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Review Article

Redox Control of Vascular Nitric Oxide Bioavailability

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INTRODUCTION

In 1980, Furchgott and Zawadski demonstrated the release of an endothelium-derived relaxing factor (EDRF) in isolated rabbit aorta with exposure to acetylcholine (Furchgott and Zawadzki, 1980). This factor, subsequently demonstrated to be nitric oxide (NO) or a closely related form of NO (Ignarro *et al.*, 1987a), is synthesized enzymatically from L-arginine and oxygen by the NO synthases, which require NADPH and tetrahydrobiopterin as cofactors. It is now known that a number of endothelial homeostatic functions, such as vasomotor tone, thrombosis, intimal growth, and leukocyte recruitment, are mediated by NO.

Endothelial dysfunction, manifested as reduced bioactivity of the NO pathway, is a common feature of many vascular diseases, including atherosclerosis and its risk factors such as hypercholesterolemia, diabetes, hypertension, cigarette smoking, and aging. Considerable evidence indicates that abnormal NO bioactivity is a contributing factor to the clinical manifestations of vascular disease, such as myocardial infarction and stroke (Levine *et al.*, 1995; Diaz *et al.*, 1997). Moreover, therapeutic maneuvers that reduce the clinical manifestations of vascular disease, such as cholesterol lowering and exercise, also improve endothelial NO bioactivity (Treasure *et al.*, 1995; Hambrecht *et al.*, 2000). On the basis of this information, endothelial function has been viewed as a potential target for altering the clinical course of vascular disease. To develop strategies to improve endothelial functions, it is necessary to understand the factors that contribute to abnormal NO bioactivity in vascular disease.

Although our understanding of the mechanism(s) responsible for impaired NO bioactivity in vascular disease is incomplete, accumulating evidence suggests that oxidative stress is an important contributing factor. In the following sections, we will review the sources of oxidative stress that have known implications for NO bioactivity. Similarly, antioxidant mechanisms that limit oxidative stress and have effects on the bioactivity of NO will also be considered. Although there are many potential sources of oxidative stress in the vasculature (Heinecke, 2000), the discussion here has been limited to sources of oxidative stress known to alter endothelial NO bioactivity.

MANIFESTATIONS OF OXIDATIVE STRESS THAT IMPAIR NO BIOACTIVITY

Superoxide

As mentioned in the introduction, atherosclerosis is associated with altered homeostasis that appears to be a function of impaired NO bioavailability. The augmented response of atherosclerotic blood vessels to vasoconstrictor stimuli has been well described (Henry and Yokoyama, 1980; Heistad *et al.*, 1984). The vasodilator response to NO release is impaired

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in both atherosclerosis (Freiman *et al.*, 1986) and hypercholesterolemia (Jayakody *et al.*, 1985), despite the increased elaboration of NO in these disease states (Minor, Jr. *et al.*, 1990). These data suggest that impaired dilation in atherosclerosis is due to inactivation of NO rather than decreased production of NO. Both atherosclerosis and hypercholesterolemia are associated with increased flux of superoxide within the vascular wall, and, indeed, superoxide appears to be an important modulator of NO bioactivity (Ohara *et al.*, 1993; Keaney, Jr. *et al.*, 1995).

The source of superoxide in the normal condition appears to be localized to the endothelium and adventitia and is principally produced by NADH and NADPH oxidoreductases (Mojazzab *et al.*, 1994). Potential sources of vascular superoxide are more numerous in pathologic conditions. Xanthine oxidase has been implicated as an important source of superoxide with hypercholesterolemia (Ohara *et al.*, 1993). In isolated segments of cholesterol-fed rabbit aorta, the increased steady-state flux of superoxide is abrogated with endothelial denudation or oxypurinol, an inhibitor xanthine oxidase (Ohara *et al.*, 1993). Consistent with this observation, hypercholesterolemic patients treated with oxypurinol demonstrate a partial improvement in NO-mediated arterial relaxation (Cardillo *et al.*, 1997). Other animal work indicates that hypercholesterolemia may be associated with increased circulating levels of xanthine oxidase that may bind to the vascular endothelium (White *et al.*, 1996). The precise role of circulating xanthine oxidase and human pathology remains to be determined.

