Strategies to reduce oxidative stress in cardiovascular disease

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Abstract
A multitude of studies in experimental animals, together with clinical data, provide evidence that increased production of ROS (reactive oxygen species) are involved in the development and progression of cardiovascular disease. As ROS appear to have a critical role in atherosclerosis, there has been considerable interest in identifying the enzyme systems involved and in developing strategies to reduce oxidative stress. Prospective clinical trials with vitamins and hormone replacement therapy have not fulfilled earlier promises, although there is still interest in other dietary supplements. Superoxide dismutase mimetics, thiols, xanthine oxidase and NAD(P)H oxidase inhibitors are currently receiving much interest, while animal studies using gene therapy show promise, but are still at an early stage. Of the drugs in common clinical use, there is evidence that ACE (angiotensin-converting enzyme) inhibitors and AT1 (angiotensin II type 1) receptor blockers have beneficial effects on oxidative stress above their antihypertensive properties, whereas statins, in addition to improving lipid profiles, may also lower oxidative stress.

Introduction
An increasing body of evidence suggests that oxidative stress is involved in the pathogenesis of a wide range of cardiovascular diseases, including hypertension, type II diabetes, hypercholesterolaemia, atherosclerosis and heart failure.

Large amounts of ROS (reactive oxygen species) are produced in vascular cells; these include the superoxide anion (O$_2^-$), H$_2$O$_2$, the hydroxyl radical and a range of lipid radicals. O$_2^-$ is the key molecule, as many other ROS are formed as a consequence of reactions involving O$_2^-$.

The principle sources of ROS in the vasculature include NAD(P)H oxidase, xanthine oxidase, uncoupled eNOS (endothelial nitric oxide synthase) and the mitochondrial respiratory chain [1,2]. NAD(P)H oxidase is reported to be the predominant source of O$_2^-$ in the human vasculature [3]. It consists of membrane-integrated cytochrome b558, which comprises gp91phox and p22phox, and at least three cytosolic subunits (p47phox, p67phox and Rac). It exists predominantly in an unassembled state in quiescent cells. In response to stimuli the oxidase is converted into an assembled form by translocation of p47phox and p67phox to the membrane. A number of pro-atherogenic stimuli have been shown to activate NAD(P)H oxidase, including LDL (low-density lipoprotein) and Ang II (angiotensin II) [4–6]. It may seem surprising that eNOS is also a source of O$_2^-$. However, studies in vitro demonstrated uncoupling of both eNOS and nNOS (neuronal NOS) such that they

Key words: cardiovascular disease, gene therapy, oxidative stress, reactive oxygen species.
Abbreviations: ACE, angiotensin-converting enzyme; Ang II, angiotensin II; AT1, angiotensin II type 1; BH4, tetrahydrobiopterin; CAT, catalase; CHF, chronic heart failure; GPx, glutathione peroxidase; LDL, low-density lipoprotein; eNOS, iNOS and nNOS, endothelial, inducible and neuronal nitric oxide synthase respectively; NYHA, New York Heart Association; ROS, reactive oxygen species; SOD, superoxide dismutase; ECSOD, extracellular SOD; SHRSP, spontaneously hypertensive stroke-prone; TBARS, thiobarbituric acid reactive substances; WHHL, Watanabe heritable hyperlipidaemic; WKY, Wistar–Kyoto.
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produce O$_2^-$, rather than NO, under certain conditions. The major determinant of NOS uncoupling appears to be availability of the co-factor tetrahydrobiopterin (BH$_4$) [7].

A number of scavenging systems are in place to limit ROS levels. O$_2^-$ may be dismutated by a family of SODs (superoxide dismutases) to H$_2$O$_2$. MnSOD (SOD2) is located in the mitochondria and two isoforms of Cu/ZnSOD are located either intracellularly (SOD1) or extracellularly (ECSOD, SOD3). H$_2$O$_2$ can be scavenged to water by CAT (catalase) or by GPx (glutathione peroxidase) in the presence of reduced gluthathione (Figure 1).

ROS are involved in intracellular signalling. However, when ROS production is enhanced, dysregulation of physiological processes occurs. O$_2^-$ and other radicals may react with NO (nitric oxide) causing endothelial dysfunction. The reaction of O$_2^-$ with NO leads to production of peroxynitrite. Peroxynitrite is itself a potent oxidant which can induce oxidation of proteins, lipids and DNA. In addition, ROS can stimulate vascular smooth muscle cell hypertrophy and hyperplasia. Furthermore, elevations in levels of ROS may, via a variety of mechanisms, initiate development of a vascular pro-inflammatory state. This pro-inflammatory state may be promoted via activation of redox-sensitive transcription factors, such as nuclear factor κB and the leucocyte adhesion molecule vascular cell adhesion molecule 1, by reduction in levels of NO or by Ang-II-dependent pathways [1,8] (Figure 2).

