Journal of Thrombosis and Haemostasis, **3**: 254–267

REVIEW ARTICLE

Oxidative stress in atherogenesis and arterial thrombosis: the disconnect between cellular studies and clinical outcomes

N. R. MADAMANCHI, Z. S. HAKIM and M. S. RUNGE
Carolina Cardiovascular Biology Center, Department of Medicine, University of North Carolina, Chapel Hill, NC, USA

To cite this article: Madamanchi NR, Hakim ZS, Runge MS. Oxidative stress in atherogenesis and arterial thrombosis: the disconnect between cellular studies and clinical outcomes. *J Thromb Haemost* 2005; **3**: 254–67.

Summary. Atherosclerosis is a multifactorial disease for which the molecular etiology of many of the risk factors is still unknown. As no single genetic marker or test accurately predicts cardiovascular death, phenotyping for markers of inflammation may identify the individuals at risk for vascular diseases. Reactive oxygen species (ROS) are key mediators of signaling pathways that underlie vascular inflammation in atherogenesis, starting from the initiation of fatty streak development through lesion progression to ultimate plaque rupture. Various animal models of atherosclerosis support the notion that ROS released from NAD(P)H oxidases, xanthine oxidase, lipoxygenases, and enhanced ROS production from dysfunctional mitochondrial respiratory chain indeed have a causatory role in atherosclerosis and other vascular diseases. Human investigations also support the oxidative stress hypothesis of atherogenesis. This is further supported by the observed impairment of vascular function and enhanced atherogenesis in animal models that have deficiencies in antioxidant enzymes. The importance of oxidative stress in atherosclerosis is further emphasized because of its role as a unifying mechanism across many vascular diseases. The main contraindicator for the role oxidative stress plays in atherosclerosis is the lack of effectiveness of antioxidants in reducing primary endpoints of cardiovascular death and morbidity. However, this lack of effectiveness by itself does not negate the existence or causatory role of oxidative stress in vascular disease. Lack of proven markers of oxidative stress, which could help to identify a subset of population that can benefit from antioxidant supplementation, and the complexity and subcellular localization of redox reactions, are among the factors responsible for the mixed outcomes in the use of antioxidants for the prevention of cardiovascular diseases. To better understand the role of oxidative stress in vascular diseases, future studies should be aimed at using advances in mouse and human genetics to define oxidative stress phenotypes and link phenotype with genotype.

Correspondence: M. S. Runge, Department of Medicine, 3033 Old Clinic Bldg., University of North Carolina, Chapel Hill, NC 27599-7005, USA.
Tel.: +1 919 843 6485; fax: +1 919 966 5775; e-mail: mrunge@med.unc.edu

Keywords: NAD(P)H oxidase, ROS, SMC, DNA damage, mitochondria, antioxidants.

The role of oxidative stress in atherogenesis and thrombosis

In all industrialized countries, including the USA, more deaths result from cardiovascular disease (CVD) than from any other cause. These diseases include coronary artery disease, hypertension, congestive heart failure, and stroke, all of which are complications of atherosclerosis. Atherogenesis is a complex process, dependent on gene–environment interactions. The search for commonalities has led many investigators to study the role of inflammation in the initiation and progression of atherosclerosis. Because no single genetic marker (or groups of markers) or non-invasive test accurately predicts cardiovascular death, markers of inflammation have been touted as a means to define a 'phenotype' of the individual at risk. However, vascular inflammation *per se* is no less complex than atherosclerosis. The inflammatory process is impacted by many cellular and humoral mediators. Although much is known about the basic pathophysiological mechanisms of atherogenesis, it is likely that a more detailed understanding will lead to the development of more precise means to identify individuals with atherosclerosis and their risk for death or serious morbidity. In this review we focus on an important component of vascular inflammation: the role of reactive oxygen species (ROS) as mediators and markers of inflammatory reactions. It is increasingly evident that ROS initiate key intracellular signals that dictate cellular responses to a variety of stresses important in atherogenesis. We review the role of ROS in these processes and the possibility that ROS measurement may define a phenotype of individuals at risk for cardiovascular complications.

© 2004 International Society on Thrombosis and Haemostasis

Atherosclerosis: an overview

In humans and animals, the earliest visible evidence of atherosclerosis is the development of 'fatty streaks' which over time evolve into atheroma. Physiological measurements suggest that even before fatty streaks are present, damage to the endothelium by circulating mediators and physical forces can result in endothelial dysfunction. Both endothelial dysfunction and fatty streaks are present in a high percentage of preadolescent children in the USA and other industrialized countries [1]. Fatty streaks are areas in the vessel wall that contain lipid deposits, but contain a paucity of the cells and cellular debris that characterize more advanced lesions. The transport of oxidized low-density lipoprotein (LDL) across the endothelium into the artery wall is necessary for the formation of fatty streaks and this transport continues as more advanced lesions develop [2,3]. Oxidized LDL (Ox-LDL) is important not only for the formation of the fatty streak but also, along with a host of other physical and/or humoral mediators, for damage to the endothelium. The damaged endothelium allows for continued transport of inflammatory cells and mediators into the vessel wall and these processes generate ROS. Indeed, there is growing evidence that endothelial cells, smooth muscle cells (SMC), and macrophages are all sources of the ROS that modify phospholipids and oxidize LDL. It is also clear that ROS are involved in signaling vascular cell migration and proliferation during the formation of atherosclerotic lesions. In addition, it has been shown that the interaction of Ox-LDL with the vessel wall results in increased expression of adhesion molecules such as P-selectin [4], and chemotactic factors such as monocyte chemoattractant protein-1 [5] and macrophage colony-stimulating factor [6]. This expression leads to tethering, activation and attachment of monocytes and T lymphocytes to the endothelial cells [7]. Under the influence of growth factors and chemoattractants secreted by endothelial cells, leukocytes and SMC, monocytes and leukocytes migrate into the subendothelial space [8]. In the presence of high concentrations of LDL (both oxidized and non-oxidized), monocytes ingest lipoproteins and become macrophages. ROS generated by macrophages convert oxidized LDL into highly oxidized LDL, which is taken up by these cells through scavenger receptors to form foam cells—all resulting in what is a vicious cycle of oxidation. Foam cells can coalesce with leukocytes, and these two are the principle cells of the fatty streak. Once a fatty streak is formed, unless the oxidative cycle is interrupted, the foam cells that are present in the fatty streak will secrete growth factors that induce SMC migration from the media into the neointima. SMC proliferation coupled with continuous influx and proliferation of monocytes and macrophages converts fatty streaks to more advanced lesions and ultimately to a fibrous plaque (Fig. 1). Fibrous plaques, by definition, encroach on the arterial lumen, causing disturbed blood flow and hemodynamic forces that further contribute to plaque growth. Calcification can occur and fibrosis continues, yielding a fibrous cap surrounding a lipid-rich core which may also contain dying or dead SMC. In acute coronary syndromes, fibrous plaques rupture and initiate thrombus formation that results in vessel occlusion.

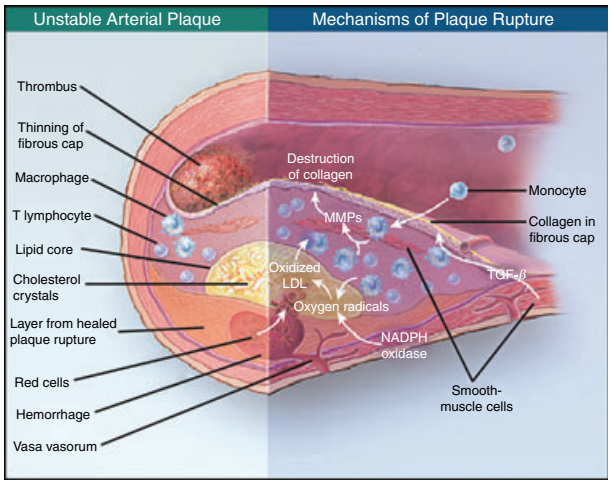

Fig. 1. An unstable arterial plaque and the mechanisms of plaque rupture ([9]; with permission from *N Engl J Med*]. Smooth muscle cell (SMC) migration from the media into the intima, induced by the growth factors secreted by foam cells, leads to the focal thickening of the intima and formation of atherosclerotic plaque. SMC proliferation and the consequent formation of connective tissue coupled with continuous influx and proliferation of monocytes and macrophages lead to more advanced lesions. Following calcification and continued fibrosis, a fibrous cap forms around a lipid-rich core; this may contain dying or dead SMC. Ultimately, the fibrous plaque protrudes into the arterial lumen. Reactive oxygen species (ROS) from many sources, including NAD(P)H oxidases of SMC and macrophages, cause necrosis of SMC. In acute coronary syndromes, matrix metalloproteinases (MMP) from macrophages and T lymphocytes digest collagen and cause thinning of the fibrous cap which eventually results in plaque rupture and thrombus formation in the vessel.

Oxidative stress and atherosclerosis

Oxygen is an abundant molecule in biological systems. Although oxygen is a radical, it is sparingly reactive because its two unpaired electrons, located in different molecular orbitals, possess parallel spins. As a result, oxygen undergoes univalent reduction to form superoxide ($O_2^{\bullet -}$) by means of enzymes such as the NADH, NAD(P)H and xanthine oxidases. Superoxide can also be formed non-enzymically by the reaction of oxygen with redox-active compounds, such as semiubiquinone, which are involved in the mitochondrial electron transport chain [10]. Superoxide anion is effervescent and under normal circumstances it is dismutated enzymically to hydrogen peroxide (H_2O_2) by the action of superoxide dismutases (SODs) [11,12]. In biological tissues, $O_2^{\bullet -}$ can also undergo non-enzymic transformation into H_2O_2 and singlet oxygen ($^1O_2$) [13]. H_2O_2 can react with other radicals such as transition metal Fe^{2+} to produced highly reactive hydroxyl radicals (Fenton reaction) capable of oxidative destruction of biomolecules. Initial oxidation of $O_2^{\bullet -}$ by Fe^{3+} generates molecular oxygen and Fe^{2+}; Fe^{2+} initiates the Fenton reaction and regenerates Fe^{3+} to propagate the reaction [14]. The oxidative potential of H_2O_2 can be amplified by myeloperoxidase,

© 2004 International Society on Thrombosis and Haemostasis

a heme protein secreted by phagocytes [15]. Hypochlorus acid (HOCl) is the major oxidant generated by the myeloperoxidase–H_2O_2–Cl^- system at physiological concentrations of Cl^- [16]. HOCl can react with $O_2^{\bullet -}$ to produce •OH [17].

$$O_2 + e^- \rightarrow O_2^{\bullet -} [10]$$

$$2O_2^{\bullet -} \xrightarrow[2H]{SOD} H_2O_2 + O_2 [11, 12]$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow {}^1O_2 + H_2O_2 [13]$$

$$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+} [14]$$

$$Fe^{2+} + H_2O_2 \rightarrow \bullet OH + OH^- + Fe^{3+} [14]$$

$$H_2O_2 + Cl^- + H^+ \xrightarrow{\text{Myeloperoxidase}} HOCL + H_2O [15, 16]$$

$$HOCL + O_2^{\bullet -} \rightarrow \bullet OH + Cl^- + O_2 [17]$$

Oxidants are produced in eukaryotic cells in normal metabolic events such as respiration and phagocytosis. In addition, low levels of ROS produced in response to growth factors and cytokines are key components of cellular metabolism. Cells have evolved both enzymic and non-enzymic mechanisms to protect against the toxic effects of oxidants. Enzymic mechanisms include SOD, catalase, and glutathione peroxidase (GPx); the non-enzymic antioxidants include glutathione, ascorbate, and α-tocopherol. Under normal conditions both types of mechanisms operate together to maintain homeostasis. However, under pathophysiological conditions ROS production exceeds the scavenging capacity of cellular antioxidant systems and the resultant oxidative stress can damage the lipids, membranes, proteins and DNA of the cell.