As another potential source of superoxide, all NOS isoforms appears capable of reducing molecular oxygen to produce superoxide, especially under limiting conditions of either L-arginine (Pou *et al.*, 1992; Xia *et al.*, 1996) or tetrahydrobiopterin (Vasquez-Vivar *et al.*, 1998; Xia *et al.*, 1998). These findings may have particular relevance for hypercholesterolemia-induced vascular superoxide generation in endothelial cells exposed to low-density lipoprotein (LDL), which exhibit enhanced superoxide anion generation from the endothelial isoform of NOS (Pritchard, Jr. *et al.*, 1995).

Peroxynitrite

One mechanism through which superoxide leads to oxidative stress is its combination with NO to yield peroxynitrite (Beckman *et al.*, 1990). This reaction occurs at the diffusion limit (Kissner *et al.*, 1997) and leads to a reduced bioactivity of NO. Because peroxynitrite is a much less potent activator of soluble guanylyl cyclase than NO, the net effect of superoxide is to reduce NO-mediated cGMP activation (Tarpey *et al.*, 1995). Peroxynitrite is also capable of transferring oxygen atoms (Beckman and Crow, 1993), oxidizing protein tyrosine residues (Landino *et al.*, 1996), oxidizing sulfhydryls (Moreno and Pryor, 1992), and initiating lipid peroxidation (Graham *et al.*, 1993). With respect to the latter, LDL isolated from atherosclerotic lesions contains nitrotyrosine (Leeuwenburgh *et al.*, 1997), suggesting that peroxynitrite is one mechanism of LDL oxidation in atherosclerosis. Thus, the formation of peroxynitrite represents an important mechanism for both propagation of superoxide-mediated oxidative stress and limitation of NO bioactivity.

Lipid peroxidation

Superoxide is a stronger reductant than oxidant at physiologic pH (Buettner, 1993) and in the presence of transition metal ions may promote lipid peroxidation (Lynch and Frei, 1997a), perhaps through the Haber-Weiss reaction and hydroxyl radical formation. Because superoxide also can reduce metal ions, only catalytic amounts of metal are needed to produce hydroxyl radical from superoxide. Atherosclerotic lesions contain catalytic amounts of iron (Smith *et al.*, 1992) and human ceruloplasmin can oxidize LDL (Ehrenwald *et al.*, 1994), suggesting two potential sources of redox-active transition metals that could support lipid peroxidation *in vivo*.

Lipids found in membrane and lipoproteins contain phospholipids and cholesterol esters with polyunsaturated fatty acids that represent attractive targets for oxidizing species. One-electron oxidation leads to the formation of a carbon-centered radical that may initiate a chain reaction of lipid peroxidation via the formation of alkoxyl and lipid peroxyl radicals

and the accumulation of lipid hydroperoxides. The accumulation of lipid peroxidation products in biological membranes and glycoproteins leads to further oxidative reactions.

Lipid peroxidation has a number of implications for NO bioactivity. Lipid peroxyl radicals can combine readily with NO to form lipid peroxynitrite derivatives (Padmaja and Huie, 1993; Rubbo *et al.*, 1994), which reduce NO bioavailability (O'Donnell *et al.*, 1997). Also, lipid peroxidation in the vascular wall leads to the oxidation of LDL (Ylä-Herttuala *et al.*, 1989) that directly inactivates NO (Chin *et al.*, 1992) and reduces expression of eNOS (Liao *et al.*, 1995). Protein kinase C (PKC) is stimulated by transfer of oxidized phospholipids from oxidized LDL to the endothelial plasma membrane (Ohgushi *et al.*, 1993), which leads to impaired G-protein-coupled signal transduction and abnormal NO-mediated arterial relaxation to receptor-dependent NO agonists (Kugiyama *et al.*, 1990).

ANTIOXIDANT STATUS AND NO BIOACTIVITY

In light of all the available evidence that oxidative stress impairs NO bioactivity, one might predict that antioxidant status has important implications for the fate of NO. Available evidence supports this position and we will now turn our attention to this evidence. We will consider, in turn, the individual antioxidant species known to alter the action and metabolism of NO.

Superoxide dismutase

The interaction between NO and superoxide effectively reduces the biologic activity of NO due to formation of peroxynitrite. Because both NO and superoxide are produced constitutively, it follows that changes in the relative flux of either species will modulate NO-dependent endothelial function. This contention is supported by the demonstration that basal endothelial superoxide production has modulates endothelium-derived NO action (Gryglewski *et al.*, 1986; Ignarro *et al.*, 1988). As a consequence, it is not surprising that exogenous superoxide dismutase (SOD) augments the endothelial-derived NO response to acetylcholine (Rubanyi and Vanhoutte, 1986).