It is still a matter of debate whether this increased oxidative stress has a primary causative role in cardiovascular disease pathogenesis or rather is a vascular sequel of disease progression. There is, however, emerging evidence for genetic components both from careful functional genomic studies [9] and from systemic evaluation of the candidate genes within the oxidative stress pathway [10]. In both cases it appears that the restoration of vascular ROS to normal is an important and frequently neglected therapeutic target.

EVIDENCE FROM ANIMAL STUDIES FOR INCREASED OXIDATIVE STRESS IN ASSOCIATION WITH CARDIOVASCULAR DISEASE

Hypertension

Endothelial dysfunction is observed in most rat models of hypertension, despite an increase in eNOS mRNA expression and protein levels. These apparently
Hypertension  Hypercholesterolaemia  Heart Failure  Diabetes  Smoking  Homocysteine

\[
O_2^{\cdot-} \rightarrow \downarrow \rightarrow NO
\]

Endothelial Dysfunction

Apoptosis  Vascular smooth muscle  Hypertrophy  Hyperplasia  Vasoconstriction  Thrombosis  Oxidation  Lipids  Proteins  DNA  Leucocyte adhesion

discordant findings may be explained by the almost universally observed increase in vascular ROS [11–14] (Figure 3). Consistent with increased ROS production being a key feature of hypertension, treatment with anti-oxidants and SOD mimetics attenuated endothelial dysfunction and lowered blood pressure in a number of rat models of the disease [15,16]. Conversely, delib-

erately increasing levels of oxidative stress by glutathione depletion can cause hypertension [17]. In the hypertensive rat it has been possible to identify eNOS and NAD(P)H oxidase as the principle sources of \( O_2^{\cdot-} \) [12,18,19]. Advantage has been taken of rat strains with genetic hypertension to search for candidate genes which may contribute to phenotypes of interest. A particularly useful approach may be microarray gene expression profiling to look for susceptibility genes whose action may be mediated at the level of variation in expression levels [20]. To detect differential expression, the mRNA profiles should be generated from both parental and the congenic strains, and genes underlying the qualitative trait loci should be differentially expressed in both the hypertensive/congenic strain combination and in the interparental strain comparisons. We used a congenic strain derived from the SHRSP (spontaneously hypertensive stroke-prone) and WKY (Wistar–Kyoto) rats combined with gene expression profiling and identified a candidate gene for hypertension, glutathione S-transferase (Gstm1) [9]. Expression of Gstm1 was reduced both in prehypertensive (5 week) and mature (15 week) animals. This gene is a putative positional and physiological candidate, and its pathophysiological role in hypertension is likely to involve defence against oxidative stress.

**Diabetes**

A role for ROS in the endothelial dysfunction associated with diabetes was proposed in the early 1990s [21]. Consistent with this, Kakkar et al. [22] reported increased
levels of TBARS (thiobarbituric acid reactive substances) in aorta, heart and blood of streptozotocin diabetic rats. Both uncoupling of eNOS and increased NAD(P)H oxidase activity have been suggested to be involved in increased O$_2^-$ production in vascular tissue [23,24]. In addition, levels of MnSOD have been reported to be decreased in streptozotocin diabetic rats [25]. Normalizing mitochondrial O$_2^-$ has been shown to block pathways involved in hyperglycaemic damage [26]. Consistent with these observations, SOD pre-treatment improved vasodilation in isolated aortic rings from streptozotocin diabetic rats [27]. Levels of O$_2^-$ are also increased in hyperinsulinaemic rats, which is believed to be related to activation of NAD(P)H oxidase [28].

**Hypercholesterolaemia**

Increased levels of O$_2^-$ generation and attenuated NO-mediated responses have been demonstrated in aortic rings from cholesterol-fed rabbits [29,30]. Treatment of the animals with polyethylene glycolated SODs improved endothelium-dependent vasodilation [31,32]. Supplementation with L-arginine has also been shown to reduce O$_2^-$ levels and restore NO-mediated responses in cholesterol-fed animals [33]. O$_2^-$ levels are also raised in WHHL (Watanabe heritable hyperlipidaemic) rabbits [34]. Multiple mechanisms appear to be involved in O$_2^-$ production in association with hypercholesterolaemia. Stepp and colleagues [35] provided evidence that in canine carotid arteries eNOS-, xanthine-oxidase- and possibly NAD(P)H-oxidase-dependent mechanisms were involved. Further evidence for the involvement of NAD(P)H oxidase was obtained in WHHL rabbits [36]. In monkeys with atherosclerosis, disease severity is related to O$_2^-$ levels, and regression of atherosclerosis is associated with decreases in O$_2^-$ levels and NAD(P)H oxidase activity [37].

