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# Forum Review

# Targeting Reactive Oxygen Species in Hypertension

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## ABSTRACT

**Oxidative stress plays an important role in the pathogenesis of hypertension. A number of sources of reactive oxygen species have been identified including NADPH oxidase, endothelial NO synthase, and xanthine oxidase. Inhibitors of these systems reduce blood pressure in experimental models. Targeted overexpression of antioxidant systems and interference with expression of oxidant systems has also been successfully used in animal models of hypertension. It is expected that these strategies will eventually be translated to human disease, but currently, the specificity and toxicity of such measures are not yet fulfilling quality criteria for treatment of humans. In the meantime, presumably nontoxic measures, such as administration of antioxidant vitamins, are the only available treatments for oxidative stress in humans. In this review, we discuss strategies to target oxidative stress both in experimental models and in humans. We also discuss how patients could be selected who particularly benefit from antioxidant treatment. In clinical practice, diagnostic procedures beyond measurement of blood pressure will be necessary to predict the response to antioxidants; these procedures will include measurement of antioxidant status and detailed assessment of vascular structure and function.** *Antioxid. Redox Signal.* 10, 1061–1077.

THE PAST decades have seen a compelling accumulation of evidence that reactive oxygen species (ROS) and reactive nitrogen species (RNS) can contribute to the pathogenesis of many cardiovascular diseases, including hypertension (Table 1). Myriad ROS and RNS are generated endogenously as byproducts of normal physiologic redox reactions (Fig. 1). However, when perturbations of normal function occur, resulting in either excess generation of ROS/RNS or in compromised endogenous antioxidant protection, many of these species are able to interact with and damage intracellular macromolecules, including proteins, lipids, and DNA. Key among these ROS and RNS are superoxide ($^{\bullet}O_2^-$), the hydroxyl radical ($^{\bullet}OH$), nitric oxide (NO), peroxynitrite ($ONOO^-$), and hydrogen peroxide ($H_2O_2$).

Superoxide is formed from the single-electron reduction of molecular oxygen by various endogenous enzyme systems as well as the spontaneous autooxidation of a number of molecules, including hemoglobin and catecholamines (52). Although its half-life is very short ($1 \times 10^{-6}$ sec), the highly reactive nature of superoxide places it as a key ROS in biologic systems, with many of its deleterious effects resulting from its conversion to or interaction with other ROS (52). Recent years have seen the emergence of the concept of superoxide as an important signaling molecule, as opposed to being simply viewed as a by-product of intracellular processes or as a cytotoxic defense molecule (32, 68, 106, 144).

The dismutation of superoxide, either spontaneously or via the actions of the superoxide dismutase family of enzymes, results in the formation of hydrogen peroxide, the two-electron reduced state of oxygen (52). The major biologic routes of removal of hydrogen peroxide are through the actions of catalase and glutathione peroxidase, with the formation of water (157). However, although it is not as reactive as superoxide *per se*, the more stable nature and membrane-crossing properties of $H_2O_2$ mean that it also plays an important role in ROS-mediated processes (156).

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Table 1. Oxidative Stress in Clinical and Experimental (Including Genetic, Environmental, and Surgical) Forms of Hypertension

| *Form of hypertension* | *Oxidative stress component?* |
|---|---|
| Human hypertension | |
| Mild essential hypertension | — |
| Severe hypertension | Y |
| Salt-sensitive hypertension | Y |
| Malignant hypertension | Y |
| Renovascular hypertension | Y |
| Preeclampsia | Y |
| Genetic models of hypertension | |
| Spontaneously hypertensive rat (SHR) | Y |
| Stroke-prone SHR (SHRSP) | Y |
| Experimentally induced models of hypertension | |
| Angiotensin II infused | Y |
| Noradrenaline infused | — |
| Salt-loaded SHRSP | Y |
| Dahl salt-sensitive rat (salt loaded) | Y |
| Lead induced | Y |
| Obesity associated | Y |
| Two-kidney, one-clip | Y |
| Postmenopausal | Y |
| Mineralocorticoid | Y |

Adapted from ref. 182, with permission.

In the presence of metal ions, hydrogen peroxide can also be further reduced *via* Fenton or Haber-Weiss chemistry to form the hydroxyl radical. This is a highly reactive radical that can attack most cellular components, as well as initiating chains of further redox reactions (32, 52). Although normally viewed in terms of its vasodilator, antithrombotic, antiatherogenic, and second-messenger activity, NO is also a radical species that can undergo redox reactions. Of particular importance in hypertension, under conditions of increased oxidative stress, NO will act avidly with superoxide to form peroxynitrite, as discussed later (11).

## SOURCES OF ROS

### *NADPH oxidase*

Over the past 10- to 15-year period, it has become increasingly well established that the (nonphagocytic) NADPH oxidase enzyme complex is one of the most important sources of superoxide in relation to cardiovascular disease, in both the vasculature (66, 181) and other key systems such as the kidney (60) (Fig. 2). A wealth of literature has arisen focusing on the NADPH oxidase systems, including recent comprehensive reviews in a level of detail beyond the scope of the present article (12).

The existence of various homologues of the gp91$^{phox}$ subunit (which together with the more conserved p22$^{phox}$ subunit comprises the catalytic cytochrome $b_{558}$ center of the complex) has been established (12), and the importance of the heterogeneous expression of these, together with the various regulatory subunits, remains to be fully determined. However, it may be that this heterogeneous pattern of expression offers the possibility of selective targeting of this key source of superoxide in particular cell or tissue types, or in specific disease states (12, 67). The close functional association between NADPH oxidase and the renin–angiotensin system (RAS) may be of particular relevance in linking oxidative stress to hypertension. Angiotensin II activates the NADPH oxidase system *via* its actions on $AT_1$ receptors and subsequent intracellular signaling pathways (99, 143, 163). This is capable of increasing ROS generation throughout the vascular wall (182). Moreover, longer-term activation of $AT_1$ receptors can result in upregulation of NADPH oxidase subunit expression (54), whereas increased ROS activity can modify $AT_1$ receptor expression (130). Thus, the potential for a deleterious positive-feedback effect of RAS activation on vascular oxidative stress clearly exists, and this may play a major role in hypertension. Other hypertension-related humoral factors shown to activate NADPH oxidase include endothelin-1, thrombin, TNF-$\alpha$, TGF-$\beta$, and PDGF (12, 64). Additionally, physical factors such as shear stress or vascular stretch can also stimulate ROS generation from NADPH oxidase (12, 13, 64).

