gori et al. 2003; Leopold and Loscalzo 2003). Several mechanisms have been suggested to explain the protective function of folates. First, the $O_2^{\bullet-}$ scavenging capability of folates could prevent NOS uncoupling. Second, folates may stabilize $H_4B$ and improve its regeneration from $H_2B$. Third, they could affect eNOS directly and increase the production of $^{\bullet}NO$, rather than of $O_2^{\bullet-}$ (Verhaar et al. 2002).

The function of NOS depends on other ROS producing enzyme systems of the vessel wall, which by providing $O_2^{\bullet-}$ and $ONOO^-$ may oxidize $H_4B$ to $H_2B$ thereby inducing NOS uncoupling. The vascular NAD(P)H oxidase, for example, is suggested to play such role by generating low amounts of $O_2^{\bullet-}$, the "kindling radical" (Landmesser et al. 2003).

*NAD(P)H oxidase*

Increased $O_2^{\bullet-}$ production in aortic rings of nitrate-tolerant rabbits can be normalized by DPI, suggesting that $O_2^{\bullet-}$ is generated by a flavoprotein (Munzel et al. 1995). Specific inhibitors of several vascular oxidases (including flavoproteins, e.g. NOS, XO, mitochondrial oxidase and CYP450), however, failed to affect $O_2^{\bullet-}$ production. In contrast, addition of enzyme substrates NADH or NADPH to nitrate-tolerant rat aorta increased $O_2^{\bullet-}$-derived chemiluminescence (Fontaine et al. 2003). The lipid-lowering statins showed beneficial effect on endothelial function in patients receiving long-term nitrate treatment (Inoue et al. 2003). Statins lower $O_2^{\bullet-}$ production in nitrate tolerant vessels without increasing eNOS abundance, indicating that they improve vasorelaxation by decreasing $O_2^{\bullet-}$ production rather than by directly increasing $^{\bullet}NO$ synthesis (Fontaine et al. 2003). Indeed, atorvastatin inhibited the expression and activation of NAD(P)H oxidase (Wassmann et al. 2002). Responses to vasodilators and $O_2^{\bullet-}$ lowering properties of statins were found impaired in the presence of NADH or NADPH (Fontaine et al. 2003). On balance, these data indicate that an NAD(P)H oxidase is activated in nitrate tolerance.

The mechanism by which NAD(P)H oxidase becomes activated may involve neurohumoral pathways (Munzel and Harrison 1997; Mollnau et al. 2002). Their activation is due to the fall in blood pressure caused by vasodilation upon nitrate intake. Increased plasma renin activity in nitrate-tolerant patients indicates renin angiotensin system (RAS) activation (Munzel et al. 1996). Angiotensin II is known to increase NAD(P)H oxidase activity (Lassegue et al. 2001), and this activation seems to require PKC (Mollnau et al. 2002). In nitrate tolerance, PKC inhibitors prevented the increased vasoconstriction to phenylephrine and improved relaxation to NTG (Zierhut and Ball 1996). Conversely, the PKC activator phorbol ester

12,13-dibutyrate (PDBu) induced significantly increased vasoconstriction in NTG tolerant compared to control arteries (Munzel and Harrison 1997).

Nonintermittent nitrate administration was accompanied by abnormal platelet activation, resulting in their increased $O_2^{\bullet -}$ release (McVeigh et al. 2002). Addition of NADH increased, whereas DPI abolished $O_2^{\bullet -}$ production. Oxypurinol, rotenone and indomethacine had no effect on platelet NADH oxidoreductase activity (McVeigh et al. 2002). These results indicate that a platelet oxidase similar to the vascular NAD(P)H oxidase may be an additional source of $O_2^{\bullet -}$ upon long-term nitrate treatment.

Thus NAD(P)H oxidases contained in the vessel wall and in platelets seem to be important contributors to elevated ROS production that accompanies nitrate tolerance. The product of these enzymes, $O_2^{\bullet -}$ as well as $ONOO^-$, uncouple eNOS by oxidizing $H_4B$, which in turn leads to increased $O_2^{\bullet -}$ production. Therefore, interaction of these enzymes is involved in development of nitrate tolerance.

## Summary and therapeutic implication

Increased ROS production complicates clinical conditions associated with I/R and also the continuous nitrate administration used for the treatment of recurrent ischemic heart attacks. The first sign of vessel impairment under these circumstances is endothelial dysfunction. ROS are known to play an important role in its development.

Several enzymes in the vessel wall are involved in $O_2^{\bullet -}$ overproduction in these conditions. Even small increases in the release of ROS, such as $O_2^{\bullet -}$ and $ONOO^-$, may have an impact on endothelial physiology as they can uncouple eNOS (Landmesser and Harrison 2001).

Antioxidant vitamins or folates, due to their low toxicity and minimal side effects, may be useful tools in preventing I/R injury and nitrate tolerance, since they reverse endothelial dysfunction (Verhaar et al. 2002; Erbs et al. 2003). SOD has also a beneficial effect in vascular pathologies accompanied by increased ROS production. However, other studies reported an inconsistent outcome of antioxidant supplementation and the native SOD has been found unsuitable for clinical use.

Recent data suggest that the production and metabolism of ROS is compartmentalized within the cell (Lassegue and Clempus 2003). One of the reasons why exogenous antioxidants are not consistently effective in restoring endothelial function in I/R or nitrate tolerance could be their low ability to penetrate cellular membranes. Newly synthetized SOD mimetics are proposed to overcome this limitation, since they are small molecules and cross membranes easily (Salvemini and Cuzzocrea 2003).

However, it is important to note that ROS have a dual function in the vasculature. This may be another reason that systemic delivery of antioxidant enzymes is not always optimal. Although ROS are often considered toxic, they also have physiological roles in the endothelium. $H_2O_2$ for example is a potential candidate for EDHF in human mesenteric (Matoba et al. 2002) and coronary circulation (Yada

et al. 2003). Besides the role of $O_2^{\bullet -}$ and $H_2O_2$ on vascular relaxation, ROS also have signaling functions (Griendling et al. 2000a), they can for example stimulate endothelial cell migration and angiogenesis (Ushio-Fukai et al. 2002). Thus, ROS can act as messengers during coronary collateral development in the ischemic myocardium, as shown with frequently repeated brief occlusions and reperfusion of the artery (Gu et al. 2003).

Targeted delivery of antioxidant drugs is an interesting approach to face the harmful effects of ROS. With cell specific antibodies (immunotargeting) antioxidant enzymes can be delivered to the sites of increased ROS production. Since the endothelium is an important source of ROS during ischemia and also in nitrate tolerant vessels, endothelial cell targeting can be used to prevent vascular dysfunction (Kozower et al. 2003). Another way of specific targeting is to direct pharmacological agents to specific enzymes (for example to inhibit oxidases responsible for ROS overproduction). Recognizing the exact enzymatic sources is therefore important for designing specific therapies.

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