**Heart failure**

In animal models of heart failure, levels of ROS are elevated and cardiac protection is observed with antioxidant treatment [38–40]. The increase in ROS associated with left ventricular hypertrophy appears to be NAD(P)H oxidase-dependent. Myocardial NAD(P)H oxidase activity is elevated and expression of p22phox, gp91phox, p67phox and p47phox is increased in left ventricular tissue from guinea-pigs after aortic banding [41]. The gp91phox-containing NAD(P)H oxidase has been shown to play an important role in the cardiac hypertrophic response to Ang II in mice [42]. It has been suggested that the increase in ROS is responsible for impaired endothelial regulation of left ventricular relaxation observed in moderate pressure overload left ventricular hypertrophy [43,44]. Increased production of ROS, decreased endothelium-dependent relaxation and NO bio-availability have also been observed in the vasculature of rats after myocardial infarction [45,46]. Moreover, O$_2^-$ contributes to impaired endothelium-dependent relaxation in coronary arteries of cardiomyopathic hamsters [47].

**EVIDENCE FROM CLINICAL STUDIES OF OXIDATIVE STRESS**

**Hypertension**

Lipid peroxidation by-products have been shown to be elevated, whereas levels/activity of anti-oxidant systems have been reported to be decreased in hypertensive subjects [48–50]. Among the latter, Redon et al. [51] observed decreases in the activities of SOD and CAT, as well as an increase in the ratio of oxidized to reduced glutathione. Moreover, many of the adverse effects of hypertension on endothelial function may be reversed by intra-arterial infusion of anti-oxidants, such as vitamin C [52,53]. Several studies have shown an increase in O$_2^-$ levels in hypertension [54,55]. Other studies implicate NAD(P)H oxidase as a source of excess O$_2^-$ [56,57]. A number of studies have described polymorphisms in the p22phox gene, which show an association with atherosclerotic disease or endothelial function [10,57,58]. Preliminary results from our group suggest that a single nucleotide polymorphism in the p22phox gene may effect arterial compliance [59]. However, treatment with the AT$_1$(angiotensin II type 1) receptor blocker Candesartan failed to improve endothelial function in one study of hypertensive patients [60], and NAD(P)H oxidase may not be the primary source of excess O$_2^-$ in all hypertensive subjects.

**Diabetes**

Many biochemical pathways strictly associated with hyperglycaemia (glucose auto-oxidation, polyol pathway, prostanooid synthesis and protein glycation) can increase the production of free radicals [61–64]. Furthermore, exposure of endothelial cells to high glucose leads to augmented production of O$_2^-$ [65,66]. In further support of the pathological role of oxidative stress, many of the adverse effects of high glucose on endothelial function, such as reduced endothelium-dependent relaxation and delayed cell replication, are reversed by anti-oxidants in vivo [67,68]. A rational extension of this proposed role of oxidative stress is the suggestion that the different susceptibility of diabetic patients to microvascular and macrovascular complications may be a function of the endogenous anti-oxidant status.

**Hyperlipidaemia**

Hypercholesterolaemia increases endothelial O$_2^-$ production and vascular oxidative stress, which may in turn contribute to impaired endothelial damage and
atherogenesis [66]. Hypercholesterolaemia has been independently associated with increased NADH-dependent superoxide production [10,66]. Endothelial cells, smooth muscle cells, neutrophils and monocytes all have the potential to oxidatively modify LDL, leading to the generation of lipid peroxidation products and ROS. Lipid peroxidation products may contribute to tissue damage through direct cytotoxic actions on endothelial cells or via reactions in which ‘modified’ LDL is generated and is selectively bound by ‘scavenger’ receptors [69]. Nourooz-Zadeh et al. [70] demonstrated that oxidative stress is increased in patients with familial hypercholesterolaemia. Anti-oxidant therapy with vitamins C and E restored endothelial function in hyperlipidaemic children [71].