### *Xanthine oxidase*

Xanthine oxidase has been characterized as another important source of ROS in the vasculature, its activity being modulated by a range of cytokines as well as physical factors, including shear stress (84). Xanthine oxidase levels are increased in a number of cardiovascular disease states, including hypertension (126).

### *eNOS*

As described later, the oxidation of the key cofactor, $BH_4$, or a lack of the L-arginine substrate can lead to "uncoupling" of eNOS and results in production of superoxide, as opposed to NO, and impaired endothelium-dependent vasodilation (5, 47). This has been demonstrated in various cardiovascular diseases, including hypertension, and can be reversed by administration of $BH_4$ (5, 47, 96).

### *Other ROS sources*

Although the main sources of ROS have been outlined, generation can also occur as byproducts of a number of endogenous enzymatic pathways, including the 5′-lipoxygenase and cyclooxygenase pathways. Additionally, although not normally expressed in vascular tissue, myeloperoxidase may be a source of superoxide generated by infiltrating inflammatory cells. "Leakage" of superoxide anions from the mitochondrial respiratory chain is likely to constitute the major nonenzymatic source of ROS. The potential for this mechanism to be involved in signaling and control of mitochondrial energetics has attracted increasing attention recently (144, 186), and the mitochondria may be the key source of superoxide, particularly in forms of hypertension in which endothelin plays a prominent role (21). These additional sources of ROS have been the subject of review elsewhere (126, 177).

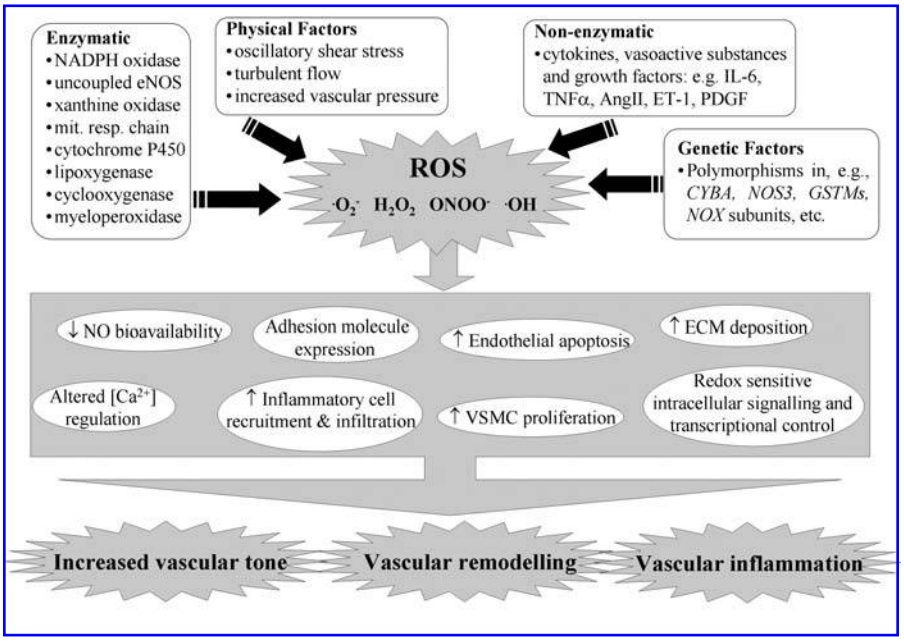


**FIG. 1. Key sources and major effects of reactive oxygen species (ROS) in the vasculature.** ROS may be generated by a number of mechanisms, including enzymatic and nonenzymatic pathways; as a response to physical changes (in, *e.g.*, blood flow), or as a result of altered transcription/activity of prooxidant or antioxidant systems due to genetic variability. Oxidative stress, where ROS generation exceeds endogenous antioxidant mechanisms, can produce a multitude of vascular changes, ultimately resulting in a loss of NO bioavailability, vascular inflammation, and long-term vascular remodelling.

## ACTIONS OF ROS IN HYPERTENSION

The deleterious actions of ROS on the cardiovascular system are widespread, including actions on the central, cardiac, neuronal, endocrine, and, in particular, the renal and vascular systems. These disparate actions of ROS have been described in detail by others (38, 73, 182, 198, 207, 212), and to review them all is beyond the scope of this article. Therefore, in the following section, we focus on the vascular effects of ROS in hypertension, before outlining the experimental evidence for targeting some of these mechanisms in the clinic.

### *Vascular tone and function*

The rapid interaction of superoxide with NO to form peroxynitrite has been established as a key mechanism in the loss of

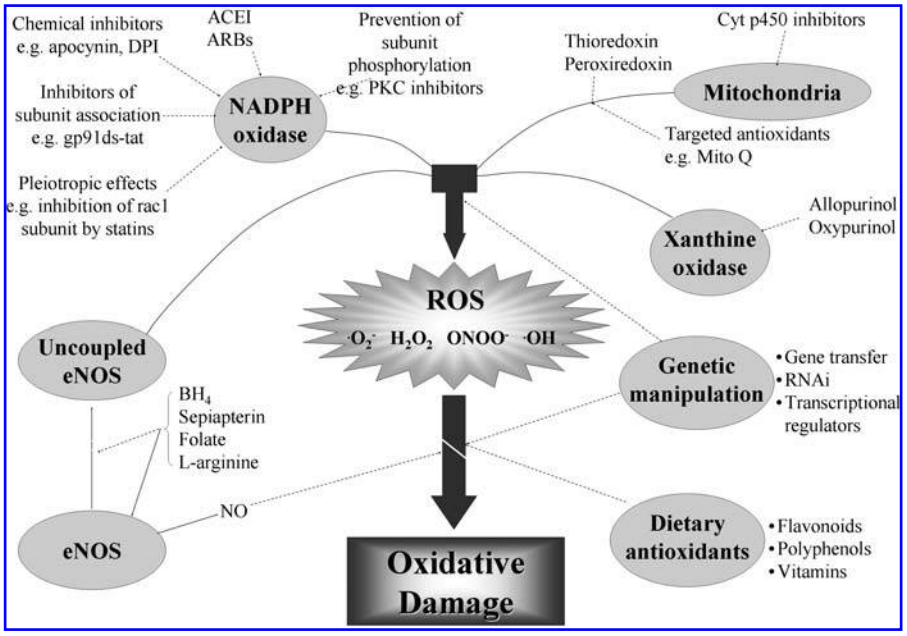


**FIG. 2. Organization and activation of the vascular NADPH oxidase enzyme complex.** The formation of a functional NADPH oxidase complex requires the assembly of at least six distinct subunits. These include the membrane-bound $p22^{phox}$ and one of the NOX isoforms, which together constitute the cytochrome $b_{558}$ catalytic centre; a $p47^{phox}$ organizational subunit; the $p67^{phox}$ subunit, which activates the complex; a p40 regulatory element; and the small GTPase, Rac 1/2. Heterogeneous expression of the various NOX isoforms occurs throughout the cardiovascular, and other, systems. Activation of the complex occurs in response to a number of processes, in particular, binding of angiotensin II to the $AT_1$ receptor. This results in the phosphorylation of the p47 subunit and translocation of the cytosolic units to the cell membrane, assembling with the NOX and $p22^{phox}$ subunits. The recruitment of the Rac subunit is also obligatory for functioning of the complex. The full intracellular signaling pathway controlling NADPH oxidase activity remains to be fully elucidated, but is known to be at least partially dependent on the activation of phospholipase D and protein kinase C in response to stimulation of $AT_1$ and other receptors.