Modulation of ROS production in vascular cells

The NAD(P)H oxidase

Many different enzyme systems are involved in regulating ROS production and degradation in vascular cells. The predominant ROS-generating system in inflammatory cells, the membrane-bound NAD(P)H oxidase, is a major source of $O_2^{\bullet -}$ generation in vascular cells as well. NAD(P)H oxidase catalyzes the reduction of molecular oxygen by transferring an electron from the substrates NADH or NADPH.

$$NAD(P)H + 2O_2 \longrightarrow NAD(P)^+ + H^+ + 2O_2^{\bullet -}$$

NADPH differs from NADH in that the 2′-hydroxyl group of its adenosine moiety is esterified with phosphate. The preference of the enzyme for the substrate is controversial. Our group [18] and most others [19,20] have shown that NADH is the preferred substrate, but some others [21] have reported that NADPH-driven $O_2^{\bullet -}$ generation predominates in vascular SMC. However, the preference of the oxidase for either of the substrates may depend on the methodology used to measure $O_2^{\bullet -}$ production and also on the cell type [22]. The NAD(P)H oxidase of inflammatory cells generates a large burst of $O_2^{\bullet -}$ that kills engulfed bacteria; however, the vascular cell NAD(P)H oxidase differs, as described below, in the rate and extent of its generation of $O_2^{\bullet -}$. The term 'vascular NAD(P)H oxidase' is used in this review in a comprehensive manner to include low $O_2^{\bullet -}$-yielding oxidases present in vascular SMC, endothelial cells and adventitial fibroblasts in contrast to the high $O_2^{\bullet -}$-yielding neutrophil NAD(P)H oxidase. The vascular NAD(P)H oxidase has a similar, but distinct structure from the phagocytic enzyme. The phagocytic oxidase contains membrane-bound subunits gp91phox (Nox2) and p22phox, the catalytic site of the oxidase and cytosolic components p47phox, p67phox and G protein rac1 or 2 [23]. Endothelial cells and adventitial fibroblasts possess all the components of phagocytic oxidase [24–26]. The most important distinction between phagocytic cells and vascular SMC is that the latter have gp91phox homologs, *N*AD(P)H *ox*idase subunits Nox1 and Nox4 [27–30], and do not appear to possess p67phox [18]. Due to their differing structures, phagocytic oxidase and vascular oxidase are active in different circumstances. The vascular oxidase is active during normal metabolism; its sustained activation occurs in response to agonists. Vascular oxidase also produces less $O_2^{\bullet -}$, by several orders of magnitude, than does phagocytic oxidase [22]. Phosphorylation and translocation of p47phox is one of the early steps in the activation of the NAD(P)H oxidase [31].

Evidence for a critical role for NAD(P)H oxidase-derived oxidative stress in atherosclerosis has come from both cell culture studies and animal models of atherosclerosis. NAD(P)H oxidase and $O_2^{\bullet -}$ production is increased in vascular cells by a variety of agonists relevant to the pathogenesis, including angiotensin II, thrombin, platelet-derived growth factor (PDGF), and tumor necrosis factor (TNF)-α [18,32–34]. Temporal regulation of NAD(P)H oxidase also occurs depending on the vascular flow conditions. Laminar shear stress, which is atheroprotective, has been reported to cause only a transient increase in the activity of the enzyme with a compensatory increase in antioxidant defenses in human umbilical vein endothelial cells. In contrast, atherogenic oscillatory shear stress appears to cause a sustained increase in oxidase activity [32]. In hypercholesterolemic rabbits prone to atherosclerosis, increased NADH-dependent vascular $O_2^{\bullet -}$ production, presumably related to the elevation of angiotensin II levels, was associated with endothelial dysfunction [35]. AT1-receptor antagonists not only inhibited the oxidase and improved endothelial function but also reduced early plaque formation. These data suggest that oxidative stress plays a crucial role in early stages of atherosclerosis.

In a different model, we have more directly demonstrated that NAD(P)H oxidase-dependent $O_2^{\bullet -}$ generation plays an important role in atherosclerosis. We used ApoE(–/–) mice deficient in NAD(P)H oxidase activity because they lack ('knockout') p47phox, an essential component of the vascular NAD(P)H oxidase. These mice had lower levels of aortic

© 2004 International Society on Thrombosis and Haemostasis

$O_2^{\bullet -}$ production compared with wild-type mice and significantly less atherosclerosis in their descending aortas [36]. Further support for NAD(P)H oxidase-induced oxidative stress in neointimal hyperplasia was observed in balloon-injured porcine [37] and rat [38] coronary arteries. In the rat balloon injury model, Nox1 and p22phox were upregulated 3–15 days; the gp91phox, 7–15 days; and the Nox4, only 15 days after injury. These findings are consistent with both spatiotemporal as well as dynamic and differential regulation of the NAD(P)H oxidase isoforms. Direct evidence has also been obtained for the role of NAD(P)H oxidase in angioplasty-induced neointimal hyperplasia using the gp91 ds-Tat peptide in a rat balloon angioplasty model [39]. The gp91 ds-Tat is a chimeric peptide consisting of a fragment from the Tat peptide of the HIV virus and a fragment of gp91phox which prevents the interaction of p47phox with Nox subunits in cell-free systems. The Tat fragment allowed the ready uptake of the peptide inhibitor into the cells and inhibited both ROS production and neointima/media area and thickness. This peptide also inhibited stretch-induced ROS generation in distended vessels and peroxynitrite formation following angioplasty. We believe that together these data strongly support the hypothesis that oxidative stress induced via activation of NAD(P)H oxidases plays a causative role in atherosclerosis.

Human studies, although they are more correlative than studies conducted in animal systems, also strongly support the hypothesis that NAD(P)H oxidase-derived oxidative stress is important in human atherosclerosis. High levels of vasoactive agonists that induce oxidative stress *in vitro* have been demonstrated in human atherosclerotic plaques by a number of investigators. Angiotensin II was observed at the shoulder regions of atherosclerotic plaques in human coronary arteries [40]. This is significant because most instances of plaque rupture, the proximate cause of myocardial infarction, occur at the shoulder of atherosclerotic plaques. Not only is angiotensin II present in these sites, but there is also an abundance of the AT1 receptor [40], to which angiotensin II binds. Similarly increased expression of a human thrombin receptor (PAR-1) was observed in human atheroma [41]. Recently, Azumi *et al.* [42] have reported that ROS production and Ox-LDL are spatially associated with NAD(P)H oxidase subunit p22phox in atherosclerotic human coronary arteries. This observation supports the notion that ROS catalyze the formation of Ox-LDL, leading to its uptake by macrophages and resulting in the formation of activated foam cells [43]. The same authors also reported that ROS production was significantly higher in patients with unstable coronary syndromes compared with patients with stable angina, suggesting that ROS might even modulate plaque stability. These results are supported by the observation that intimal SMC, but not medial SMC and macrophages, express high levels of NAD(P)H oxidase subunits [44]. ROS induce plaque instability by inducing the expression of matrix-degrading enzymes such as matrix metalloproteinase (MMP)-2 and MMP-9 [45] and inducing endothelial and SMC apoptosis via Ox-LDL [42]. Although the causal link is more difficult to establish in human studies, in concert with findings in animal models of atherosclerosis, these studies suggest that ROS are important in human atherosclerosis also.

Mitochondrial ROS generation

It is becoming increasingly clear that under pathological conditions, mitochondrial oxidative phosphorylation can become uncoupled and results in the generation of $O_2^{\bullet -}$. The paradigm that mitochondrial ROS are key determinants of myocardial dysfunction has been addressed by a number of investigators in both experimental animal systems and humans. For example, in the rabbit heart, myocardial ischemia decreased cytochrome oxidase activity [46,47], whereas inhibition of this enzyme enhanced myocardial damage [48]; this suggests that disruption of electron flow at cytochrome oxidase can lead to both enhanced ROS production and myocardial damage. Increased mitochondrial ROS production also correlated well with reduced oxidative capacity and development and progression of left ventricular remodeling and failure in mice [49]. Clinical manifestations of the disorders of oxidative phosphorylation invariably include neuropathy, myopathy and cardiomyopathy (reviewed in [50]). Examination of oxidative phosphorylation enzyme activities in patients with cardiomyopathy revealed defects in respiratory chain complexes I, III, IV and V. Similarly, a significant decrease in the activity of complex I was observed in the failing myocardium of explanted hearts compared with donor hearts [51]. Thus, although it has been shown that increased myocardial damage and diminished mitochondrial function correlate with abnormalities in electron transport complexes [52], the decline in mitochondrial function further exacerbates ROS production which leads to a vicious cycle [53] (Fig. 2).

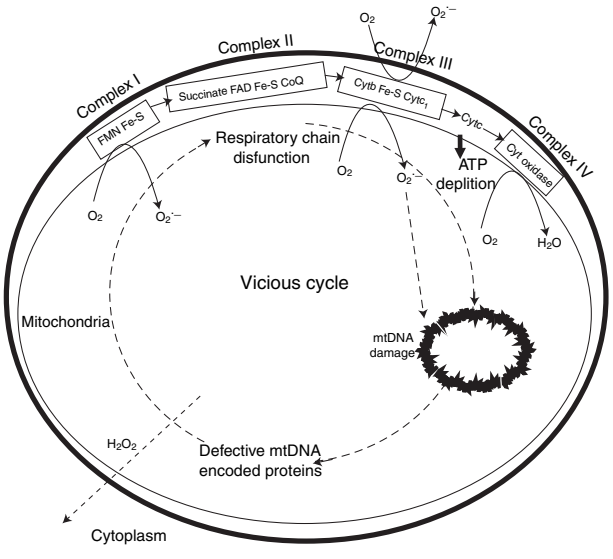

Fig. 2. In cardiovascular pathological conditions, increased reactive oxygen species (ROS) production leads to respiratory chain dysfunction and mtDNA damage. Mitochondrial respiratory chain dysfunction, in turn, results in increased ROS production, thus completing a vicious cycle.