Acute hypertriglyceridaemia has been shown to cause endothelial dysfunction via enhanced oxidant stress [72]. Depressed vitamin E levels have also been found in diabetic patients with increased lipid peroxides [73] and when associated with hypertriglyceridaemia [74,75]. In vitro data would seem to indicate that phospholipid changes (via triacylglycerol-enriched very low-density lipoprotein) increase the oxidative milieu present in the arterial wall [75]. Monocytes and polymorphonuclear cells release more O$_2^-$ when exposed to plasma from hypertriglyceridaemic patients [76,77], a phenomenon also positively correlated with plasma triacylglycerol (triglyceride) and negatively with high-density lipoprotein. Furthermore, leucocyte activation secondary to hypertriglyceridaemia may contribute to the increased oxidative stress seen in Type II diabetes [78].

**CHF (chronic heart failure)**

In patients with CHF, TBARS and 8-isoprostaglandin F2α, major biochemical consequences of ROS generation, have been found to be elevated in plasma and pericardial fluid respectively [79,80]. Studies in vivo of endothelium-dependent and -independent coronary vasodilation in patients with dilated cardiomyopathy have demonstrated a selective impairment of acetylcholine-induced increase in coronary blood flow, compared with that obtained after infusion of adenosine [81]. These original observations suggest that endothelial dysfunction may be evident in CHF patients, possibly due to enhanced ROS activity.

Studies by McMurray et al. [82] confirmed that ROS activity was increased in plasma of patients with heart failure secondary to coronary artery disease, compared with controls. Plasma malondialdehyde, a marker of lipid peroxidation, is elevated in CHF [83] and is related to exercise intolerance [84]. In other studies, increased concentrations of malondialdehyde and decreased concentrations of glutathione, vitamins C and E were correlated with both NYHA (New York Heart Association) functional class [85,86] and plasma concentrations of the cytokine, tumour necrosis factor α [86]. Lower levels of ECSOD have also been reported in subjects with coronary artery disease [87]. CHF is a state characterized by a number of processes that may promote ROS generation in vivo. These pro-oxidant pathways include cytokine activation [88–90], mitochondrial dysfunction [91], recurrent hypoxia–reperfusion [92], possibly genetic susceptibilities [10] and activation of the renin–angiotensin system [88]. There are a number of potential cellular sources implicated in enhanced ROS generation in CHF. It has recently been demonstrated that CHF patients may have increased leucocyte O$_2^-$ production, which is, in turn, related to severity of disease, as measured by NYHA functional class [93]. Other sources of enhanced ROS generation in human CHF are both the myocardium [94] and peripheral blood vessels [83]. Increased activity of myocardial NADPH oxidase has been reported in heart failure [95].

**Hyperhomocysteinaemia**

Clinical studies have shown that patients with hyperhomocysteinaemia exhibit endothelial dysfunction and elevated oxidative stress both in vitro and in vivo [96,97], however, the mechanisms by which homocysteine affects endothelial function are unclear. It is important to consider that most studies have used concentrations of homocysteine that exceed that observed in vivo. The experimental increase of plasma homocysteine concentration by methionine loading rapidly impairs endothelial function and ROS in healthy humans. Increased oxidant stress appears to play a key role in the deleterious endothelial effects of homocysteine, because the administration of an anti-oxidant completely prevents these processes [96].

**Cigarette smoking**

Serum activities of the anti-oxidant enzymes GPx, glutathione reductase and ECSOD are reported to be lower in smokers than in non-smokers [98]. Serum ascorbic acid and folic acid concentrations also are lower in smokers than in non-smokers, whereas serum malondialdehyde and TBARS are higher [98,99]. Activities of SOD and CAT in erythrocytes are significantly lower in heavy smokers, light smokers and passive smokers than in non-smokers. It has been observed that passive smokers are affected by the environmental smoke to the same extent as active smokers [98,100]. Cigarette smoking cessation increases plasma levels of several anti-oxidant micronutrients and improves resistance towards oxidative challenge [101].

A plethora of evidence suggests that administration of anti-oxidants, such as vitamins C and E, suppresses increased smoking-related lipid peroxidation markers in cigarette smokers [102–104].
PHARMACOLOGICAL APPROACHES TO LOWER OXIDATIVE STRESS

NAD(P)H oxidase inhibition

NAD(P)H oxidase has been reported to be the major source of $O_2^-$ in vascular tissue [3]. However, there is a lack of effective inhibitors targeting the NAD(P)H oxidase system. Although diphenyleneiodonium is frequently used, it can inhibit a broad range of flavin-containing enzymes.