endothelial NO bioavailability that occurs in hypertension (11). Although peroxynitrite possesses vasodilator activity (171), it is much weaker than that of NO. In addition to the net resultant loss of vasodilatory tone, peroxynitrite is itself capable of undergoing further redox reactions to generate further ROS, with subsequent downstream effects, including the oxidation of $BH_4$, which leads to the "uncoupling" of eNOS and further superoxide generation (47). The zinc-binding center of eNOS can also be oxidized by peroxynitrite, leading to the loss of the $BH_4$-binding domain and inhibiting generation of NO. Furthermore, it has been documented that superoxide can have direct effects on calcium mobilization and homeostasis, resulting in modulation of vascular tone (1).

In contrast to the vasoconstrictor effects of superoxide, hydrogen peroxide has been described as having vasodilator properties (202). Given the dismutation of superoxide to form hydrogen peroxide, it could be suggested at a simplistic level that the opposite vasomotor effects of these two ROS may act to offset each other. However, because of the interplay between many different ROS and numerous redox pathways potentially available, the specific effect of any given ROS on vascular tone may be difficult to interpret. This is further compounded by the differential effects of vascular ROS between differing species and vascular beds (180, 181).

### *Inflammation and remodelling*

In addition to effects on vascular endothelial function, oxidative stress has been clearly implicated in longer-term physical remodelling of the vasculature, which occurs in a number of cardiovascular diseases, including hypertension.

Oxidative vascular damage can result in expression of various well-characterized inflammatory cytokines, including interleukin-6 and TNF-$\alpha$, which can in turn influence the expression of adhesion molecules, including ICAM-1 and VCAM-1, in a ROS-sensitive manner (136, 182). This has the effect of increasing endothelial layer permeability, further inhibiting normal endothelial function, and promoting recruitment and transmigration of polymorphonuclear leukocytes (136, 182). These cells can differentiate into activated macrophages, which have a much higher capacity for ROS production than do resident cells, further exacerbating oxidative damage (136).

In addition to inflammation, vascular growth and proliferation of smooth muscle cells are key features of vascular remodelling in hypertension. Early studies defined a clear relation between levels of ROS and vascular growth responses, showing, for example, that catalase can inhibit PDGF-stimulated DNA synthesis in cultured VSMCs (92, 170). Subsequently, other proliferative agents, including thrombin and catecholamines, have been shown to require the activation of NADPH-oxidases to produce their effects, which are sensitive to antioxidants (17, 139). More-recent studies suggest that PDGF-stimulated proliferative responses can be inhibited with newer agents that have no effect on ROS production (137). In contrast, migration of VSMCs can be inhibited by NADPH-oxidase inhibition, but with no effect on the synthesis of DNA (178). Taken together, it seems likely that both ROS-sensitive and ROS-insensitive pathways may be involved in the growth and proliferative response of vascular cells in cardiovascular diseases. The differential effects of ROS on vascular growth further complicate dissection of this role, as low concentrations of $H_2O_2$ can promote growth, whereas higher concentrations can be proapoptotic (37, 105).

Turnover of vascular extracellular matrix is also modified by increased levels of ROS in hypertension, further contributing to vascular remodelling. Studies on matrix metalloproteinases have shown that the activities of MMP2 and MMP9 (which are involved in the breakdown of basement membrane and elastin) are sensitive to ROS generated by endothelial, vascular smooth muscle, and macrophage cells. Moreover, this sensitivity to ROS can result in either stimulatory or inhibitory effects, depending on the concentration of ROS present (147). In addition to modifying activity of MMPs, ROS can modify their expression (112).

## TARGETING OXIDATIVE STRESS

The previous section outlined the various sources of ROS and their effects on the vasculature in relation to hypertension. Experimental evidence has shown that manipulation of these systems may offer potential therapeutic strategies for combating the compounding effects of oxidative stress in the clinic. This evidence is reviewed here (Fig. 3).

### *NADPH oxidase system*

Given the importance of NADPH oxidase as a source of ROS, this system has attracted much interest as a potential therapeutic target (see Fig. 2). Two of the most widely used inhibitors of the NADPH oxidase complex, apocynin and diphenylene iodonium (DPI), have been most used as research tools and have been shown to inhibit many of the deleterious effects of the NADPH oxidase–derived ROS outlined earlier, in many *in vitro* and *ex vivo* studies. However, these agents may lack selectivity, and in the case of apocynin, require to be metabolized by a peroxidase enzyme to become active. Nonetheless, the ability of apocynin significantly to reduce vascular oxidative stress, increase the activity of the glutathione system, and moreover, to attenuate blood-pressure increases in the DOCA salt-sensitive rat model of hypertension (60) underlines the therapeutic potential of targeting the NADPH oxidase system. Peptide inhibitors, chiefly the Gp91ds-tat molecule, may offer an alternative to chemical inhibitors of NADPH oxidase. These inhibitors are effective in preventing the assembly of the NADPH oxidase complex and have produced effects similar to those of chemical inhibitors on blood pressure and vascular ROS production in *in vivo* models of hypertension (67, 149), although their clinical use may be limited because of difficulties of administration. A further approach attracting interest is in the inhibition of the assembly of the intact oxidase by prevention of one of the key first steps—phosphorylation of the $p47^{phox}$ subunit—*via* protein kinase C inhibitors. This approach has shown promise in animal and in *in vitro* human studies and is now subject to clinical trials (10, 69, 124). Successful demonstrations of RNAi-based approaches to silence specific NADPH oxidase subunits selectively have already been published. These range from mechanistic, *in vitro* studies on the role of the subunits (28, 107, 108) to *in vivo* studies showing a significant reduction in blood pressure in the angiotensin II rat model of hy-