© 2004 International Society on Thrombosis and Haemostasis

Other oxidase systems

Xanthine oxidase (XO) generates $O_2^{\bullet -}$ by the catalysis of hypoxanthine and xanthine to uric acid. This is another major source of vascular oxidative stress under pathophysiological conditions [10]. Xanthine oxidase is present in plasma and endothelial cells but not in SMC [54]. In hypercholesterolemic rabbits, diet-induced atherosclerosis has been attributed to increased oxidative stress caused by the activation of XO [55]. In hypercholesterolemic patients, administration of the XO inhibitor oxypurinol improved vasodilation. Several additional lines of evidence support an important role for XO in atherosclerosis [56]. These include: (i) electron spin resonance studies show significant activation of both NAD(P)H oxidase and XO in the coronary arteries of patients with coronary artery disease; (ii) endothelial XO is inversely proportional to and positively related to the effect of vitamin C on endothelium-dependent vasodilation in these patients; and (iii) increase of vascular XO activity is an early event in asymptomatic young individuals with familial hypercholesterolemia.

Another important ROS-generating system is represented by the lipoxygenases (LO). The LO are non-heme-containing dioxygenases that oxidize polyunsaturated fatty acids to generate hydroperoxy fatty acid derivatives; these are another important source of ROS production in the vascular wall [10]. Leukocyte-type 12/15-LO and its products, 12(S)-hydroxy-eicosatetraenoic acid [12(s)-HETE] and 15(S)-HETE, have been implicated in atherogenesis. In ApoE(–/–) mice, homozygous deletion of the 12/15-LO results in a marked inhibition of early atherosclerosis [57]. Moreover, inhibition of 12/15-LO reduced hypertension in hypertensive rats [58] and prevented intimal hyperplasia in balloon-injured rat carotid arteries [59], which bolsters the role of this enzyme in vascular pathology. Further, in cultured cells, 12/15-LO activation leads to SMC growth, hypertrophy, and inflammatory gene expression; and SMC deficient for this enzyme demonstrate impaired mitogen-induced ROS generation [60,61].

Nitric oxide synthases (NOS), and in particular endothelial NOS (eNOS) can also be sources of $O_2^{\bullet -}$ under certain pathophysiological conditions [54]. These enzymes transfer electrons from a heme group in the oxygenase domain to the substrate L-arginine to form L-citrulline and nitric oxide (NO) in the presence of a cofactor 5,6,7,8-tetrahydrobiopterin (BH_4). If either BH_4 or L-arginine availability decreases, eNOS switches from a coupled state (generates NO) to an uncoupled state (generates $O_2^{\bullet -}$) as the electrons from the heme reduce oxygen to form $O_2^{\bullet -}$ [62]. Peroxynitrite oxidation of BH_4 was shown to be the main mechanism for its deficiency and pathogenic uncoupling of NOS [63]. It was further reported that oxidation of BH_4 requires NAD(P)H oxidase, as this process does not occur in p47phox(–/–) mice that are made hypertensive [64]. The majority of studies report that induction of NO by L-arginine supplementation decreases atherosclerosis in hypercholesterolemic animal models and improves vascular function in hypercholesterolemic or hyperhomocysteinemic humans [65], whereas other studies fail to show such effects [66].

ROS scavenging systems

Just as there are many systems within the cell that generate ROS, there are many cellular systems that can degrade ROS. Catalase, SODs, and disulfide reduction reactions (GPx/glutathione reductase/GSH) have all been shown to be important (Fig. 3). Although beyond the scope of this review, the degradation of ROS is equally important to the generation of ROS in regulating ambient levels of ROS in the cell.

Oxidative stress and aging

Age is a risk factor for many vascular diseases and adults over 65 are two and one-half times more likely to suffer from hypertension and four times more likely to suffer from coronary heart disease than are those in the 40–49 years age group [67]. Age–risk factor and age–disease interactions are two very plausible explanations for the increased incidence of CVD in the elderly. In the age–risk factor hypothesis, the longer the individual is exposed to age-dependent risk factors such as hypertension, diabetes, obesity, and sedentary life style, the greater the likelihood that clinically important CVD will develop. In the second hypothesis, cardiovascular structure and function change during the aging process and specific pathophysiological mechanisms linked to experimental hypothesis, when superimposed on this altered substrate, result in clinically manifest CVD.

Evidence for increased oxidative stress with aging and its involvement in greater susceptibility to vascular disease is

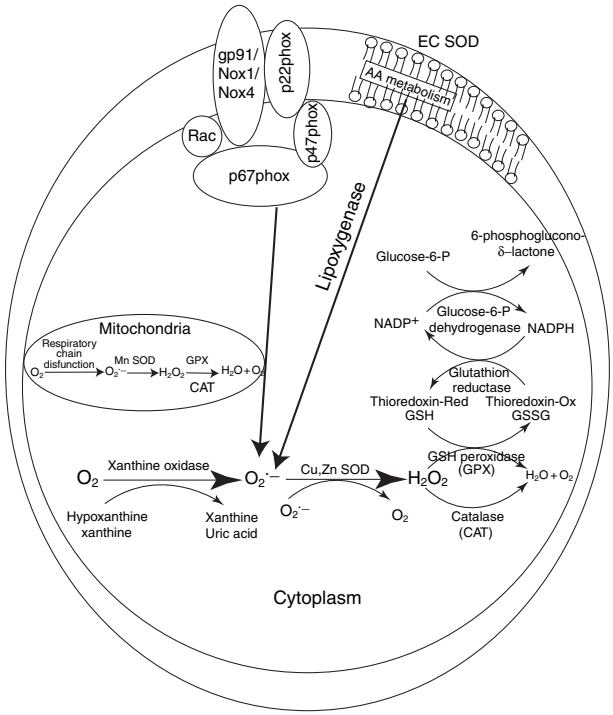

Fig. 3. Reactive oxygen species (ROS) producing and scavenging enzymes. Glucose-6-P, Glucose-6-phosphate; GSH, glutathione; GSSG, glutathione disulfide; SOD, superoxide dismutase.

© 2004 International Society on Thrombosis and Haemostasis

obtained from biochemical, physiological and genetic studies. Arterial remodeling in rats includes progressive dilation of vessels associated with intimal thickening [68]. Alteration in the collagen and elastin content of aorta from aged rats coincides with decreased arterial compliance [69]. Increased expression of transforming growth factor-β, intercellular adhesion molecule-1, and MMP-2 staining and activity, often localized near the breaks in internal elastic membrane, was observed in the thickened intima of aged rats compared with younger rats [70]. Arteriopathy associated with aging was linked to phenotypic alteration of vascular SMC when MMP-2 secretion, in response to cytokine treatment, was greater in the aortic SMC from aged rats compared with those of young rats. Age-associated arterial remodeling can be attributed to enhanced oxidative stress [71,72]. Interestingly, it was recently shown that homozygous deletion of p66shc, a gene involved in systemic and tissue oxidative stress, not only decreased early atherogenesis in mice fed a high-fat diet [73], but also extended their lifespan [74]. This suggests that steady-state levels of intracellular oxidants and oxidative damage are genetically determined and are regulated by environmental cues [75].

The effect of aging-associated oxidative stress on vascular disease can be inferred from the study of antioxidant enzyme activities and the beneficial effects of antioxidants in alleviating disease symptoms. In the cerebral cortex and hippocampus of stroke-prone spontaneously hypertensive rats, activity of the antioxidant enzymes SOD and NOS decreased with age and was correlated with cerebral lesions [76]. Arterial pressure was increased, phenylephrine-induced vascular contraction was enhanced, and acetylcholine-induced vascular relaxation was reduced in aged spontaneously hypertensive rats (SHR) compared with young ones [77]. Tempol, an SOD mimic, reduced arterial pressure and phenylephrine-induced vascular contraction in aged SHR rats. In contrast, in aged SHR rats treated with tempol and vitamin E + C, L-NAME (N^{ω}-nitro-L-arginine methyl ester), a NOS inhibitor, and ODQ (1H-[1,2,4] oxadiazolo [4,3] quinoxalin-1-one), an inhibitor of cGMP (guanosine 3′,5′-cyclic monophosphate) production in SMC, inhibited acetylcholine-induced relaxation, and enhanced phenylephrine-induced vascular contraction. These data suggest that aging is associated with impaired vascular relaxation pathways and that oxidative stress is involved in the reduction of vascular relaxation, promotion of vascular contraction, and hypertension in aged rats [77].

Similar age-related adaptive changes also occur in the vascular structure of normal humans. These changes include increases in arterial stiffening; increases in aortic root size and aortic wall thickness; and measurable abnormalities in vascular function such as enhanced arterial systolic pressure, pulse pressure, and pulse wave velocity [78]. Collagen content is increased but elastin content is decreased [79]. As in animal models, age-related endothelium-dependent vasodilation was observed in humans [80]. Acetylcholine-induced NOS-dependent vasodilation decreased with age in normotensive individuals whereas it was present in younger hypertensive subjects (age < 30 years). Vitamin C enhanced acetylcholine-dependent vasodilation in both normotensive and hypertensive individuals but it was evident in much older normotensive individuals (age > 60 years) compared with younger hypertensive individuals (age > 30 years). These results suggest that early dysfunction of the NO system is later followed by age-related oxidative stress-impaired endothelium-dependent vasorelaxation. The prevalence of non-fatal myocardial infarction increases with age and is more significant in individuals with low-activity antioxidant paraoxonase1 polymorphic allele [81].

Oxidative stress and DNA damage

DNA damage is another purported mechanism of oxidative stress-induced vascular disease. Evidence for DNA damage in atherosclerosis includes the presence of 8-hydroxy-2′-deoxyguanosine (8-OH-dG), a typical indicator of oxidative DNA damage and DNA adducts in atherosclerotic lesions and tissues [82–84]. Patients with atherosclerosis have significantly higher levels of 8-OH-dG in leukocytes compared with healthy subjects [85,86]. A similar increase in 8-OH-dG levels was observed in macrophage-derived foam cells in the atherosclerotic plaques of rabbits fed a cholesterol-rich diet [87]. DNA strand breaks and DNA repair enzymes, which were elevated in cholesterol-fed animals, were reduced with dietary lipid lowering.