Recently several pharmacological and molecular approaches to directly target the NAD(P)H oxidase enzyme have been proposed. Apocynin, a methoxy-substituted catechol, has been used by Peruvian Indians as an anti-inflammatory agent. It acts by blocking the assembly of p47phox into the membrane complex [105]. We have shown that apocynin decreases $O_2^-$ production in rat and human vascular rings, increases NO production in cultured human endothelial cells, and improves endothelial function ex vivo in human arteries and veins, as well as arteries from WKY and SHRSP rats [106] (Figure 4). Interestingly, effects of apocynin in young WKY rats (low oxidative stress) were minimal when compared with effects in age-matched SHRSP rats (high oxidative stress). It has also been reported that administration in vivo of apocynin to deoxycorticosterone-acetate-salt hypertensive rats decreased both vascular $O_2^-$ production and blood pressure [107]. Although apocynin appears to be an effective NAD(P)H oxidase inhibitor in vascular tissue from both rats and man, it needs to be present in relatively high concentrations to be effective. Rey et al. [108] have also considered disruption of the active NAD(P)H oxidase complex as a means of reducing oxidative stress. They used a chimaeric peptide (gp91ds-tat) designed to cross cell membranes and then inhibit p47phox association with gp91phox. Infusion of this peptide into mice significantly inhibited Ang-II-induced rises in blood pressure and vascular $O_2^-$ production. Another recently developed compound, S17834, a benzo(b)pyran-4-one, has been shown to inhibit NAD(P)H oxidase activity, $O_2^-$ production and attenuate atherosclerotic lesions in apolipoprotein-E-deficient mice [109]. However, its exact mechanism of action remains to be elucidated.

Several studies have suggested 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) have...
inhibitory actions on $O_2^−$ production from NAD(P)H oxidase independent of LDL reduction [110,111]. It has been shown recently that both $O_2^−$ and $H_2O_2$ production by vascular tissue and leucocytes are inhibited by simvastatin in Ang-II-infused rats [112]. Prevention of $O_2^−$ production by statins may be linked to prenylation-dependent Rac translocation and NAD(P)H oxidase inhibitor [113].

**Inhibition of the renin–angiotensin system**

Ang II has been shown to be a potent stimulation of NAD(P)H oxidase activity in vascular smooth muscle, fibroblasts and endothelial cells and cardiomyocytes. Infusions of Ang II have been shown to cause upregulation of the subunits of NAD(P)H oxidase and increase $O_2^−$ levels in animal studies [114–116]. There is accumulating evidence that Ang II is also an important stimulant of NAD(P)H oxidase activity and $O_2^−$ production in man [6,66,117]. In addition to its interactions with NAD(P)H oxidase, Ang II has been shown to induce LOX-1 expression, the human endothelial receptor for oxidized LDL [118]. Thus it is not surprising that ACE inhibition and Ang-II-receptor antagonisms may play a key role in reducing levels of oxidative stress.

It has been commonly postulated since the Heart Outcomes Protection Study [119] that some of the beneficial effects of ACE (angiotensin-converting enzyme) inhibitors are independent of their effect on blood pressure. ACE inhibition as an anti-oxidant strategy has been suggested as part of the explanation for this. Consistent with this hypothesis, ACE inhibition has been shown to improve endothelial function in patients with coronary artery disease [120]. Additionally AT1 receptor antagonists have been shown to be anti-oxidant and vasoprotective in patients with coronary artery disease, again downregulating vascular NAD(P)H oxidase expression [121]. Treatment with either an ACE inhibitor or an AT1-receptor antagonist resulted in lower levels of vascular $O_2^−$ [122].

It has been shown that calcium channel blockers, beta blockers and alpha receptor blockers have anti-oxidant effects in conditions *in vitro*. However, although a recent study by Baykal and colleagues [123] demonstrated a reduction in malondialdehyde and an increase in erythrocyte levels of SOD in hypertensives taking the ACE inhibitor ramipril or the AT1 receptor blocker valsartan, no improvement in the anti-oxidant status was observed in patients taking amlopidine (calcium channel blocker), metoprolol (beta blocker) or doxazosin (alpha blocker).