Vascular tissue contains considerable intra- and extracellular SOD activity as protection against superoxide leakage from normal oxidative metabolism. Therefore, the finding that vascular SOD activity exerts considerable influence of the local activity of endothelium-derived NO (Omar *et al.*, 1991) and NO-independent vasodilators (Mugge *et al.*, 1991a) is not surprising. Inhibition of copper-zinc superoxide dismutase produces less NO-mediated arterial relaxation than untreated cells despite similar production of inactive NO, suggesting that SOD is necessary for the release of biologically active NO (Mugge *et al.*, 1991a). Similarly, inhibition of copper-zinc SOD in bovine coronary arteries results in impaired NO-mediated relaxation to acetylcholine, and an impaired response to the NO-independent vasodilators nitroprusside or nitroglycerin (Omar *et al.*, 1991). These data suggest that intact SOD activity is required for normal NO-mediated arterial relaxation. Further support comes from a rat model of dietary copper deficiency characterized by impaired copper-zinc SOD activity (Sarvazyan *et al.*, 1995; Lynch *et al.*, 1997b). In this model, there is an increase in vascular superoxide flux (Lynch *et al.*, 1997b) and, as a consequence, impaired NO-mediated arterial relaxation (Schuschke *et al.*, 1992; Lynch *et al.*, 1997b). These data indicate that the balance between NO and superoxide modulates NO bioactivity.

In hypercholesterolemic rabbits, blood vessels produce excess superoxide compared to normal controls (Ohara *et al.*, 1993; Keaney, Jr. *et al.*, 1995; Lynch *et al.*, 1997b) and dietary treatment of hypercholesterolemia normalizes both the vascular superoxide flux and the bioactivity of endothelium-derived NO (Ohara *et al.*, 1995). These observations suggest that increased vascular SOD activity should improve NO bioactivity in disease states characterized by excess superoxide. This contention is borne out in the available data; atherosclerotic rabbits treated for 1 week with polyethylene-glycolated SOD demonstrated increased vascular SOD activity and improved NO-mediated arterial relaxation to acetylcholine compared to untreated

animals (Mugge *et al.*, 1991b). Also, acute treatment of arterial segments from atherosclerotic rabbits with liposome-encapsulated SOD improves NO-mediated arterial relaxation (White *et al.*, 1994). The ability of SOD manipulation to restore endothelium-derived NO bioactivity in hypercholesterolemia and atherosclerosis appears highly dependent on the disease stage. In contrast to early disease, advanced atherosclerosis is largely insensitive to the status of vascular SOD activity (Kagota *et al.*, 1998), perhaps due to limited access of SOD to the vessel wall or irreversible oxidative damage.

Impairment of NO activity in diabetes is also linked to excess vascular superoxide. High levels of serum glucose are characteristic of diabetes mellitus. The α-hydroxy aldehyde structure of glucose is subject to enediol rearrangement and the formation of an enediol radical anion (Wolff and Dean, 1987). One potential outcome of enediol radical formation is the subsequent reduction of molecular oxygen to form superoxide (Wolff and Dean, 1987), an event with obvious implications for NO bioactivity. The exposure of isolated rabbit arterial segments to pathophysiologic levels of glucose results in impaired NO-mediated arterial relaxation to acetylcholine that is normalized by SOD (Tesfamariam and Cohen, 1992). However, the effects of hyperglycemia on NO-mediated arterial relaxation can not be explained simply by the autoxidation of glucose. Isolated arteries from diabetic animals are characterized by impaired NO-mediated arterial relaxation even under normal glucose conditions (Pieper and Gross, 1988; Hattori *et al.*, 1991; Tesfamariam *et al.*, 1992), and this defect is partially restored by SOD. These latter observations suggest that hyperglycemia may provoke intracellular signals that stimulate endogenous superoxide production.