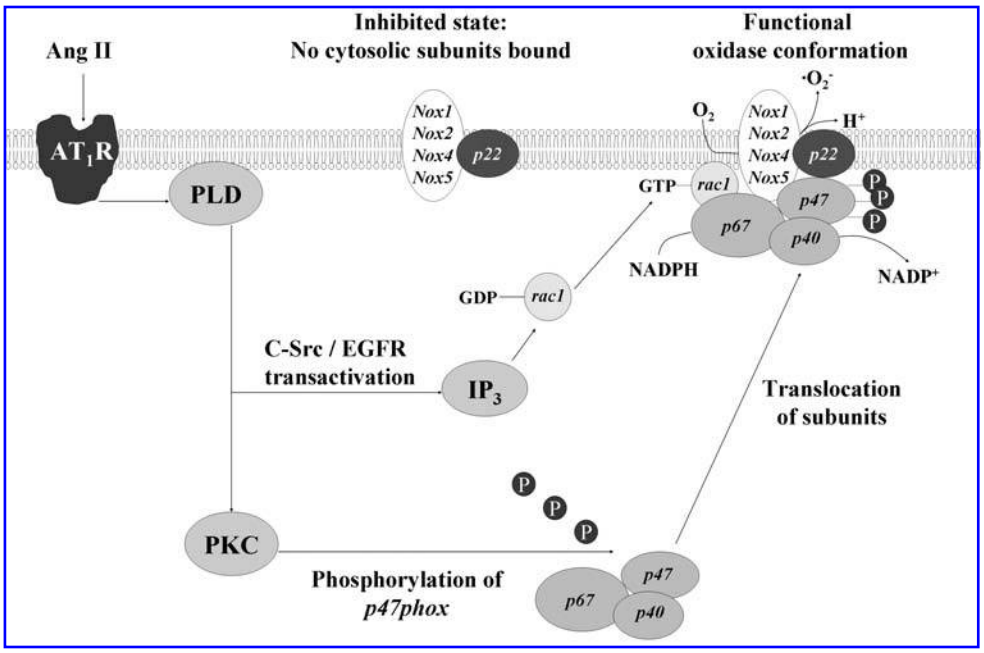


**FIG. 3. Targeting oxidative stress in hypertension.** Prooxidant pathways are depicted as solid lines, and negative regulators, as dotted lines. A number of clinical and experimental strategies have arisen to combat oxidative stress in hypertension. These include the use of inhibitors of the key enzymatic sources of ROS; development of tissue, cellular, and even organelle-specific antioxidants; administration of substrates and cofactors to prevent uncoupling of eNOS; and dietary supplementation of antioxidants and vitamins. An ongoing challenge is the successful translation of many of these encouraging strategies into the clinic. In terms of future approaches, the refinement of targeted gene transfer and RNA-interference approaches offers the tantalizing prospect of highly selective activation or silencing of particular pro- and antioxidants.

pertension after silencing of $p22^{phox}$ (123). The ability to silence expression of individual NADPH oxidase subunits, particularly given the heterogeneous expression of the NOX subunits, seems likely to be a key target of future studies.

A final area in which the activity of NADPH oxidase system can be modulated is through pleiotropic actions of drugs directed primarily at other targets. As described earlier, the RAS exerts a strong influence over the NADPH oxidase system *via* the actions of angiotensin II on $AT_1$ receptors. It is, therefore, perhaps unsurprising that drugs acting on this system, including ACE inhibitors and angiotensin-receptor blockers, have been found to have additional beneficial effects on endothelial function by inhibiting NADPH oxidase activity, reducing the expression of the complex's subunits and reducing vascular oxidative stress (19, 131, 146). A number of members of the statin lipid-lowering family of drugs have also been found to have beneficial effects on the NADPH oxidase system, reducing, for example, the activity and also the expression of NOX subunits in cultured human endothelial cells (151). Furthermore, administration of atorvastatin to the spontaneously hypertensive rat *in vivo* produced a reduction in the expression of NADPH oxidase subunits and reduced vascular superoxide levels (193). Interestingly, in some studies, pleiotropic effects of statins have been shown at subtherapeutic doses, or at time points earlier than the development of their main lipid-lowering effect. Although such effects of statins initially seem less intuitive than the antioxidant effects of ACEI and ARBS, it has subsequently been determined that the same inhibition of geranylgeranylation that underlies their main therapeutic effect also prevents the association of Rac1 with the NADPH oxidase complex, thus inhibiting its activity (193). Agents with combined effects may offer additional protective benefits on the cardiovascular system. Work from our group has shown that omapatrilat, a combined ACEI and neutral endopeptidase inhibitor, not only can improve vascular function and reduce blood pressure in the SHRSP, but may also attenuate end-organ damage such as left ventricular hypertrophy (63). Recently the combined administration of ARB and statin was shown to provide enhanced vascular protection *via* their differential pleiotropic effects in a rat model of salt-sensitive hypertension (203). The potential of targeting the NADPH oxidase system has been the subject of recent detailed review (67).

## *Superoxide dismutase and SOD mimetics*

Studies stretching back to the point at which the main EDRF was identified as NO have shown clearly that administration of native superoxide dismutase (52) or SOD mimetics (113) can reduce superoxide levels and improve endothelial function and vasodilation in functional studies (127). Extension of these studies into *ex vivo* and *in vivo* settings is problematic with native SOD as, because of its relatively high molecular weight, it exhibits poor membrane permeability. However, *in vivo*-tolerated SOD-mimetic antioxidants such as tempol (138, 197) showed significant reductions in superoxide levels and related phenotypes, including blood pressure and vascular remodelling in animal models. Work by our group and others has further demonstrated the potential of SOD-replacement strategies as a

therapy in hypertension *via* adoption of gene-transfer techniques in animal models (4, 22, 27, 45, 213). It should be noted that compartmentalization is an important consideration when attempting to overexpress SODs, as (*e.g.*, extracellular SOD) has been found to be effective in improving vascular function, whereas MnSOD and CuZnSOD were not (4). The importance of appropriate localization of expression was reinforced when a soluble, heparin-binding domain–deleted version of extracellular SOD was found to be ineffective in reducing blood pressure in the SHR, in contrast to the native version of the protein (27).