**Vitamins and dietary anti-oxidants**

A wealth of data from epidemiological studies suggest that a greater intake of anti-oxidant vitamins, such as vitamin E, vitamin C and beta carotene, are associated with a reduced risk of cardiovascular disease [124]. Numerous animal studies support this hypothesis [125–127], as do a number of relatively short-term functional studies in man, although many of these studies employed supra-physiological concentrations of vitamins. Vitamin E has been shown to decrease LDL oxidation [128,129] and to improve endothelial function [130,131]. Similarly vitamin C administration has been shown to improve endothelium-dependent vasodilatation [53,132,133]. The exact molecular mechanisms underlying these beneficial effects are not fully understood, but some recent studies are beginning to elucidate potential pathways. Ulker and colleagues [134] reported that 24 h exposure to vitamin C (10–100 $\mu$M) or vitamin E (100 $\mu$M) enhanced NOS activity and attenuated NAD(P)H oxidase activity in rat aorta. It has been suggested that the vitamin C-mediated increase in NOS activity could be related to alterations in BH$_4$ levels [135,136]. Consistent with this hypothesis, long-term treatment of apolipoprotein-E-deficient mice with vitamin C resulted in a decrease in levels of 7,8-dihydrobiopterin (BH$_2$), an oxidized form of BH$_4$, and an improvement in the ratio of BH$_4$/BH$_2$ [137].

Despite strong evidence demonstrating anti-oxidant effects of vitamins C and E in animals, and acutely in man, prospective randomized clinical trials have produced contrasting results. Of the larger trials, Gruppo Italiano per lo Studio della Sopravvivenza nell’Infarto miocardico [138], the Heart Outcomes Prevention Evaluation [119], the Heart Protection Study [139] and the Primary Prevention Project [140] failed to show any benefit. In contrast, the Cambridge Heart Anti-oxidant Study [141] and, most recently, the Anti-oxidant Supplementation in Atherosclerosis Prevention Study [142] report positive results. Data from these and some smaller trials have been elegantly summarized in an editorial by Jialal and Devary [128]. Numerous explanations have been proposed for the lack of observed benefit in the majority of randomized trials. They include oxidant stress status of the participants and dose and combination of vitamins administered. Vitamins C and E reside in different cellular compartments, supporting the concept of combined therapy. Moreover, vitamin E may be oxidized to form the tocopheroxyl radical. This radical can enhance lipid peroxidation and needs to be converted back into the reduced form by other anti-oxidants [143].

Although the role of the anti-oxidant vitamins remains controversial, it is widely accepted that a ‘healthy diet’ has an important role in prevention of cardiovascular disease. Two recent studies emphasize this. In one randomized placebo-controlled trial in which participants were encouraged to increase fruit and vegetable consumption both systolic and diastolic blood pressure were significantly lower in the intervention group [144]. In the second study, 6 weeks of ‘Mediterranean diet’, but not oral vitamin C, was shown to improve vascular function [145]. It is probable that anti-oxidant vitamins in the
‘healthy diet’ act in synergy with other anti-oxidants, such as flavonoids and other phenolic compounds, to provide a better anti-oxidant environment than that achieved with vitamin supplementation alone. Recently, the beneficial effects of polyphenols, particularly from red wine, have received much attention [146]. Several studies have demonstrated anti-oxidant properties of red wine and purple grape juice [147,148]. It has also been suggested that red wine polyphenols could act to improve endothelial function by increasing eNOS expression [149]. However, it must be remembered that other beverages, including beer and green tea, have been reported to have oxidative potential, as have a range of food stuffs ranging from olive oil to nuts [150]. Such data support the recommendation of a diet rich in fruits, vegetables, whole grain, oils and nuts for cardiovascular protection.

**L-Arginine**

Numerous studies in both experimental animals and man have shown acute and chronic administration of L-arginine improves vascular function in hypercholesterolaemia and other forms of cardiovascular disease [33,151,152]. The availability of L-arginine for reaction with eNOS should not be rate limiting, as intracellular levels of L-arginine are in the millimolar range, whereas the $K_m$ for the substrate is in the micromolar range. This apparent discrepancy is frequently referred to as the ‘L-arginine paradox’. Explanations for this paradox include decreased $O_2^-$ production, decreased transport of arginine into endothelial cells, increased levels of asymmetric dimethylarginine and increased insulin release [153]. Most recently it has been suggested that translational control of NOS expression by arginine can explain the arginine paradox, at least for iNOS (inducible NOS) [154].

**Thiol-containing compounds**

Over the years a number of thiol-containing compounds have been used experimentally to inhibit LDL oxidation and reduce oxidative stress. Recent studies would support the continued investigation of such compounds. In glucose-fed rats α-lipoic acid attenuated hypertension, insulin resistance and oxidative stress [155], and in another study was shown to lower blood pressure in spontaneously hypertensive rats [156]. In man, the classical sulphhydryl compound N-acetylcysteine reduced cardiovascular events in patients with end-stage renal failure [157].