Hypertension is yet another vascular disease linked to excess superoxide. Treatment of hypertensive rats with SOD produced a marked reduction in arterial pressure not observed in normotensive rats, suggesting that superoxide may play a role in the impaired bioactivity of basal endothelial NO release in hypertension (Nakazono *et al.*, 1991). The demonstration that eNOS-deficient mice are hypertensive (Shesely *et al.*, 1996) and excess vascular superoxide characterizes some forms of hypertension (Laursen *et al.*, 1997) support such speculation. Angiotensin II-mediated hypertension clearly involves some component of oxidative stress manifested by an increased ambient vascular flux of superoxide (Griendling *et al.*, 1994). Infusion of angiotensin II in rats resulted in increased in steady-state vascular superoxide and impaired NO-mediated arterial relaxation, an effect that is attenuated by liposome-encapsulated SOD (Rajagopalan *et al.*, 1996). However, similar increases in blood pressure from chronic norepinephrine infusion are not characterized by an increase vascular superoxide (Laursen *et al.*, 1997), suggesting that this phenomenon is specific to angiotensin II-mediated hypertension. One effect of angiotensin II germane to NO bioactivity is the induction of an NADH/NADPH oxidase-like activity in vascular smooth muscle cells (Griendling *et al.*, 1994). Observations that liposome-encapsulated SOD lowers blood pressure in angiotensin II-treated animals (Rajagopalan *et al.*, 1996) suggest that superoxide is critical to the manifestations of hypertension due to angiotensin II. In patients with essential hypertension, however, the situation appears more complex. Infusion of SOD in such patients has no effect on NO-mediated arterial relaxation response to acetylcholine (Garcia *et al.*, 1995).

Catalases

Hydrogen peroxide (H_2O_2) does not react directly with NO; as a consequence, the local concentration of H_2O_2 does not appear to modulate directly the action of NO in the same manner as superoxide. However, under specific conditions H_2O_2 does appear to result in impaired NO-mediated arterial relaxation. For example, transient exposure of cat cerebral arterioles to physiologically relevant concentrations of H_2O_2 impaired NO-mediated arterial relaxation response to acetylcholine, sodium nitroprusside, or exogenous NO; both SOD and catalase reversed this effect (Wei and Kantos, 1990). Nitric oxide production by cultured endothelial cells was impaired as the result of exposure to H_2O_2, despite removal of residual H_2O_2 (Marczin *et al.*, 1992). Two potential mechanisms may explain the effect of H_2O_2 on

NO production. The first involves H_2O_2-mediated oxidation of intracellular thiols that may be required for normal endothelial NO production (Ghigo *et al.*, 1993; Marczin *et al.*, 1993). In fact, eNOS contains reduced thiols that are critical for enzymatic activity (Zhang *et al.*, 1996). Alternatively, H_2O_2 treatment of endothelial cells may also result in an increase in tyrosine phosphorylation of eNOS associated with a 50% decrease in the specific activity of the enzyme (Garcia-Cardena *et al.*, 1996). Catalase would be expected to prevent any effect of H_2O_2 on these critical thiols. In the absence of H_2O_2-induced stress, however, endothelial cell catalase activity does not seem to be required for normal NO production (Mugge *et al.*, 1991a).

Glutathione peroxidase

Organic hydroperoxides are detoxified principally by enzymes of the glutathione peroxidase family, a family of selenocysteine-containing proteins. Glutathione peroxidases reduce H_2O_2 in lipid hydroperoxides to the corresponding alcohol using glutathione is a source of reducing equivalents. This function of glutathione peroxidase may be relevant for NO bioactivity, because organic hydroperoxides are an important source of the lipid peroxyl radicals that combine readily with NO leading to the formation of lipid peroxynitrite derivatives (Rubbo *et al.*, 1994, 1995). This reaction between NO and lipid peroxyl radicals is thought to underlie the observation that NO inhibits lipid peroxidation in cells (Wink *et al.*, 1993a) and lipoproteins (Hogg *et al.*, 1993). The role of lipid peroxyl radicals in modulating NO bioactivity has only recently been examined. Inhibition of platelet aggregation by NO is potentiated by glutathione peroxidase, suggesting that reduction of lipid hydroperoxides enhances NO bioactivity (Freedman *et al.*, 1995). Observations that lipid peroxyl radicals react rapidly with NO also supports the notion that NO bioactivity is altered by the availability of lipid hydroperoxides (O'Donnell *et al.*, 1999).