### *Endothelial NO synthase and nitric oxide*

As discussed earlier, one of the key mechanisms by which NO activity is compromised is caused by the rapid interaction with superoxide under conditions of oxidative stress. It is, therefore, clear that approaches to reduce oxidative stress can broadly be expected to improve NO bioavailability and vascular function. As described earlier, this has been demonstrated in a number of *in vitro* studies of isolated tissue preparations, as well as in *in vivo* studies after antioxidant therapy (113, 127, 138, 197).

Other studies have shown that the reduced NO-generating capacity of uncoupled eNOS can be addressed *via* administration of the substrate L-arginine (25) or the $BH_4$ cofactor (5, 30). Furthermore, gene transfer of the GTP cyclohydrolase gene, an enzyme crucial to the synthesis of $BH_4$, was found to rescue $BH_4$ levels, increase NO generation, reduce ROS, and improve endothelial function in the DOCA salt-sensitive rat (211). Potentially more applicable from a clinical perspective, oral administration of a $BH_4$ analogue, sepiapterin, has shown promise in reducing oxidative damage and attenuating endothelial dysfunction in resistance vessels in a mouse model of diabetes (134). The suitability of such an approach in human cardiovascular diseases has yet to be fully determined.

The other main approach to reconstituting normal NO activity in hypertension has been the direct gene transfer of the eNOS gene. We have previously shown that local adenoviral-mediated transfer of eNOS *in vivo* can improve NO bioavailability in the carotid artery of the SHRSP (4). Furthermore, eNOS gene transfer can produce reductions in systolic blood pressure (109). We have found that specific targeting of eNOS overexpression to the vascular endothelium is necessary to demonstrate functional effects, including improved NO bioavailability and attenuation of systolic blood pressure increases in the SHRSP (122). A number of studies have shown sustained blood pressure–reducing effects of eNOS gene delivery into the cardiovascular control centers of the brain in animal models (90, 175), demonstrating the importance of central effects of NO in regulating blood pressure. However, the mechanisms underlying these centrally derived effects are likely to be different from the blood pressure–reducing effects seen with peripheral overexpression of eNOS, and are also less likely to be applicable for translational studies in humans.

### *Glutathione system*

The glutathione and glutathione peroxidase systems have been clearly defined as playing an important role in detoxification of many substances, including phase II byproducts of oxidative attack and direct effects on ROS such as $H_2O_2$ (39). Studies regarding the *specific* targeting of individual components of the glutathione systems in hypertension are limited (24). However, it is clear that interventions aimed at improving overall antioxidant status may also exert their effects at least in part *via* increased activity of this system. For example, in the DOCA salt-sensitive rat model of hypertension, prolonged treatment with the antioxidant quercetin produced a number of beneficial effects, including increasing total GSH levels in heart and liver, improving liver glutathione-*S*-transferase (GST) and glutathione peroxidase function, and improving renal GST function (56).

Strategies aimed at enhancing the activity of glutathione pathways include the administration of *N*-acetylcysteine, a known antioxidant that can improve the GSSG/GSH ratio by promoting the synthesis of GSH by acting as a source of L-cysteine (the rate-limiting substrate in GSH synthesis) and also by upregulation of glutathione peroxidase and GSSG reductase (20). *N*-acetylcysteine administration *in vivo* reduced vascular lipid peroxidation, ROS levels, and systolic blood pressure in the SHR rodent model of genetic hypertension (20, 140). In contrast to the potentially therapeutic benefits to be derived from stimulating glutathione pathways, work from our laboratory and others has identified that derangement of the GST system may underlie a number of pathologies. Studies in the cancer field have identified that mutations in GST enzymes can result in compromised antioxidant protection and may be linked to the susceptibility to certain types of carcinoma (120). Moreover, we recently identified that reduced expression of *Gstm1* in the kidney of the stroke-prone spontaneously hypertensive rat (SHRSP) may be linked to elevated oxidative stress and systolic blood pressure in this model (117, 118). Ongoing work by our group will determine whether targeted modulation of *Gstm1* can improve the oxidative-stress and blood-pressure profiles of the SHRSP. Translational studies on human homologues of rat *Gstm1* may help to clarify the importance of this enzyme family in clinical hypertension, for which conflicting reports have been published (87, 115).

### *Xanthine oxidase*

Experimental studies showing a beneficial effect of xanthine oxidase inhibition on oxidative stress and hypertension are limited, with some studies showing no beneficial effects (95). In contrast, some benefit of xanthine oxidase inhibition has been demonstrated in experimental models in which other cardiovascular insults in addition to hypertension are present, such as vascular injury (205) or a high-fat diet (43). Interestingly, this may be mirrored in human studies, with allopurinol appearing to be useful in situations with comorbidity, such as heart failure (44). However, extensive human studies of the potential benefits of allopurinol are somewhat limited (51) and may warrant further investigation.

### *Mitochondrial ROS*

Although a number of studies aimed at attenuating hypertension have reported effects on mitochondrial ROS generation (21), few have focused specifically on this area as their central aim. Recent studies suggest that the activity of a mitochondrial-

specific thioredoxin system can reduce oxidative stress, improve NO bioavailability, and may protect against vascular damage in atherosclerosis (210). In contrast, previous work from our laboratory found that adenoviral mediated *in vivo* overexpression of mitochondrial superoxide dismutase had no effect on vascular dysfunction in intact SHRSP blood vessels (45). A promising area may be the development of mitochondria-targeted antioxidants, which have shown promise in *in vitro* and *in vivo* studies and which are being evaluated for early clinical studies (2, 129).

### *Dietary antioxidants*

The potential for dietary manipulation to have beneficial effects on oxidative stress and cardiovascular disease has attracted increasing attention over the past decades, at times not without controversy. However, in recent years, this area has become the subject of more-detailed study, with reports of potentially beneficial effects of a number of compounds now emerging, including phytoestrogens such as genistein (189, 201) and phenolic compounds including catechins (80, 104) and quercetin (155, 204). Although promising in animal studies, in which the effects of life-long administration of antioxidant-rich diets can be relatively easily assessed (208), the benefits of these and other related compounds remain to be confirmed in human studies. Given the general failure (to date, at least) of vitamin compounds successfully to translate their beneficial effects from animal to human studies (78), this may not be a trivial task.

The preceding sections outlined the key sources of ROS as determined from preclinical and mechanistic studies, the mechanisms through which they exert their deleterious effects on the vasculature in hypertension, and strategies aimed at combating this facet of cardiovascular disease. We now examine the translation of these concepts into human hypertension.