**Oestrogen and hormone replacement therapy**

Pre-menopausal women are at a lower risk of atherosclerosis and have a lower incidence of coronary heart disease and myocardial infarction than post-menopausal women or age-matched men [158,159]. Acute oestrogen administration has been reported to improve vasoreactivity in healthy post-menopausal women [160, 161]. Epidemiological studies suggested that hormone replacement therapy reduced the morbidity and mortality associated with cardiovascular disease [158]. Innumerable animal studies have also shown favourable effects of oestrogen on the cardiovascular system [162,163]. However, controversy exists over the mechanisms underlying the beneficial effects of oestrogen. Some groups have cited decreased $O_2^-$ production as a primary cause [163,164], and others increased expression of NOS by genomic or non-genomic pathways [162,165–167]. In addition, oestrogens may activate the gene encoding cyclo-oxygenase and decrease production of the potent vasoconstrictor endothelin [168,169]. Surprisingly, against this background, data from recently published randomized prospective-controlled clinical trials failed to show cardiovascular benefit from hormone replacement therapy (Heart and Estrogen Progesterone Replacement Study [170], Estrogen Replacement and Atherosclerosis [171] and the Women’s Health Initiative Randomised Controlled Trial [172]). Part of this apparent contradiction may relate to the cohorts studied. Most animal studies and most of the early observational studies used healthy cohorts which may not be representative of the general population. Women with existing cardiovascular disease may not show the same beneficial effects of oestrogen on endothelial function as demonstrated in healthy cohorts. In such women the adverse effects of oestrogen, such as the increase in triacylglycerol levels and C-reactive protein, may out weight the benefits [170].

**SOD mimetics**

Endogenous $O_2^-$ is dismutated to $H_2O_2$ by a family of SODs. In general, studies, both in vivo and in vitro, aimed at reducing oxidative stress by increasing levels of Cu/Zn SOD have proved disappointing. This may be because Cu/Zn SOD does not gain access to the appropriate cellular compartments. However, a number of SOD mimetics are available that cross the membrane and have proved more successful in decreasing oxidative stress and improving endothelial function [15,173].

**Xanthine oxidase inhibition**

Xanthine oxidase has been proposed to be an important source of $O_2^-$ in man [6]. The enzyme exists in two isoforms, xanthine oxidase and xanthine dehydrogenase. Activity of the former may be increased in ischaemia reperfusion injury and inflammation. Cardillo et al. [174] reported that the xanthine oxidase inhibitor oxypurinol improved endothelial function in hypercholesterolaemic, but not hypertensive, subjects. More recently, another xanthine oxidase inhibitor allopurinol has been shown to improve endothelial function in Type II diabetes, CHF and cigarette smokers [99,175,176]. However, it must be
Table 1  Studies of NOS and SOD gene transfer in cardiovascular disease

<table>
<thead>
<tr>
<th>Gene</th>
<th>Disease model</th>
<th>Blood vessel</th>
<th>Phenotype</th>
<th>Effect</th>
<th>Reference</th>
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<td>Carotid artery</td>
<td>Vasodilation</td>
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<td>[177,178]</td>
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<tr>
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<td>SHRSP rat</td>
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<td>Vasodilation</td>
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<td>Vasodilation</td>
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<td>[181]</td>
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<td>Vasodilation</td>
<td>↑</td>
<td>[182]</td>
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<td>Neointima</td>
<td>↓</td>
<td>[184]</td>
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<tr>
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<td>Neointima</td>
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<td>↑</td>
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<td>[190]</td>
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<tr>
<td>Cu/ZnSOD</td>
<td>SHRSP rat</td>
<td>Carotid artery</td>
<td>Endothelial function</td>
<td>=</td>
<td>[180]</td>
</tr>
<tr>
<td>Cu/ZnSOD</td>
<td>Alloxan diabetic rabbit</td>
<td>Carotid artery</td>
<td>Vasodilation</td>
<td>=</td>
<td>[181]</td>
</tr>
<tr>
<td>Cu/ZnSOD</td>
<td>Ang-II-infused rabbit</td>
<td>Aorta</td>
<td>Vascular function</td>
<td>=</td>
<td>[183]</td>
</tr>
<tr>
<td>Cu/ZnSOD</td>
<td>WHHL rat</td>
<td>Aorta</td>
<td>Vasodilation</td>
<td>=</td>
<td>[34]</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Cholesterol-fed rabbit</td>
<td>Carotid artery</td>
<td>Vascular function</td>
<td>=</td>
<td>[192]</td>
</tr>
<tr>
<td>MnSOD</td>
<td>SHRSP rat</td>
<td>Carotid artery</td>
<td>Endothelial function</td>
<td>=</td>
<td>[193]</td>
</tr>
<tr>
<td>ECSD</td>
<td>Ang-II-infused rabbit</td>
<td>Aorta</td>
<td>Vascular function</td>
<td>=</td>
<td>[179]</td>
</tr>
<tr>
<td>ECSD</td>
<td>WHHL rat</td>
<td>Aorta</td>
<td>Vasodilation</td>
<td>=</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>Rabbit subarachnoid haemorrhage</td>
<td>Basilar artery</td>
<td>Vasospasm</td>
<td>↓</td>
<td>[194]</td>
</tr>
<tr>
<td></td>
<td>Balloon injury (rabbit)</td>
<td>Aorta</td>
<td>Neointima</td>
<td>↓</td>
<td>[195]</td>
</tr>
<tr>
<td></td>
<td>SHRSP rat</td>
<td>Carotid artery</td>
<td>Endothelial function</td>
<td>↑</td>
<td>[193]</td>
</tr>
<tr>
<td></td>
<td>SHR rat</td>
<td>In vivo</td>
<td>Blood pressure</td>
<td>↓</td>
<td>[196]</td>
</tr>
</tbody>
</table>