The role of glutathione peroxidase in modulating NO bioactivity is not restricted to the reduction of organic hydroperoxides. The reduction of H_2O_2 by glutathione peroxidase proceeds in the presence of either authentic glutathione or *S*-nitroso-glutathione and with the latter, NO is liberated (Scorza *et al.*, 1997). Recent data indicate that this phenomenon is not specific for either glutathione peroxidase or *S*-nitroso-glutathione. A number of selenium-containing compounds such as selenocysteine and selenocystamine also catalyze the decomposition of several different *S*-nitrosothiols resulting in the liberation of NO. Moreover, the composition of *S*-nitroso-glutathione by glutathione peroxidase does not appear to require the presence of any peroxide. Thus, glutathione peroxidase may modulate the bioactivity NO either through the reduction of organic H_2O_2 or the composition of endogenous *S*-nitrosothiols resulting in the liberation of NO.

Lipid-soluble antioxidants

Given that lipid peroxidation impairs NO bioactivity, any reduction of lipid peroxidation via lipid-soluble antioxidants should attenuate this effect. Lipid-soluble antioxidants are localized mainly to the plasma membrane and lipoproteins. The three major lipid-soluble antioxidants in humans are α-tocopherol, γ-tocopherol, and ubiquinol-10 (Keaney, Jr. and Frei, 1994a). Probucol is a synthetic lipid-soluble compound with cholesterol-lowering properties that has also been used extensively in animal models.

α-Tocopherol: The effects of α-tocopherol on endothelium-dependent NO (EDNO) bioactivity have been examined in the cholesterol-fed rabbit. Rabbits consuming a 1% cholesterol diet typically demonstrate marked impairment of endothelium-dependent NO-mediated arterial relaxation (Jayakody *et al.*, 1985; Verbeuren *et al.*, 1986) linked to increased vascular oxidative stress (Ohara *et al.*, 1993). Dietary supplementation with α-tocopherol resulted in near normal NO-mediated arterial relaxation in response to acetylcholine and A23187 (Keaney, Jr. *et al.*, 1993a). This effect was not explained by any change in the lipoprotein profiles or the extent of atherosclerosis (Keaney, Jr. *et al.*, 1993a). Similar findings have been reported in the coronary (Andersson *et al.*, 1994) and carotid (Stewart-Lee *et al.*, 1994) arteries of cholesterol-fed rabbits as well as the aorta of rats

fed a high-fat diet (Lutz *et al.*, 1995). In hypercholesterolemic rats, the combined deficiency of α-tocopherol and selenium, an essential cofactor for glutathione peroxidase, is associated with impaired NO-mediated arterial relaxation (Raij *et al.*, 1993). These data suggest that lipid-soluble antioxidant protection is important in maintaining normal NO bioactivity in the setting of hypercholesterolemia or atherosclerosis.

Lipid-soluble antioxidants such as α-tocopherol localize mainly to membranes and lipoproteins where they serve to limit lipid peroxidation damage. Because LDL oxidation produces impaired NO bioactivity (Kugiyama *et al.*, 1990), and α-tocopherol can inhibit low-density lipoprotein (LDL) oxidation (Dieber-Rotheneder *et al.*, 1991), lipid-soluble antioxidants may be expected to preserve NO bioactivity through decreased oxidative modification of LDL. To address this issue, cholesterol-fed rabbits were treated with two different dietary regimens of α-tocopherol. As expected, the lowest dose of α-tocopherol reversed the effect of cholesterol feeding on NO-mediated arterial relaxation and protected LDL from copper-mediated oxidation *ex vivo* (Keaney, Jr. *et al.*, 1993a, 1994b). In contrast, a 10-fold greater dose of α-tocopherol actually worsened NO bioactivity, despite continued protection of LDL against *ex vivo* copper-mediated oxidation (Keaney, Jr. *et al.*, 1994b). Thus, antioxidant protection of LDL is not sufficient to preserve NO-mediated bioactivity in the setting of cholesterol feeding.