Compared with nuclear DNA, mitochondrial DNA (mtDNA) is more prone to oxidative DNA damage because of its proximity to the sources of ROS generation in the mitochondrial inner membrane, its lack of protective histone-like proteins, and its poor DNA damage-repair activity [88]. MtDNA damage eventually leads to decreased mtRNA transcription and the consequent loss of function [89]. Consistent with this, we have shown that exogenous ROS cause mtDNA damage and decrease mtDNA-encoded gene transcription in a dose-dependent manner [90]. The resultant decrease in cellular ATP levels and mitochondrial redox function in vascular cells indicates how mtDNA damage affects vascular cell function in the setting of atherogenesis. Decreases in mtRNA and protein levels that lead to mitochondrial dysfunction in heart failure could also arise from a decrease in mtDNA copy number [91]. The generation of oxidative energy in mitochondria is essential for normal cardiac function; deficiencies in respiratory enzyme activities and oxidative phosphorylation associated with enhanced oxidative stress are observed in heart failure [92]. We have demonstrated that mtDNA damage not only correlated with the extent of atherosclerosis in human specimens and the aortas from ApoE(–/–) mice, but also preceded atherogenesis in ApoE(–/–) mice [93]. ApoE(–/–) mice deficient in manganese SOD (SOD2), a mitochondrial antioxidant enzyme, exhibited early increases in mitochondrial DNA damage and a phenotype of accelerated atherogenesis at arterial branch points. Enhanced mtDNA damage, as measured by increases in protective 8-oxo-dGTPase levels, was observed in post-myocardial infarction mice hearts, which suggests that mtDNA damage is important in heart failure [94].

© 2004 International Society on Thrombosis and Haemostasis

Oxidative stress and heart failure

Oxidative stress is a hallmark of chronic heart failure [95,96]. Antioxidants prevent the progression of several pathologic processes—such as cardiac hypertrophy, cardiac myocyte apoptosis, ischemia-reperfusion, and myocardial stunning—which lead to heart failure in animal models. TNF-α and angiotensin II induced hypertrophy in rat cardiac myocytes in a ROS-dependent manner and this was prevented in the presence of the antioxidants butylated hydroxyanisole, vitamin E, and catalase [97]. In vascular SMC, angiotensin II-induced hypertrophy was significantly reduced by overexpression of catalase, and transfection with antisense p22phox inhibited angiotensin II-induced H_2O_2 production, suggesting that NAD(P)H oxidase-induced oxidative stress was an underlying cause of hypertrophy [98]. NAD(P)H oxidase-dependent ROS production increased progressively during compensated hypertrophy and peaked at the stage of decompensated heart failure in guinea pig, indicating that ROS may be important mediators of heart failure [99]. Structural damage and contractile dysfunction in the failing heart might also arise from xanthine oxidase [100] and mitochondria [101]. Enhanced ROS production may reduce NO bioavailability and impair diastolic function [102], and enhanced levels of peroxynitrite may cause cytokine-induced myocardial contractile failure by inactivating sarcoplasmic Ca^{2+}-ATPase and dysregulating Ca^{2+} homeostasis [103,104].

Emerging evidence indicates that oxidative stress in general and NAD(P)H oxidase-derived ROS in particular are important in heart failure. Upregulation of NAD(P)H oxidase-derived ROS was observed in the failing myocardium of patients with ischemic or dilated cardiomyopathy [105]. In patients with heart failure, plasma TNF-α levels and platelet-derived NAD(P)H oxidase activity were elevated [106]. Recently, the presence and activation of NAD(P)H oxidase along with increased translocation of regulatory p47phox from the cytosol to the sarcolemmal membrane was established in human cardiomyocytes [107]. Together, these results suggest that oxidative stress contributes to the pathophysiology of cardiac dysfunction in heart failure.

Oxidative stress and stroke

Oxidative stress has been implicated in brain injury following ischemia and reperfusion, and free radicals produced during cerebral ischemia induce lipid peroxidation, protein oxidation, and DNA damage [108–110]. The best evidence for oxidative stress in ischemia-induced brain injury was obtained from genetic manipulation of antioxidant enzymes in rodents. Exacerbated infarct size [111], increased mitochondrial cytochrome c release, and DNA fragmentation [112] were observed in SOD2 knockout mice following permanent focal cerebral ischemia, whereas mice that overexpress SOD2 showed neuronal protection after transient focal cerebral ischemia [113]. In consonance with this, following photothrombotic ischemia, a reduction in blood–brain barrier disruption and infarct size along with decreased oxidative DNA damage and DNA fragmentation were observed in copper/zinc SOD (SOD1) transgenic mice compared with wild-type mice [114]. This suggests that $O_2^{\cdot -}$ plays a critical role in oxidative cellular injury. In a mouse ischemia-reperfusion model, deficiency of the antioxidant enzyme GPx-1 resulted in a 3-fold increase in brain infarct volume when compared with wild-type mice [115]. Increased infarct volume was associated with early activation of caspase-3 expression and enhanced apoptosis. Consistent with this, infusion of ebselen, a GPx mimic, before and during middle cerebral artery occlusion in rats conferred significant protection against ischemic damage [116]. Support for the role of oxidative stress in cerebral ischemia was also obtained from mice deficient in NOS isoforms. A reduction in infarct size was noted following permanent focal cerebral ischemia in mice deficient in neuronal (nNOS) [117,118] and inducible (iNOS) [119] isoforms, whereas an increase in lesion volume was observed in endothelial-type isoform (eNOS) knockout mice [120]. Electron spin resonance spectroscopy studies revealed high oxidative stress in the brains of stroke-prone spontaneously hypertensive rats compared with Wistar-Kyoto rats [121]. A decrease in glutathione concentration preceded cerebral infarction in severe transient focal cerebral ischemia in rats, suggesting that early oxidative stress might contribute to cerebral damage in stroke [122].

Significant increases in plasma homocystine, lipid peroxide, and NO and a decrease in ascorbate levels were observed in stroke patients compared with healthy controls [123]. Circulating phagocytes showed enhanced ROS production by opsonin receptor-dependent and opsonin receptor-independent mechanisms in patients with ischemic stroke compared with healthy individuals [124]. Together, these observations suggest that oxidative stress plays a distinct role in the pathogenesis of ischemic brain injury.

Oxidative stress and arterial thrombosis

Thrombosis, a consequence of plaque disruption, is the late complication of atherosclerosis and arterial thrombi are primarily composed of platelets [125]. Endothelial denudation and high shear force around the swollen plaque are also potent stimuli for coronary thrombosis [126]. Arterial thrombosis is a major pathogenic mechanism in acute coronary syndromes [127,128] and involves cross-talk among platelets, leukocytes and endothelial cells [129]. A plethora of stimulatory or inhibitory agonists may influence the formation of platelet–leukocyte aggregates which may facilitate leukocyte tethering, rolling and migration and enhance thrombin generation [130,131]. Leukocyte-released $O_2^{\cdot -}$ is one of the factors that could induce platelet–leukocyte aggregation [132]. Procoagulant activities of monocytes/macrophages are mediated by tissue factor [133], which is the receptor and cofactor for plasma factor VII(a), which, in turn, initiates the coagulation cascade leading to thrombogenesis *in vivo* [134]. It has also been shown that polymorphonuclear leukocytes modulate tissue factor expression by mononuclear cells via ROS, suggesting

© 2004 International Society on Thrombosis and Haemostasis

that oxidative stress induced by polymorphonuclear monocytes plays an important role in the pathogenesis of thrombosis and atherosclerosis [135]. Emerging evidence supports the presence of NAD(P)H oxidase in platelets that can be activated via protein kinase C [136–138]. In fact, NAD(P)H oxidase activation and ROS generation were involved in tissue factor upregulation in activated platelets [139,140]. Superoxide formation by hyperactive platelets during hyperhomocysteinemia may be one mechanism that contributes to arterial thrombosis [141,142].

In pathophysiological conditions such as hyperglycemia, upregulation of $O_2^{\bullet -}$ production from hyperactive platelets might form the physiological basis for prolonged thrombus formation [136]. The ROS-dependent enhancement of the activation of the platelets is kept in check by endothelium- and platelet-derived NO. eNOS overexpression in endothelial cells inhibits platelet aggregation *in vitro* [143]. In addition, NO can inhibit platelet 12-LO, an enzyme involved in the synthesis of the radical 12(S)-HETE [144]. NO can react with $O_2^{\cdot -}$ to form the radical peroxynitrite and, paradoxically, localized peroxynitrite generation can limit thrombosis in several ways. These include: (i) limiting available $O_2^{\cdot -}$; (ii) impairing thromboxane A_2 generation by inactivating cyclooxygenanse-1 [145]; (iii) S-nitrosothiol-dependent inhibition of thromboxane A_2 synthesis [146]; and (iv) inhibiting cyclooxygenase-1 [147]. Then platelet-derived arterial thrombosis is both a cause and a consequence of oxidative stress in the vasculature [148]. These *in vitro* observations suggest that platelet–leukocyte interactions and platelet-derived ROS are important mediators of thrombosis and atherosclerosis.

The importance of oxidative stress in arterial thrombosis is also obtained from experimental animal models of thrombosis and clinical investigations. A deficiency of bioactive NO is associated with arterial thrombosis in mice that lacked functional eNOS (NOSIII). These mice were deficient in platelet-derived NO also and had shorter bleeding times compared with wild-type mice [149]. In addition, thrombocytopenic wild-type mice transfused with eNOS-deficient platelets had significantly shorter bleeding times compared with mice transfused with wild-type platelets, which suggests that lack of platelet-derived NO alters the *in vivo* hemostatic response by increasing platelet recruitment. In an experimental mouse thrombosis model a moderate iron overload markedly accelerated thrombus formation, impaired vasoreactivity, enhanced production of ROS and systemic markers of oxidative stress also [150]. ROS scavenger DL-cysteine completely abrogated the iron load-induced thrombus formation which corroborates the role of oxidative stress, at least in specific thrombotic events. Antioxidant vitamin E has been shown to decrease arterial $O_2^{\bullet -}$ production, increase platelet NO release, and delay intra-arterial thrombus formation [151,152]. Also supporting the role of oxidative stress in arterial thrombosis is the observation that ROS upregulate the expression of plasminogen activator inhibitor (PAI)-1 in endothelial cells [153] and enhanced PAI-1 expression in vasculature promotes prothrombotic phenotype and atherosclerosis in ApoE(–/–) mice [154].

Evidence for a causal relationship between oxidative stress and arterial thrombosis was obtained from investigation of patients with childhood stroke [155,156]. These patients had deficiency of plasma GPx-3 and platelet inhibitory activity of the NO donors to the plasma of these patients was restored only upon exogenous addition of GPx [155]. In addition, P-selectin expression in response to thrombin treatment on platelets in these patients correlated well with the extent of GPx-3 deficiency [156]. NO production is impaired from the aggregating platelets of patients with acute coronary syndromes [157]. Although the exact mechanism for decreased NO release from the platelets of these patients has not been established, oxidative stress was implicated in the altered function of the platelets. Together, these observations indicate a strong correlation between oxidative stress and arterial thrombosis.