noted that the patient numbers in all these studies were low (11 patients or less).

GENE TRANSFER STRATEGIES FOR LOWERING OXIDATIVE STRESS

The principle of viral vectors to deliver genes efficiently to the vasculature is well established in severe single gene disorders and cancer studies. In recent years a number of studies have applied these techniques in animal models of cardiovascular disease with varying degrees of success. The majority of these studies have attempted to increase NO bio-availability directly via increased expression of NOS genes or indirectly via increased expression of SOD genes. Details of a broad selection of these studies are given in Table 1.

Both eNOS and nNOS consistently improved endothelial function in the range of models examined, whereas overexpression of iNOS produced more varied effects possibly due to a concomitant increase in O₂⁻ production. Overexpression of Cu/ZnSOD and MnSOD generally resulted in little improvement in vascular function, whereas overexpression of ECSOD improved vascular function in the majority of studies (Figure 5). Fennel et al. [193] proposed a number of explanations for this apparent discrepancy, including the longer half-life of ECSOD and the wider sphere of influence of ECSOD compared with intracellular Cu/ZnSOD and mitochondrial MnSOD.

Studies aimed at overexpression of other anti-oxidant genes are limited, although adenoirus-mediated transfer of GPx has been shown to rescue homocysteine-induced endothelial dysfunction [197], whereas adenovirus-mediated transfer of CAT has been shown to inhibit...
endothelial cell proliferation [198] and protect endothelial cells from oxidative stress [199].

Despite the promise shown in many of the studies outlined above, to date the vast majority of studies of vascular gene transfer have been carried out either in vitro or following local in vivo administration. These approaches may allow sufficient gene transfer to eventually prove a useful intervention for oxidative stress associated with certain vascular pathologies, e.g., during vein graft procedures or as an adjunct to balloon angioplasty and stenting. However, in many conditions, oxidative stress is widespread throughout the vasculature. Thus there is a need to be able to specifically target vectors to desired vascular beds. Some studies have reported sustained systemic effects following injection of either naked transgene DNA or vectors encoding for transgenes [196,200]. However, in practice, most studies of this type are hampered by the natural tropism of most viral vectors for certain tissues, such as the liver. Many groups are currently focusing efforts on techniques to de-target viral vectors away from their natural targets to defined vascular cells and regions [201–204]. In addition to providing more exact control over transgene expression, targeting strategies will also allow the administration of lower doses of vector, thus enhancing the safety profile of these agents.

**CONCLUSION**

This review summarizes the extensive array of experimental animal and human data describing pharmacological and molecular approaches to reducing oxidative stress with the aim of improving clinical outcome in patients with cardiovascular disease.

The clinical importance of hormone replacement therapy and anti-oxidant vitamin supplementation have not been confirmed by large controlled trials. To the practising physician, the benefits of ACE inhibition and AT1 receptor blockers have been clearly described and the elucidation that, in part, some of the benefit may be derived from optimizing the ‘oxidant milieu’ leads to the pursuit of other drugs or indeed molecular therapies that act in the same way. The promise of the SOD mimetics, xanthine oxidase inhibitors, NAD(P)H oxidase inhibitors and gene transfer strategies to enhance the bioactivity of NO may soon provide clinicians with additional therapeutic options in the treatment of cardiovascular disease which will lower oxidative stress, a process which is becoming increasingly recognized as critical in the pathophysiology of vascular disease.

**REFERENCES**


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