## SITUATION IN HUMANS: EVIDENCE FOR A ROLE OF OXIDATIVE STRESS IN CARDIOVASCULAR DISEASE

A large body of evidence suggests that oxidative stress is involved in human cardiovascular disease. However, definition of phenotypes, options to dissect the role of individual oxidative-stress pathways, and options to modify oxidative stress are limited in humans. Many of these findings have the potential to be translated into human cardiovascular disease and will eventually improve diagnosis, prevention, and therapy of oxidative stress–related disorders in humans. It is reasonable to assume that within the near future, strategies to modify oxidative stress in humans will be based on available data from clinical studies rather than on novel insights from experimental models.

We therefore briefly review the existing evidence of the role of oxidative stress in human hypertension and cardiovascular disease. We then examine potential antioxidant strategies and their effect on hypertension and intermediate phenotypes in well-defined functional studies before we examine whether results from these studies hold true in large-scale trials. We finally develop future strategies to address oxidative stress in hypertension and cardiovascular disease.

### *Markers of oxidative stress*

A number of circulating markers of oxidative stress have been examined in patients at all stages of cardiovascular disease. Evidence exists of increased oxidative stress and reduced antioxidant potential in hypertension (26, 148). An unfavorable antioxidant/prooxidant balance is already present in children and adolescents with hypertension (185), suggesting a role of oxidative stress in early stages of the disease process. Whereas circulating markers reflect only some of the aspects of oxidative stress (76), it is reassuring that these data are supported by more-direct measurements of ROS production, such as superoxide release from phagocytic cells (48). In more advanced cardiovascular disease, circulating markers of oxidative stress are altered in parallel to increased vascular superoxide production (3).

### *Reactive oxygen species and intermediate cardiovascular phenotypes*

Intermediate phenotypes along the cardiovascular continuum are associated with increased oxidative stress. This provides functional and therefore strong evidence for the role of oxidative stress in pathogenesis and development of cardiovascular disease. We give three examples of vascular phenotypes that are associated with both hypertension and oxidative stress: endothelial dysfunction, carotid intima–media thickness and plaques, and vascular stiffness.

*Endothelial dysfunction.* ROS scavenge endothelium-derived NO and thereby lead to impaired endothelium-dependent vasodilation (71, 97). In humans, conflicting data concern whether hypertension *per se* causes endothelial dysfunction, as assessed by response of forearm blood flow in response to intraarterial infusion of acetylcholine into the brachial artery (28, 135). However, more recently, Higashi *et al*. (75) demonstrated improved endothelial function of the forearm vasculature after reducing oxidative stress and blood pressure by treatment of renal artery stenosis with angioplasty. Also, the antioxidant vitamin C restores endothelial function in patients with essential hypertension (172), particularly in elderly subjects (174). In patients with coronary artery disease, extracellular superoxide dismutase activity determines endothelium-dependent vasodilation of the forearm vasculature (98).

*Carotid intima–media thickness and plaques.* Oxidized LDL cholesterol has a greater proatherogenic potential compared with native LDL cholesterol (72). Levels of oxidized LDL cholesterol are increased in hypertension (53), and, in parallel, carotid intima–media thickness, an early marker of the atherogenic process, is increased (188), and greater prevalence of carotid plaques is found in patients with borderline hypertension compared with normotensive subjects (102). Moreover, carotid intima–media thickness is directly related to ROS production in hypertension (206).

*Vascular stiffness.* Blood pressure is a major determinant of vascular stiffness (133). We and others demonstrated a relation between vascular superoxide production and vascular stiffness (35, 200). In healthy volunteers, short-term adminis-

tration of the antioxidant vitamin C reduces vascular stiffness (199). Pretreatment with vitamin C prevents the smoking-induced increase in pulse-wave velocity in healthy volunteers (88). Vitamin C has also been shown to reduce vascular stiffness in patients with type 2 diabetes (128). However, not all studies have been positive. For example, Zureik *et al*. (214) failed to show a beneficial effect of antioxidant vitamins on pulse-wave velocity in a large cohort of apparently healthy study participants.

### *Evidence from genetic studies*

Polymorphisms of oxidative stress–related genes have been associated with hypertension in a number of studies. One of the most robust candidates is the C242T polymorphism of *CYBA*, the gene encoding the p22$^{phox}$ subunit of NADPH oxidase. The C allele is associated with increased vascular superoxide production (70) and has also been found to be associated with hypertension (125). Evidence also suggests an association between polymorphisms of *NOS3*, the gene encoding endothelial NO synthase, and hypertension (79, 141), but this evidence is less robust, and a number of conflicting data are available from other studies (23). We are currently in the process of translating these findings on glutathione *S*-transferases into human hypertension and have recently reported an association between polymorphisms in human *GSTM5* and hypertension in the British Genetics of Hypertension Study (33). These and other data provide direct evidence for a role of oxidative stress in the pathogenesis of hypertension.

### *Evidence from association and epidemiologic studies*

The majority of these studies have been carried out by using composite cardiovascular end points or cardiovascular phenotypes other than hypertension. However, given the importance of hypertension as a cardiovascular risk factor and its involvement in virtually every cardiovascular trait, it is safe to draw conclusions from these studies to hypertension. Levels of antioxidants and markers of oxidative stress are associated with blood pressure and cardiovascular outcome (9, 31, 55, 85, 89, 93). Some studies demonstrated that increased antioxidant intake from dietary sources (57, 62, 89, 91, 94) and from nutritional supplements (55, 94, 150, 166) is associated with reduced cardiovascular risk. More recently, activity of the antioxidant enzyme system, glutathione peroxidase 1, has been demonstrated to predict cardiovascular events in patients with coronary artery disease (16).

## ANTIOXIDANT STRATEGIES IN HUMANS

These data clearly demonstrate the role of oxidative stress in the pathogenesis of human hypertension and other cardiovascular diseases and justify antioxidant treatment strategies as part of the preventive and therapeutic regimens. However, only limited treatment options with proven or suspected positive safety profile are available to be used in humans. Most of them have to be regarded as unspecific (*e.g*., vitamins) or modify other systems in addition to reducing ROS production (*e.g*., statins). Novel and potentially more-specific and also more-efficient methods, such as modifying expression of oxidative stress–related genes by use of gene therapy and RNAi, are not yet available for widespread use in humans. Local *ex vivo* gene transfer to reduce oxidative damage and to improve endothelial function of vein grafts might, however, be a therapeutic option in the not-so-distant future (59). In this section, we summarize treatment strategies that have been used to modify oxidative stress in hypertension and other cardiovascular diseases and evidence from functional studies in favor of these strategies.