Antioxidants: the good, the bad and the ugly

Despite the preponderance of evidence for the association of increased oxidative stress with various vascular diseases, the outcomes of the use of antioxidants for prevention of CVD have been mixed [158,159]. Of the 12 studies that used antioxidant vitamins at varying concentrations and follow-up times, five showed benefit with regard to their respective primary endpoints. In the *C*ambridge *H*eart *A*nti*o*xidant *S*tudy (CHAOS), natural alphatocopherol (RRR-AT) at a dose of either 400 or 800 IU day^{-1} caused a significant reduction in the combined primary endpoints of cardiovascular death and non-fatal myocardial infarction [160]. In the *S*econdary *P*revention with *A*ntioxidants of *C*ardiovascular disease in *E*nd-stage renal disease (SPACE) study, RRR-AT at 800 IU day^{-1} significantly reduced the composite primary endpoint—which includes fatal and non-fatal myocardial infarction, ischemic stroke, peripheral vascular disease, and unstable angina—in hemodialysis patients with pre-existing CVD [161]. Investigation of transplant-associated atherosclerosis with a small sample size (total $N = 40$) revealed inhibition of progression of coronary intimal index (plaque area/vessel area) with combined supplementation of RRR-AT (800 IU day^{-1}) and ascorbic acid (AA; 1000 mg day^{-1}) [162]. In the *A*ntioxidant *S*upplementation in *A*therosclerosis *P*revention (ASAP) Study ($N = 440$), a combination of RRR-AT (272 IU day^{-1}) and slow-release AA (500 mg day^{-1}) significantly decreased carotid intimal medial thickness in hypercholesterolemic males [163]. During 16 years of follow-up with the women in the Nurses' Health Study ($N = 85\,118$), vitamin C intake of > 359 mg day^{-1} from diet plus supplements or supplement use alone was associated with a significant reduction in non-fatal and fatal myocardial infarction [164]. Contrary to these positive studies, seven other antioxidant supplementation studies did not show any effect on the primary endpoint of cardiovascular events (reviewed in [158]).

There could be several reasons for the apparent lack of effectiveness of antioxidants in preventing CVD. Atherosclerosis is a multifactorial disease and oxidative stress may be the predominant effector of pathology only in a subset of patient

© 2004 International Society on Thrombosis and Haemostasis

population. This could be the reason for the apparent lack of efficacy of antioxidants tested in large, prospective cardiovascular clinical trials. The second reason could relate to the optimum dose and the type of antioxidants that are used. In this regard, a dose of 800 IU day^{-1} of RRR-AT and 500 mg day^{-1} of AA have been suggested as effective threshold doses [158,165]. However, a recent meta-analysis of data from 19 clinical trials revealed that high doses (≥ 400 IU/d) may slightly increase all-cause mortailty (Miller *et al*, AHA meeting, New Orleans, LA, USA). Antioxidant formulation is also important as five out of seven antioxidant supplementation trials that are ineffective on primary endpoints used all-racemic-AT (all-rac-AT), whereas four trials described above that had positive results used RRR-AT [158]. The third reason for antioxidants' inefficacy in CVD prevention could be the complexity of redox reactions *in vivo* and the possibility of a paradoxical increase in oxidant generation by antioxidants. For example, high doses of vitamin C supplementation increased free radical-induced DNA damage in healthy volunteers [166]. Similarly β-carotene was linked to an increased risk of ischemic heart disease [167] and it could be that this antioxidant acts as an oxidant under certain conditions [168]. It is hypothesized that transition metal ions are released from metalloproteins after initial oxidative stress and the transition metals can act as catalysts in the presence of antioxidants to exacerbate the free radical damage [169].

Until our understanding of the nexus between oxidative stress and CVD advances to the extent that permits the effective use of antioxidant vitamins for primary prevention of vascular disease, we must rely on a combination of preventive measures such as healthy diet and lifestyle, and proven pharmacological agents such as statins and ACE inhibitors, to combat vascular disease.

Conclusions

Investigation of animal models of atherosclerosis and correlative data from human studies implicate oxidative stress in the development of atherosclerosis and other vascular diseases. However, our understanding of the ROS-dependent signal transduction mechanisms, their localization, and the integration of both ROS-dependent transcriptional and signaling pathways in vascular pathophysiology is limited. In order to develop effective pharmacological interventions, the oxidative stress phenotypes that underlie various vascular pathologies will need to be defined and a phenotype–genotype linkage that incorporates the recent advances in mouse and human genetics will need to be devised. These should be complemented by studies that phenotype vascular cells and their progenitors, which will further contribute to the development of regenerative therapies. Ultimately, remedial measures for oxidative stress-induced vascular malfunction will employ a combination of preventive and regenerative therapies.

References

1 Stary HC. Changes in the cells of atherosclerotic lesions as advanced lesions evolve in coronary arteries of children and young adults. In: Glagov S, Newman WP, Scaffer SA, eds. *Pathobiology of the Human Atherosclerotic Plaque*. New York: Springer, 1990: 93–106.

2 Young SG, Parthasarathy S. Why are low-density lipoproteins atherogenic? *West J Med* 1994; **160**: 153–64.

3 Navab M, Berliner JA, Watson AD, Hama SY, Territo MC, Lusis AJ, Shih DM, Van Lenten BJ, Frank JS, Demer LL, Edwards PA, Fogelman AM. The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. *Arterioscler Thromb Vasc Biol* 1996; **16**: 831–42.

4 Vora DK, Fang ZT, Liva SM, Tyner TR, Parhami F, Watson AD, Drake TA, Territo MC, Berliner JA. Induction of P-selectin by oxidized lipoproteins. Separate effects on synthesis and surface expression. *Circ Res* 1997; **80**: 810–8.

5 Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci USA* 1990; **87**: 5134–8.

6 Rajavashisth TB, Andalibi A, Territo MC, Berliner JA, Navab M, Fogelman AM, Lusis AJ. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. *Nature* 1990; **344**: 254–7.

7 McEver RP. Leukocyte–endothelial cell interactions. *Curr Opin Cell Biol* 1992; **4**: 840–9.

8 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; **362**: 801–9.

9 Heistad DD. Unstable coronary-artery plaques. *N Engl J Med* 2003; **349**: 2285–7.

10 Dröge W. Free radicals in the physiological control of cell function. *Physiol Rev* 2002; **82**: 47–95.

11 Fridovich I. The biology of oxygen radicals. *Science* 1978; **201**: 875–80.

12 Deby C, Goutier R. New perspectives on the biochemistry of superoxide anion and the efficiency of superoxide dismutases. *Biochem Pharmacol* 1990; **39**: 399–405.

13 Steinbeck MJ, Khan AU, Karnovsky MJ. Extracellular production of singlet oxygen by stimulated macrophages quantified using 9,10-diphenylanthracene and perylene in a polystyrene film. *J Biol Chem* 1993; **268**: 15649–54.

14 Kehrer JP. The Haber–Weiss reaction and mechanisms of toxicity. *Toxicology* 2000; **149**: 43–50.

15 Schraufstatter IU, Browne K, Harris A, Hyslop PA, Jackson JH, Quehenberger O, Cochrane CG. Mechanisms of hypochlorite injury of target cells. *J Clin Invest* 1990; **85**: 554–62.

16 Foote CS, Goyne TE, Lehrer RI. Assessment of chlorination by human neutrophils. *Nature* 1983; **301**: 715–6.

17 Herdener M, Heigold S, Saran M, Bauer G. Target cell-derived superoxide anions cause efficiency and selectivity of intercellular induction of apoptosis. *Free Radic Biol Med* 2000; **29**: 1260–71.

18 Patterson C, Ruef J, Madamanchi NR, Barry-Lane P, Hu Z, Horaist C, Ballinger CA, Brasier AR, Bode C, Runge MS. Stimulation of vascular cell NAD(P)H oxidase by thrombin; evidence that $p47^{phox}$ may participate in forming this oxidase *in vitro* and *in vivo*. *J Biol Chem* 1999; **274**: 19814–22.

19 Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 1994; **74**: 1141–8.

20 Mohazzab KM, Kaminski PM, Wolin MS. NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *Am J Physiol* 1994; **266**: H2568–H2572.

21 Pagano PJ, Ito Y, Tornheim K, Gallop PM, Tauber AI, Cohen RA. An NADPH oxidase superoxide-generating system in the rabbit aorta. *Am J Physiol* 1995; **268**: H2274–H2280.

22 Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 2000; **86**: 494–501.