### *Lifestyle modification*

Certainly the safest antioxidant strategy to reduce cardiovascular risk is lifestyle modification. Reduction in body weight and increased physical activity have been shown not only to reduce blood pressure (184) but also to reduce oxidative stress (121). Recent studies have also demonstrated that not only endurance training but also isometric exercise reduces blood pressure and oxidative stress (142). Dietary approaches to change blood pressure have also been successful when diets rich in antioxidants have been prescribed (111).

### *Antioxidant vitamins*

Antioxidant vitamins such as vitamin C improve endothelial function in a number of vascular beds and cardiovascular disorders. The majority of functional studies examined the effect of short-term administration, particularly of infusion, of vitamins on endothelium-dependent vasodilation. Brief infusion of antioxidant vitamins rapidly improves endothelial function in the coronary (74, 81, 103), renal (34, 162), and forearm circulation (172). Possibly by improvement of endothelium-dependent vasodilation, vitamin C also improves vascular stiffness, as discussed earlier (199). Some studies suggest a blood pressure–reducing effect of antioxidant vitamins such as vitamin C (40, 49).

### *Statins*

In addition to their lipid-lowering potential, statins reduce NADPH oxidase–related ROS production, possibly by reduction in oxLDL levels and inhibition of Rac1 (151, 176). A number of studies therefore demonstrated improvement of endothelial function of the forearm vasculature with very short term (as few as 3 days of treatment) statin therapy, although the full lipid-reducing effect was not yet established (82, 83, 194). Statins improve markers of inflammation and oxidative stress, such as C-reactive protein and urinary 8-isoprostane levels, after 4 weeks of treatment (169). Even in patients with mean pretreatment serum LDL cholesterol concentrations in the normal range, statins improved endothelial function and reduced oxidative stress after 6 weeks of treatment (194).

### *Allopurinol*

We previously demonstrated a role of xanthine oxidase as an endothelial source of superoxide in cardiovascular disease (3). The xanthine oxidase inhibitor allopurinol has been shown to

improve endothelial function and to reduce oxidative stress in a number of conditions, including heart failure (44), sleep apnea (41) and diabetes (36). The effect of allopurinol is at least in part independent of the drug's effect on serum uric acid levels (58). Long-term therapy with high-dose allopurinol has been shown to reduce mortality in patients with chronic heart failure (168).

### *Other antioxidant strategies*

A number of other strategies to reduce oxidative stress in cardiovascular disease have been used in functional studies in humans. L-Arginine deficiency leads to uncoupling of endothelial NO synthase and production of superoxide anion from this source (71). Hypertension is characterised by impaired L-arginine transport (159) and increased arginase activity (209), both of which reduce L-arginine availability to endothelial cells for NO synthesis. These mechanisms may explain why L-arginine has been shown to restore endothelium-dependent vasodilation in patients with hypertension (158). Another antioxidant, *N*-acetylcysteine, has been shown to improve endothelial function (161), renal function (179), and to enhance the effect of ACE inhibitors on blood pressure (8) in patients with hypertension. Folic acid alone or in combination with other antioxidants also improves endothelial function in elderly patients with hyperhomocysteinemia and in patients with hypertension (22, 167). It should also be mentioned that numerous naturally occurring antioxidants have been studied in hypertension and other cardiovascular diseases. These include tomato extract (42), green tea (77), garlic (145), and pomegranate juice (7). Absence of hypertension in Kuna Indians has been attributed to regular consumption of flavanol-rich cocoa (119). Probably the most important measure to reduce oxidative stress in hypertension is to reduce blood pressure, and the beneficial effect of lower blood pressure on oxidative stress appears to be independent of the choice of antihypertensives (50, 152, 173).

## DISAPPOINTING RESULTS FROM LARGE-SCALE OUTCOME STUDIES

Although excellent evidence exists from association studies and well-defined functional studies on the role of oxidative stress in cardiovascular disease and on potential strategies to modify oxidative stress, these strategies have to be tested in adequately powered trials against hard end points. Several short-term interventions mentioned previously have been tested with regard to longer-term outcome, and the results have been almost universally negative. An example is the study on green tea by Hodgson *et al*. (77), who observed an immediate blood pressure-lowering effect within 30 min after ingestion of green tea but no change in 24-h ambulatory blood pressure after 7 days of regular green tea consumption. Intake of fat-soluble vitamins correlates with concentrations in adipose tissue (86), which makes intervention, particularly with vitamin E, attractive. Unfortunately, results from large-scale trials with well-defined intervention with antioxidant vitamins were mostly disappointing and were recently analyzed and reviewed by Vivekananthan (191) and Bjelakovic *et al*. (14).

Possible reasons for these negative results were discussed in detail by Griendling (64) and Touyz (183). These studies may have chosen patients at relatively advanced stages of cardiovascular disease, whereas oxidative stress probably plays the most important role in earlier stages of disease. Patients were not selected for antioxidant status and were not controlled for other dietary sources of antioxidants. Markers of oxidative stress have not been measured in any of the large-scale trials (190). With regard to hypertension, we must emphasize that this has not been the primary end point of any of these studies but has either been part of a composite end point or subject to *post hoc* analysis in patients selected for other cardiovascular conditions. It has also been discussed that the choice of antioxidants in these trials might not be ideal in terms of mode of administration, dose, and the substances *per se* (164, 183). Most commonly, vitamins C and E have been studied, and these vitamins have been shown to exert prooxidant effects *in vivo* (101, 187).

## IDENTIFYING PATIENTS WHO BENEFIT MOST FROM ANTIOXIDANT STRATEGIES AND POTENTIAL OUTCOME MEASURES

Probably the most crucial issues are selection of patients who might benefit most from treatment with antioxidants and measurement of an adequate outcome parameter. Although no scientist would, for example, examine the effect of blood pressure–reducing therapy in the general population without measuring blood pressure and thereby without identifying hypertensive patients, this is exactly what happened in antioxidant trials. Studies have been carried out in patients not being selected for high levels of oxidative stress, and only outcomes such as cardiovascular events but not changes in phenotypes directly related to oxidative stress have been assessed. In this section, we discuss phenotypes and conditions that might serve for both patient selection and measurement of outcome.

### *Antioxidant status*

It has been suggested to measure antioxidant status in patients before they undergo antioxidant therapy (190). This would be an extremely useful selection criterion, because one would avoid supplementing those patients with vitamins or treating those with other antioxidant strategies who already have a high intake of vitamins through their diet or have increased antioxidant capacity because of genetic predisposition. Unfortunately, assessment of antioxidant status in serum or plasma is relatively expensive and mainly determined by uric acid, for which it would have to be corrected. The fact that neither antioxidant status nor any other marker of oxidative stress provides comprehensive information of global and local oxidant burden (76, 114) would lead to measurement of a panel of oxidative-stress markers with enormous logistic and financial consequences. It is unlikely that this effort will be made in large-scale studies in the near future.