© 2004 International Society on Thrombosis and Haemostasis

23 Babior BM. NADPH oxidase: an update. *Blood* 1999; **93**: 1464–76.
24 Jones SA, O'Donnell VB, Wood JD, Broughton JP, Hughes EJ, Jones OTG. Expression of phagocyte NAD(P)H oxidase components in human endothelial cells. *Am J Physiol* 1996; **271**: H1626–H1634.
25 Bayraktutan U, Draper N, Lang D, Shah AM. Expression of functional neutrophil-type NADPH oxidase in cultured rat coronary microvascular endothelial cells. *Cardiovasc Res* 1998; **38**: 256–62.
26 Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM, Quinn MT. Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. *Proc Natl Acad USA* 1997; **94**: 14483–8.
27 Suh YA, Arnold RS, Lassègue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD. Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 1999; **401**: 79–82.
28 Lambeth JD, Cheng G, Arnold RS, Edens WA. Novel homologs of gp91phox. *Trends Biochem Sci* 2000; **25**: 459–61.
29 Sorescu D, Weiss D, Lassègue B, Clempus RE, Szöcs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK. Superoxide production and expression of Nox family proteins in human atherosclerosis. *Circulation* 2002; **105**: 1429–35.
30 Grote K, Flach I, Luchtefeld M, Akin E, Holland SM, Drexler H, Schieffer B. Mechanical stretch enhances mRNA expression and proenzyme release of matrix metalloproteinase-2 (MMP-2) via NAD(P)H oxidase-derived reactive oxygen species. *Circ Res* 2003; **92**: e80–e86.
31 Sellmayer A, Obermeier H, Danesch U, Aepfelbacher M, Weber PC. Arachidonic acid increases activation of NADPH oxidase in monocytic U937 cells by accelerated translocation of p47-phox and co-stimulation of protein kinase C. *Cell Signal* 1996; **8**: 397–402.
32 De Keulenaer GW, Alexander RW, Ushio-Fukai M, Ishizaka N, Griendling KK. Tumor necrosis factor-α activates a p22phox-based NADH oxidase in vascular smooth muscle cells. *Biochem J* 1998; **329**: 653–7.
33 Sundaresan M, Yu Z-X, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* 1995; **270**: 296–9.
34 Bhunia AK, Arai T, Bulkley G, Chatterjee S. Lactosylceramide mediates tumor necrosis factor-alpha-induced intercellular adhesion molecule-1 (ICAM-1) expression and the adhesion of neutrophil in human umbilical vein endothelial cells. *J Biol Chem* 1998; **273**: 34349–57.
35 Warnholtz A, Nickenig G, Schulz E, Macharzina R, Brasen JH, Skatchkov M, Heitzer T, Stasch JP, Griendling KK, Harrison DG, Bohm M, Meinertz T, Munzel T. Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation* 1999; **99**: 2027–33.
36 Barry-Lane PA, Patterson C, van der Merwe M, Hu Z, Holland SM, Yeh ET, Runge MS. p47phox is required for atherosclerotic lesion progression in ApoE(–/–) mice. *J Clin Invest* 2001; **108**: 1513–22.
37 Shi Y, Niculescu R, Wang D, Patel S, Davenpeck KL, Zalewski A. Increased NAD(P)H oxidase and reactive oxygen species in coronary arteries after balloon injury. *Arterioscler Thromb Vasc Biol* 2001; **21**: 739–45.
38 Szöcs K, Lassègue B, Sorescu D, Hilenski LL, Valppu L, Couse TL, Wilcox JN, Quinn MT, Lambeth JD, Griendling KK. Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury. *Arterioscler Thromb Vasc Biol* 2002; **22**: 21–7.
39 Jacobson GM, Dourron HM, Liu J, Carretero OA, Reddy DJ, Andrzejewski T, Pagano PJ. Novel NAD(P)H oxidase inhibitor suppresses angioplasty-induced superoxide and neointimal hyperplasia of rat carotid artery. *Circ Res* 2003; **92**: 637–43.
40 Schieffer B, Schieffer E, Hilfiker-Kleiner D, Hilfiker A, Kovanen PT, Kaartinen M, Nussberger J, Harringer W, Drexler H. Expression of angiotensin II and interleukin 6 in human coronary atherosclerotic plaques: potential implications for inflammation and plaque instability. *Circulation* 2000; **101**: 1372–8.
41 Nelken NA, Soifer SJ, O'Keefe J, Vu TK, Charo IF, Coughlin SR. Thrombin receptor expression in normal and atherosclerotic human arteries. *J Clin Invest* 1992; **90**: 1614–21.
42 Azumi H, Inoue N, Ohashi Y, Terashima M, Mori T, Fujita H, Awano K, Kobayashi K, Maeda K, Hata K, Shinke T, Kobayashi S, Hirata K, Kawashima S, Itabe H, Hayashi Y, Imajoh-Ohmi S, Itoh H, Yokoyama M. Superoxide generation in directional coronary atherectomy specimens of patients with angina pectoris: important role of NAD(P)H oxidase. *Arterioscler Thromb Vasc Biol* 2002; **22**: 1838–44.
43 Channon KM. Oxidative stress and coronary plaque stability. *Arterioscler Thromb Vasc Biol* 2002; **22**: 1751–2.
44 Kalinina N, Agrotis A, Tararak E, Antropova Y, Kanellakis P, Ilyinskaya O, Quinn MT, Smirnov V, Bobik A. Cytochrome b558-dependent NAD(P)H oxidase-phox units in smooth muscle and macrophages of atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2002; **22**: 2037–43.
45 Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases *in vitro*. Implications for atherosclerotic plaque stability. *J Clin Invest* 1996; **98**: 2572–9.
46 Lesnefsky EJ, Tandler B, Ye J, Slabe TJ, Turkaly J, Hoppel CL. Myocardial ischemia decreases oxidative phosphorylation through cytochrome oxidase in subsarcolemmal mitochondria. *Am J Physiol* 1997; **273**: H1544–H1554.
47 Lesnefsky EJ, Slabe TJ, Stoll MS, Minkler PE, Hoppel CL. Myocardial ischemia selectively depletes cardiolipin in rabbit heart subsarcolemmal mitochondria. *Am J Physiol Heart Circ Physiol* 2001; **280**: H2770–H2778.
48 Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP, Tritto I, Cirillo P, Condorelli M, Chiariello M, Flaherty JT. Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. *J Biol Chem* 1993; **268**: 18532–41.
49 Ide T, Tsutsui H, Hayashidani S, Kang D, Suematsu N, Nakamura K, Utsumi H, Hamasaki N, Takeshita A. Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction. *Circ Res* 2001; **88**: 529–35.
50 Fosslien E. Review: Mitochondrial medicine—cardiomyopathy caused by defective oxidative phosphorylation. *Ann Clin Lab Sc* 2003; **33**: 371–95.
51 Scheubel RJ, Tostlebe M, Simm A, Rohrbach S, Prondzinsky R, Gellerich FN, Silber RE, Holtz J. Dysfunction of mitochondrial respiratory chain complex I in human failing myocardium is not due to disturbed mitochondrial gene expression. *J Am Coll Cardiol* 2002; **40**: 2174–81.
52 Williams MD, Van Remmen H, Conrad CC, Huang TT, Epstein CJ, Richardson A. Increased oxidative damage is correlated to altered mitochondrial function in heterozygous manganese superoxide dismutase knockout mice. *J Biol Chem* 1998; **273**: 28510–5.
53 Ide T, Tsutsui H, Kinugawa S, Suematsu N, Hayashidani S, Ichikawa K, Utsumi H, Machida Y, Egashira K, Takeshita A. Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. *Circ Res* 2000; **86**: 152–7.
54 Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol* 2003; **91**: 7A–11A.
55 Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest* 1993; **91**: 2546–51.
56 Spiekermann S, Landmesser U, Dikalov S, Bredt M, Gamez G, Tatge H, Reepschlager N, Hornig B, Drexler H, Harrison DG. Electron spin resonance characterization of vascular xanthine and

© 2004 International Society on Thrombosis and Haemostasis

NAD(P)H oxidase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. *Circulation* 2003; **107**: 1383–9.

57 Cyrus T, Witztum JL, Rader DJ, Tangirala R, Fazio S, Linton MF, Funk CD. Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. *J Clin Invest* 1999; **103**: 1597–604.

58 Nozawa K, Tuck ML, Golub M, Eggena P, Nadler JL, Stern N. Inhibition of lipoxygenase pathway reduces blood pressure in renovascular hypertensive rats. *Am J Physiol* 1990; **259**: H1774–H1780.

59 Gu JL, Pei H, Thomas L, Nadler JL, Rossi JJ, Lanting L, Natarajan R. Ribozyme-mediated inhibition of rat leukocyte-type 12-lipoxygenase prevents intimal hyperplasia in balloon-injured rat carotid arteries. *Circulation* 2001; **103**: 1446–52.

60 Reddy MA, Thimmalapura PR, Lanting L, Nadler JL, Fatima S, Natarajan R. The oxidized lipid and lipoxygenase product 12(S)-hydroxyeicosatetraenoic acid induces hypertrophy and fibronectin transcription in vascular smooth muscle cells via p38 MAPK and cAMP response element-binding protein activation. Mediation of angiotensin II effects. *J Biol Chem* 2002; **277**: 9920–8.

61 Reddy MA, Kim YS, Lanting L, Natarajan R. Reduced growth factor responses in vascular smooth muscle cells derived from 12/15-lipoxygenase-deficient mice. *Hypertension* 2003; **41**: 1294–300.

62 Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA Jr. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci USA* 1998, **95**: 9220–5.

63 Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpey M, Fukai T, Harrison DG. Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation* 2001; **103**: 1282–8.

64 Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 2003; **111**: 1201–9.

65 Cooke JP, Sydow K. A peculiar result and a fanciful hypothesis regarding L-arginine. *Arterioscler Thromb Vasc Biol* 2003; **23**: 1128.

66 Chen J, Kuhlencordt P, Urano F, Ichinose H, Astern J, Huang PL. Effects of chronic treatment with L-arginine on atherosclerosis in apoE knockout and apoE/inducible NO synthase double-knockout mice. *Arterioscler Thromb Vasc Biol* 2003; **23**: 97–103.

67 Lakatta EG, Levy D. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part I. aging arteries: a 'set up' for vascular disease. *Circulation* 2003; **107**: 139–46.

68 Michel JB, Heudes D, Michel O, Poitevin P, Philippe M, Scalbert E, Corman B, Levy BI. Effect of chronic ANG I-converting enzyme inhibition on aging processes. II. Large arteries. *Am J Physiol* 1994; **267**: R124–R135.

69 Fornieri C, Quaglino D Jr, Mori G. Role of the extracellular matrix in age-related modifications of the rat aorta. Ultrastructural, morphometric, and enzymatic evaluations. *Arterioscler Thromb* 1992; **12**: 1008–16.

70 Li Z, Froehlich J, Galis ZS, Lakatta EG. Increased expression of matrix metalloproteinase-2 in the thickened intima of aged rats. *Hypertension* 1999; **33**: 116–23.

71 Alexander RW. The Jeremiah Metzger Lecture. Pathogenesis of atherosclerosis: redox as a unifying mechanism. *Trans Am Clin Climatol Assoc* 2003; **114**: 273–304.

72 Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ Res* 2002; **90**: 251–62.

73 Napoli C, Martin-Padura I, de Nigris F, Giorgio M, Mansueto G, Somma P, Condorelli M, Sica G, De Rosa G, Pelicci P. Deletion of the p66Shc longevity gene reduces systemic and tissue oxidative stress, vascular cell apoptosis, and early atherogenesis in mice fed a high-fat diet. *Proc Natl Acad Sci USA* 2003; **100**: 2112–6.

74 Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L, Pelicci PG. The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature* 1999; **402**: 309–13.

75 Trinei M, Giorgio M, Cicalese A, Barozzi S, Ventura A, Migliaccio E, Milia E, Padura IM, Raker VA, Maccarana M, Petronilli V, Minucci S, Bernardi P, Lanfrancone L, Pelicci PG. A p53-p66Shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis. *Oncogene* 2002; **21**: 3872–8.

76 Kimoto-Kinoshita S, Nishida S, Tomura TT. Age-related change of antioxidant capacities in the cerebral cortex and hippocampus of stroke-prone spontaneously hypertensive rats. *Neurosci Lett* 1999; **273**: 41–4.

77 Payne JA, Reckelhoff JF, Khalil RA. Role of oxidative stress in age-related reduction of NO-cGMP-mediated vascular relaxation in SHR. *Am J Physiol Regul Integr Comp Physiol* 2003; **285**: R542–R551.

78 Lakatta EG. Cardiovascular regulatory mechanisms in advanced age. *Physiol Rev* 1993; **73**: 413–67.

79 Robert L. Aging of the vascular wall and atherogenesis: role of the elastin-laminin receptor. *Atherosclerosis* 1996; **123**: 169–79.

80 Taddei S, Virdis A, Ghiadoni L, Salvetti G, Bernini G, Magagna A, Salvetti A. Age-related reduction of NO availability and oxidative stress in humans. *Hypertension* 2001; **38**: 274–9.

81 Senti M, Tomas M, Marrugat J, Elosua R, REGICOR Investigators. Paraoxonase1-192 polymorphism modulates the nonfatal myocardial infarction risk associated with decreased HDLs. *Arterioscler Thromb Vasc Biol* 2001; **21**: 415–20.

82 De Flora S, Izzotti A, Walsh D, Degan P, Petrilli GL, Lewtas J. Molecular epidemiology of atherosclerosis. *FASEB J* 1997; **11**: 1021–31.

83 Van Schooten FJ, Hirvonen A, Maas LM, De Mol BA, Kleinjans JC, Bell DA, Durrer JD. Putative susceptibility markers of coronary artery disease: association between VDR genotype, smoking, and aromatic DNA adduct levels in human right atrial tissue. *FASEB J* 1998; **12**: 1409–17.

84 Binkova B, Smerhovsky Z, Strejc P, Boubelik O, Stavkova Z, Chvatalova I, Sram RJ. DNA-adducts and atherosclerosis: a study of accidental and sudden death males in the Czech Republic. *Mutat Res* 2002; **501**: 115–28.

85 Gackowski D, Kruszewski M, Jawien A, Ciecierski M, Olinski R. Further evidence that oxidative stress may be a risk factor responsible for the development of atherosclerosis. *Free Radic Biol Med* 2001; **31**: 542–7.

86 Botto N, Masetti S, Petrozzi L, Vassalle C, Manfredi S, Biagini A, Andreassi MG. Elevated levels of oxidative DNA damage in patients with coronary artery disease. *Coron Artery Dis* 2002; **13**: 269–74.

87 Martinet W, Knaapen MW, De Meyer GR, Herman AG, Kockx MM. Oxidative DNA damage and repair in experimental atherosclerosis are reversed by dietary lipid lowering. *Circ Res* 2001; **88**: 733–9.

88 Clayton DA. Transcription of the mammalian mitochondrial genome. *Annu Rev Biochem* 1984; **53**: 573–94.

89 Wallace DC. Mitochondrial diseases in man and mouse. *Science* 1999; **283**: 1482–8.

90 Ballinger SW, Patterson C, Yan CN, Doan R, Burow DL, Young CG, Yakes FM, Van Houten B, Ballinger CA, Freeman BA, Runge MS. Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circ Res* 2000; **86**: 960–6.

91 Marin-Garcia J, Goldenthal MJ, Moe GW. Mitochondrial pathology in cardiac failure. *Cardiovasc Res* 2001; **49**: 17–26.

92 Marin-Garcia J, Goldenthal MJ, Moe GW. Selective endothelin receptor blockade reverses mitochondrial dysfunction in canine heart failure. *J Card Fail* 2002; **8**: 326–32.

© 2004 International Society on Thrombosis and Haemostasis

93 Ballinger SW, Patterson C, Knight-Lozano CA, Burow DL, Conklin CA, Hu Z, Reuf J, Horaist C, Lebovitz R, Hunter GC, McIntyre K, Runge MS. Mitochondrial integrity and function in atherogenesis. *Circulation* 2002; **106**: 544–9.

94 Tsutsui H, Ide T, Shiomi T, Kang D, Hayashidani S, Suematsu N, Wen J, Utsumi H, Hamasaki N, Takeshita A. 8-oxo-dGTPase, which prevents oxidative stress-induced DNA damage, increases in the mitochondria from failing hearts. *Circulation* 2001; **104**: 2883–5.

95 Belch JJ, Bridges AB, Scott N, Chopra M. Oxygen free radicals and congestive heart failure. *Br Heart J* 1991; **65**: 245–8.

96 Mallat Z, Philip I, Lebret M, Chatel D, Maclouf J, Tedgui A. Elevated levels of 8-iso-prostaglandin F2alpha in pericardial fluid of patients with heart failure: a potential role for *in vivo* oxidant stress in ventricular dilatation and progression to heart failure. *Circulation* 1998; **97**: 1536–9.

97 Nakamura K, Fushimi K, Kouchi H, Mihara K, Miyazaki M, Ohe T, Namba M. Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-alpha and angiotensin II. *Circulation* 1998; **98**: 794–9.

98 Zafari AM, Ushio-Fukai M, Akers M, Yin Q, Shah A, Harrison DG, Taylor WR, Griendling KK. Role of NADH/NADPH oxidase-derived H_2O_2 in angiotensin II-induced vascular hypertrophy. *Hypertension* 1998; **32**: 488–95.

99 Li JM, Gall NP, Grieve DJ, Chen M, Shah AM. Activation of NADPH oxidase during progression of cardiac hypertrophy to failure. *Hypertension* 2002; **40**: 477–84.

100 Ekelund VE, Harrison RW, Shokek O, Thakkar RN, Tunin RS, Senzaki H, Kass DA, Marban E, Hare JM. Intravenous allopurinol decreases myocardial oxygen consumption and increases mechanical efficiency in dogs with pacing-induced heart failure. *Circ Res* 1999; **85**: 437–45.

101 Ide T, Tsutsui H, Kinugawa S, Utsumi H, Kang D, Hattori N, Uchida K, Arimura K, Egashira K, Takeshita A. Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. *Circ Res* 1999; **85**: 357–63.

102 Grocott-Mason R, Anning P, Evans H, Lewis MJ, Shah AM. Modulation of left ventricular relaxation in isolated ejecting heart by endogenous nitric oxide. *Am J Physiol* 1994; **267**: H1804–H1813.

103 Klebl BM, Ayoub AT, Pette D. Protein oxidation, tyrosine nitration, and inactivation of sarcoplasmic reticulum Ca2+-ATPase in low-frequency stimulated rabbit muscle. *FEBS Lett* 1998; **422**: 381–4.

104 Ferdinandy P, Danial H, Ambrus I, Rothery RA, Schulz R. Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. *Circ Res* 2000; **87**: 241–7.

105 Maack C, Kartes T, Kilter H, Schafers HJ, Nickenig G, Bohm M, Laufs U. Oxygen free radical release in human failing myocardium is associated with increased activity of rac-1 GTPase and represents a target for statin treatment. *Circulation* 2003; **108**: 1567–74.

106 De Biase L, Pignatelli P, Lenti L, Tocci G, Piccioni F, Riondino S, Pulcinelli FM, Rubattu S, Volpe M, Violi F. Enhanced TNF alpha and oxidative stress in patients with heart failure: effect of TNF alpha on platelet O2- production. *Thromb Haemost* 2003; **90**: 317–25.

107 Heymes C, Bendall JK, Ratajczak P, Cave AC, Samuel JL, Hasenfuss G, Shah AM. Increased myocardial NADPH oxidase activity in human heart failure. *J Am Coll Cardiol* 2003; **41**: 2164–71.

108 Love S. Oxidative stress in brain ischemia. *Brain Pathol* 1999; **9**: 119–31.

109 Traystman RJ, Kirsch JR, Koehler RC. Oxygen radical mechanisms of brain injury following ischemia and reperfusion. *J Appl Physiol* 1991; **71**: 1185–95.

110 Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. *J Cereb Blood Flow Metab* 2001; **21**: 2–14.

111 Murakami K, Kondo T, Kawase M, Li Y, Sato S, Chen SF, Chan PH. Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. *J Neurosci* 1998; **18**: 205–13.

112 Fujimura M, Morita-Fujimura Y, Kawase M, Copin JC, Calagui B, Epstein CJ, Chan PH. Manganese superoxide dismutase mediates the early release of mitochondrial cytochrome C and subsequent DNA fragmentation after permanent focal cerebral ischemia in mice. *J Neurosci* 1999; **19**: 3414–22.

113 Keller JN, Kindy MS, Holtsberg FW, St Clair DK, Yen HC, Germeyer A, Steiner SM, Bruce-Keller AJ, Hutchins JB, Mattson MP. Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J Neurosci* 1998; **18**: 687–97.

114 Kim GW, Lewen A, Copin J, Watson BD, Chan PH. The cytosolic antioxidant, copper/zinc superoxide dismutase, attenuates blood–brain barrier disruption and oxidative cellular injury after photothrombotic cortical ischemia in mice. *Neuroscience* 2001; **105**: 1007–18.

115 Crack PJ, Taylor JM, Flentjar NJ, de Haan J, Hertzog P, Iannello RC, Kola I. Increased infarct size and exacerbated apoptosis in the glutathione peroxidase-1 (Gpx-1) knockout mouse brain in response to ischemia/reperfusion injury. *J Neurochem* 2001; **78**: 1389–99.

116 Imai H, Graham DI, Masayasu H, Macrae IM. Antioxidant ebselen reduces oxidative damage in focal cerebral ischemia. *Free Radic Biol Med* 2003; **34**: 56–63.

117 Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* 1994; **265**: 1883–5.

118 Shimizu-Sasamata M, Bosque-Hamilton P, Huang PL, Moskowitz MA, Lo EH. Attenuated neurotransmitter release and spreading depression-like depolarizations after focal ischemia in mutant mice with disrupted type I nitric oxide synthase gene. *J Neurosci* 1998; **18**: 9564–71.

119 Iadecola C, Zhang F, Casey R, Nagayama M, Ross ME. Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. *J Neurosci* 1997; **17**: 9157–64.

120 Huang Z, Huang PL, Ma J, Meng W, Ayata C, Fishman MC, Moskowitz MA. Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. *J Cereb Blood Flow Metab* 1996; **16**: 981–7.

121 Miyazaki H, Shoji H, Lee MC. Measurement of oxidative stress in stroke-prone spontaneously hypertensive rat brain using *in vivo* electron spin resonance spectroscopy. *Redox Rep* 2002; **7**: 260–5.

122 Lerouet D, Beray-Berthat V, Palmier B, Plotkine M, Margaill I. Changes in oxidative stress, iNOS activity and neutrophil infiltration in severe transient focal cerebral ischemia in rats. *Brain Res* 2002; **958**: 166–75.

123 El Kossi MM, Zakhary MM. Oxidative stress in the context of acute cerebrovascular stroke. *Stroke* 2000; **31**: 1889–92.

124 Alexandrova ML, Bochev PG, Markova VI, Bechev BG, Popova MA, Danovska MP, Simeonova VK. Changes in phagocyte activity in patients with ischaemic stroke. *Luminescence* 2001; **16**: 357–65.

125 Reitsma PH. Is hypercoagulability an issue in arterial thrombosis? No. *J Thromb Haemost* 2004; **2**: 692–4.

126 Conde ID, Kleiman NS. Arterial thrombosis for the interventional cardiologist: from adhesion molecules and coagulation factors to clinical therapeutics. *Catheter Cardiovasc Interv* 2003; **60**: 236–46.