### *Comorbidities*

An easier way to identify candidates for antioxidant-treatment strategies is clinically to select patients at high cardio-

vascular risk in the hope that they will benefit most. It has been shown that patients with end-stage renal disease and cardiovascular disease benefit from antioxidant therapy with the antioxidant vitamin E, which reduces a composite cardiovascular end point (18). One study in patients with type 2 diabetes showed reduction of blood pressure and vascular stiffness after 4 weeks of treatment with vitamin C (128). Vitamin E improves endothelial function in patients with type 1 diabetes after 3 months of therapy (165). However, it is also possible that antioxidant treatment strategies come too late in patients at advanced stages of cardiovascular disease. This may explain why not all studies have shown beneficial effects of vitamins in patients at increased cardiovascular risk. Indeed, some studies have even shown increased blood pressure (192) and increased incidence of heart failure (110) after treatment with antioxidant vitamins.

### *Intermediate vascular phenotypes*

Intermediate phenotypes within the cardiovascular continuum may be better suited to assess risk of vascular damage and potential benefit of treatment in individual patients. Endothelial dysfunction (74), vascular stiffness (15), and carotid intima–media thickness (132) have been shown to predict cardiovascular events independent of other cardiovascular risk factors. In other words, they provide information on top of classic cardiovascular risk factors and integrate a number of pathogenetic factors, including oxidative stress. Modification of intermediate phenotypes by blood pressure reducing and antioxidant treatment is well established. These phenotypes may therefore help to monitor treatment effects and, provided they are easy to assess, may also be used in large-scale studies.

*Endothelial dysfunction.* Excellent outcome data support assessment of endothelial function to predict cardiovascular outcome (74). By reflecting impaired NO bioavailability through increased ROS production, endothelial dysfunction directly relates to oxidative stress. However, endothelial function is also determined by factors other than oxidative stress, particularly by cholesterol (3) and triglyceride levels (160). A number of studies demonstrate that antioxidant regimens have the potential to modify endothelium-dependent vasodilation. However, assessment of endothelium-dependent vasodilation is not a trivial task and involves invasive techniques, special equipment such as ultrasound devices and related skills, or is very expensive. It is hardly possible to use endothelial function as intermediate phenotype for oxidative stress in large-scale multicenter studies.

*Carotid intima–media thickness.* Again, this phenotype has been assessed in numerous studies and is clearly linked to cardiovascular risk factors (153) and outcome (132). Increased intima–media thickness is an early manifestation of atherosclerosis and therefore closely related to oxidative stress (6, 48, 195) and oxidative stress–induced modification of LDL cholesterol toward the even more proatherogenic oxidized LDL cholesterol (188). Carotid intima–media thickness is a reversible phenotype (7) and therefore well suited to monitor treatment effects. Assessment and data analysis again require special skills, but one of the few long-term studies that showed positive outcome with antioxidant treatment was able to demonstrate these benefits by assessment of carotid intima–media thickness (154). It is certainly worth considering assessment of this measure in future studies on antioxidant treatment in cardiovascular disease.

*Vascular stiffness.* Vascular stiffness can be assessed by a number of methods reviewed elsewhere (100). Carotid–femoral pulse wave velocity is regarded as the gold standard in assessment of vascular stiffness (100) and is relatively easy to measure. Vascular stiffening is a reversible process, which supports assessment of vascular stiffness to monitor treatment effects. Previous studies have demonstrated reduction of vascular stiffness with short-term antioxidant therapy (128, 199), and establishment of measures of vascular stiffness in studies into antioxidants in cardiovascular disease is clearly warranted.

### *"Omics" and oxidative stress*

Oxidative stress affects a huge number of systems within the organism, and it is difficult to determine its precise contribution in individual patients. For example, even patients carrying an unfavourable allele of an oxidative stress–related gene might be at low risk when this is counterbalanced by other genes and environmental factors. Enormous expectations have been held that broader genomic approaches examining a multitude of genetic risk factors will allow us to estimate an individual's cardiovascular risk and potential benefit of treatment more precisely, but only very recently has the technology for these undertakings become available (66, 116). Possibly even more important than genomic approaches examining hundreds of thousands of polymorphisms are proteomic approaches examining expression profiles of millions of proteins and peptides. These analyses are still extremely expensive but can be carried out in body fluids such as plasma or urine (46, 157). Already very limited data exist on the effect of treatment with vitamin C on polypeptide fingerprints in patients with end-stage renal disease (196). Analysis of these expression patterns my help to unravel underlying pathways but also to identify patients who are likely to benefit from vitamin C treatment.

## CONCLUSIONS

A number of strategies to reduce oxidative stress, including overexpression of antioxidant systems and interference with expression of oxidant systems, have been successfully used in animal models of hypertension. Despite this success, treatment of oxidative stress in human hypertension is currently limited to blood pressure reduction and life-style modification in parallel with administration of antioxidant vitamins. Even the cheap and relatively unspecific treatment with antioxidants should be targeted to those patients in whom antioxidant status is low or who have vascular phenotypes that are likely to be related to increased oxidative stress. Diagnostic procedures before commencing antioxidant therapy, irrespective of whether it is unspecific administration of vitamins or more-targeted future strategies such as gene therapy, will require diagnostic proce-

dures beyond measurement of blood pressure. These procedures will include measurement of antioxidant status and detailed assessment of vascular structure and function.

## ABBREVIATIONS

ACEI, angiotensin-converting enzyme inhibitor; $BH_4$, tetrahydrobiopterin; DNA, desoxyribonucleic acid; DOCA, deoxycorticosterone acetate; DPI, diphenylene iodonium; EDRF, endothelium-derived relaxation factor; eNOS, endothelial nitric oxide synthase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; ICAM-1, intercellular adhesion molecule-1; LDL, low-density lipoprotein; MMP, matrix metalloproteinase; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; NO, nitric oxide; NOX, NADPH oxidase; PDGF, platelet-derived growth factor; RAS, renin–angiotensin system; RNS, reactive nitrogen species; ROS, reactive oxygen species; SHRSP, stroke-prone spontaneously hypertensive rat; SOD, superoxide dismutase; TGF-$\beta$, transforming growth factor-beta; TNF-$\alpha$, tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule-1; VSMC, vascular smooth muscle cell.

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