127 Willerson JT, Golino P, Eidt J, Campbell WB, Buja LM. Specific platelet mediators and unstable coronary artery lesions. Experimental evidence and potential clinical implications. *Circulation* 1989; **80**: 198–205.

128 Fuster V. Lewis A. Conner Memorial Lecture. Mechanisms leading to myocardial infarction: insights from studies of vascular biology. *Circulation* 1994; **90**: 2126–46.

129 Li N, Hu H, Lindqvist M, Wikstrom-Jonsson E, Goodall AH, Hjemdahl P. Platelet–-leukocyte cross talk in whole blood. *Arterioscler Thromb Vasc Biol* 2000; **20**: 2702–8.

© 2004 International Society on Thrombosis and Haemostasis

130 Diacovo TG, Puri KD, Warnock RA, Springer TA, von Andrian UH. Platelet-mediated lymphocyte delivery to high endothelial venules. *Science* 1996; **273**: 252–5.
131 Fernvik E, Lundahl J, Magnusson CG, Hallden G. The effect of *in vitro* activation and platelet interaction on the CD9 distribution and adhesion properties of human eosinophils. *Inflamm Res* 1999; **48**: 28–35.
132 Bazzoni G, Dejana E, Del Maschio A. Platelet–neutrophil interactions. Possible relevance in the pathogenesis of thrombosis and inflammation. *Haematologica* 1991; **76**: 491–9.
133 McGee MP, Li LC. Functional difference between intrinsic and extrinsic coagulation pathways. Kinetics of factor X activation on human monocytes and alveolar macrophages. *J Biol Chem* 1991; **266**: 8079–85.
134 Rapaport SI, Rao LV. Initiation and regulation of tissue factor-dependent blood coagulation. *Arterioscler Thromb* 1992; **12**: 1111–21.
135 Cadroy Y, Dupouy D, Boneu B, Plaisancie H. Polymorphonuclear leukocytes modulate tissue factor production by mononuclear cells: role of reactive oxygen species. *J Immunol* 2000; **164**: 3822–8.
136 Leo R, Pratico D, Iuliano L, Pulcinelli FM, Ghiselli A, Pignatelli P, Colavita AR, FitzGerald GA, Violi F. Platelet activation by superoxide anion and hydroxyl radicals intrinsically generated by platelets that had undergone anoxia and then reoxygenated. *Circulation* 1997; **95**: 885–91.
137 Tajima M, Sakagami H. Tetrahydrobiopterin impairs the action of endothelial nitric oxide via superoxide derived from platelets. *Br J Pharmacol* 2000; **131**: 958–64.
138 Seno T, Inoue N, Gao D, Okuda M, Sumi Y, Matsui K, Yamada S, Hirata KI, Kawashima S, Tawa R, Imajoh-Ohmi S, Sakurai H, Yokoyama M. Involvement of NADH/NADPH oxidase in human platelet ROS production. *Thromb Res* 2001; **103**: 399–409.
139 Gorlach A, Brandes RP, Bassus S, Kronemann N, Kirchmaier CM, Busse R, Schini-Kerth VB. Oxidative stress and expression of p22phox are involved in the up-regulation of tissue factor in vascular smooth muscle cells in response to activated platelets. *FASEB J* 2000; **14**: 1518–28.
140 Herkert O, Diebold I, Brandes RP, Hess J, Busse R, Gorlach A. NADPH oxidase mediates tissue factor-dependent surface procoagulant activity by thrombin in human vascular smooth muscle cells. *Circulation* 2002; **105**: 2030–6.
141 Krotz F, Sohn HY, Gloe T, Zahler S, Riexinger T, Schiele TM, Becker BF, Theisen K, Klauss V, Pohl U. NAD(P)H oxidase-dependent platelet superoxide anion release increases platelet recruitment. *Blood* 2002; **100**: 917–24.
142 Signorello MG, Pascale R, Leoncini G. Reactive oxygen species accumulation induced by homocysteine in human platelets. *Ann NY Acad Sci* 2002; **973**: 546–9.
143 Kader KN, Akella R, Ziats NP, Lakey LA, Harasaki H, Ranieri JP, Bellamkonda RV. eNOS-overexpressing endothelial cells inhibit platelet aggregation and smooth muscle cell proliferation *in vitro*. *Tissue Eng* 2000; **6**: 241–51.
144 Fujimoto Y, Tagano S, Ogawa K, Sakuma S, Fujita T. Comparison of the effects of nitric oxide and peroxynitrite on the 12-lipoxygenase and cyclooxygenase metabolism of arachidonic acid in rabbit platelets. *Prostaglandins Leukot Essent Fatty Acids* 1998; **59**: 95–100.
145 Marks DS, Vita JA, Folts JD, Keaney JF Jr, Welch GN, Loscalzo J. Inhibition of neointimal proliferation in rabbits after vascular injury by a single treatment with a protein adduct of nitric oxide. *J Clin Invest* 1995; **96**: 2630–8.
146 Tsikas D, Ikic M, Tewes KS, Raida M, Frolich JC. Inhibition of platelet aggregation by S-nitroso-cysteine via cGMP-independent mechanisms: evidence of inhibition of thromboxane A2 synthesis in human blood platelets. *FEBS Lett* 1999; **442**: 162–6.
147 Boulos C, Jiang H, Balazy M. Diffusion of peroxynitrite into the human platelet inhibits cyclooxygenase via nitration of tyrosine residues. *J Pharmacol Exp Ther* 2000; **293**: 222–9.
148 Loscalzo J. Nitric oxide insufficiency, platelet activation, and arterial thrombosis. *Circ Res* 2001; **88**: 756–62.
149 Freedman JE, Sauter R, Battinelli EM, Ault K, Knowles C, Huang PL, Loscalzo J. Deficient platelet-derived nitric oxide and enhanced hemostasis in mice lacking the NOSIII gene. *Circ Res* 1999; **84**: 1416–21.
150 Day SM, Duquaine D, Mundada LV, Menon RG, Khan BV, Rajagopalan S, Fay WP. Chronic iron administration increases vascular oxidative stress and accelerates arterial thrombosis. *Circulation* 2003; **107**: 2601–6.
151 Freedman JE, Li L, Sauter R, Keaney JF Jr. alpha-Tocopherol and protein kinase C inhibition enhance platelet-derived nitric oxide release. *FASEB J* 2000; **14**: 2377–9.
152 Saldeen T, Li D, Mehta JL. Differential effects of alpha- and gamma-tocopherol on low-density lipoprotein oxidation, superoxide activity, platelet aggregation and arterial thrombogenesis. *J Am Coll Cardiol* 1999; **34**: 1208–15.
153 Swiatkowska M, Szemraj J, Al-Nedawi KN, Pawlowska Z. Reactive oxygen species upregulate expression of PAI-1 in endothelial cells. *Cell Mol Biol Lett* 2002; **7**: 1065–71.
154 Schafer K, Muller K, Hecke A, Mounier E, Goebel J, Loskutoff DJ, Konstantinides S. Enhanced thrombosis in atherosclerosis-prone mice is associated with increased arterial expression of plasminogen activator inhibitor-1. *Arterioscler Thromb Vasc Biol* 2003; **23**: 2097–103.
155 Freedman JE, Loscalzo J, Benoit SE, Valeri CR, Barnard MR, Michelson AD. Decreased platelet inhibition by nitric oxide in two brothers with a history of arterial thrombosis. *J Clin Invest* 1996; **97**: 979–87.
156 Kenet G, Freedman J, Shenkman B, Regina E, Brok-Simoni F, Holzman F, Vavva F, Brand N, Michelson A, Trolliet M, Loscalzo J, Inbal A. Plasma glutathione peroxidase deficiency and platelet insensitivity to nitric oxide in children with familial stroke. *Arterioscler Thromb Vasc Biol* 1999; **19**: 2017–23.
157 Freedman JE, Ting B, Hankin B, Loscalzo J, Keaney JF Jr, Vita JA. Impaired platelet production of nitric oxide predicts presence of acute coronary syndromes. *Circulation* 1998; **98**: 1481–6.
158 Jialal I, Devaraj S. Antioxidants and atherosclerosis: don't throw out the baby with the bath water. *Circulation* 2003; **107**: 926–8.
159 Patterson C, Madamanchi NR, Runge MS. The oxidative paradox: another piece in the puzzle. *Circ Res* 2000; **87**: 1074–6.
160 Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 1996; **347**: 781–6.
161 Boaz M, Smetana S, Weinstein T, Matas Z, Gafter U, Iaina A, Knecht A, Weissgarten Y, Brunner D, Fainaru M, Green MS. Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. *Lancet* 2000; **356**: 1213–8.
162 Fang JC, Kinlay S, Beltrame J, Hikiti H, Wainstein M, Behrendt D, Suh J, Frei B, Mudge GH, Selwyn AP, Ganz P. Effect of vitamins C and E on progression of transplant-associated arteriosclerosis: a randomised trial. *Lancet* 2002; **359**: 1108–13.
163 Salonen RM, Nyyssonen K, Kaikkonen J, Porkkala-Sarataho E, Voutilainen S, Rissanen TH, Tuomainen TP, Valkonen VP, Ristonmaa U, Lakka HM, Vanharanta M, Salonen JT, Poulsen HE; Antioxidant Supplementation in Atherosclerosis Prevention Study. Six-year effect of combined vitamin C and E supplementation on atherosclerotic progression: the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) Study. *Circulation* 2003; **107**: 947–53.
164 Osganian SK, Stampfer MJ, Rimm E, Spiegelman D, Hu FB, Manson JE, Willett WC. Vitamin C and risk of coronary heart disease in women. *J Am Coll Cardiol* 2003; **42**: 246–52.
165 Frei B. To C or not to C, that is the question! *J Am Coll Cardiol* 2003; **42**: 253–5.

© 2004 International Society on Thrombosis and Haemostasis

166 Podmore ID, Griffiths HR, Herbert KE, Mistry N, Mistry P, Lunec J. Vitamin C exhibits pro-oxidant properties. *Nature* 1998; **392**: 559.

167 Rapola JM, Virtamo J, Ripatti S, Huttunen JK, Albanes D, Taylor PR, Heinonen OP. Randomised trial of alpha-tocopherol and beta-carotene supplements on incidence of major coronary events in men with previous myocardial infarction. *Lancet* 1997; **349**: 1715–20.

168 Burton GW, Ingold KU. beta-Carotene: an unusual type of lipid antioxidant. *Science* 1984; **224**: 569–73.

169 Halliwell B. The antioxidant paradox. *Lancet* 2000; **355**: 1179–80.

© 2004 International Society on Thrombosis and Haemostasis