Although α-tocopherol is an efficient scavenger lipid peroxyl radicals, it is now clear that lipid peroxidation does occur in the vascular wall during atherosclerosis despite the presence of α-tocopherol (Suarna *et al.*, 1995). One potential activity of α-tocopherol may be the prevention of deleterious effects on vascular cells due to oxidized LDL. There is now considerable evidence to support this contention. Isolated arterial segments from α-tocopherol-deficient animals demonstrate impaired NO-mediated arterial relaxation after exposure to oxidized LDL (Keaney, Jr. *et al.*, 1996). Arterial segments derived from animals supplemented with α-tocopherol, however, exhibit marked resistance to endothelial dysfunction from oxidized LDL. The same holds true for isolated endothelial cells in culture (Jay *et al.*, 1997). This impairment NO-mediated arterial relaxation by oxidized LDL results from protein kinase C (PKD) stimulation, a known consequence of oxidized LDL exposure to vascular tissue (Kugiyama *et al.*, 1992; Sugiyama *et al.*, 1994). Incorporation of α-tocopherol into endothelial cells prevents PKC stimulation by oxidized LDL (Keaney, Jr. *et al.*, 1996), thereby preserving NO-mediated arterial relaxation. A similar effect on PKC activity has been observed with α-tocopherol incorporation into smooth muscle cells (Boscoboinik *et al.*, 1991) and platelets (Freedman *et al.*, 1996).

The role of α-tocopherol in preserving NO bioactivity is not limited to models of atherosclerosis and hypercholesterolemia. Diabetes mellitus is another chronic disease that involves hyperglycemia, increased oxidative stress, and impaired NO-mediated arterial relaxation. Chronic treatment of streptozotocin-induced diabetic rats with vitamin E preserved NO-mediated arterial relaxation isolated aorta and perfused coronary arteries, but not mesenteric arteries (Keegan et al., 1995; Karasu *et al.*, 1997a, b). There is also some role for vitamin E in preserving NO action in the absence of vascular disease. Vitamin E-deprived rats demonstrated impaired NO-mediated arterial relaxation that appears to result from an increase in vascular superoxide (Davidge *et al.*, 1998).

Probucol: Probucol is a lipid-soluble cholesterol-lowering compound transported in lipoproteins (Marshall, 1982) and accumulates in the vascular wall (Keaney, Jr. *et al.*, 1995; Shaish *et al.*, 1995). Probucol is a potent inhibitor of LDL oxidation (Marshall, 1982; Parthasarathy *et al.*, 1986) and it inhibits atherosclerosis in both hypercholesterolemic rabbits (Carew *et al.*, 1987; Kita *et al.*, 1987; Shaish *et al.*, 1995) and cholesterol-fed primates (Sasahara *et al.*, 1994).

The effect of probucol on endothelium-derived NO action has been examined in cholesterol-fed (Simon *et al.*, 1993; Keaney, Jr. *et al.*, 1995; Inoue *et al.*, 1998) and LDL receptor-deficient (Hoshida *et al.*, 1997) rabbits. In these studies, probucol treatment did not significantly alter plasma cholesterol, but did prevent increase in plasma in aortic lipid peroxides associated with cholesterol feeding. Probucol treatment was associated with preserved NO bioactivity as assessed by endothelium-depen-

dent arterial relaxation in response to acetylcholine or A23187 (Simon *et al.*, 1993; Keaney, Jr. *et al.*, 1995; Inoue *et al.*, 1998). In normocholesterolemic animals, probucol did not alter NO bioactivity (Simon *et al.*, 1993), suggesting that the effect of probucol is specific for mechanisms that depend on increased oxidative stress. This contention is also supported by observations that probucol treatment of rabbits with alloxan-induced diabetes is also associated with improved NO bioactivity as determined by endothelium-dependent arterial relaxation (Foubert *et al.*, 1992).

The mechanism of probucol action in cholesterol-fed rabbits appears unique compared to that of the other lipid-soluble compounds discussed above. Cholesterol feeding in rabbits is associated with an increase in the steady-state vascular flux of superoxide (Ohara *et al.*, 1993; Keaney, Jr. *et al.*, 1995; Inoue *et al.*, 1998). Probucol treatment prevents this increase in the vascular flux of superoxide (Keaney, Jr. *et al.*, 1995; Inoue *et al.*, 1998), thereby improving NO-mediated arterial relaxation. This reduction in vascular superoxide inhibits lipid peroxidation and lysophosphatidylcholine accumulation in the arterial wall (Keaney, Jr. *et al.*, 1995; Inoue *et al.*, 1998), two effects that would also be expected to increase NO bioactivity.

Human studies with lipid-soluble antioxidants: Human studies of lipid-soluble antioxidants in NO bioactivity have been both sparse and mixed. Hypercholesterolemic patients treated with vitamin E or probucol did not demonstrate any improvement in NO-mediated arterial relaxation of forearm resistance vessels (McDowell *et al.*, 1994). Similarly, hypercholesterolemic patients treated with a cocktail of vitamin E, vitamin C, and beta-carotene for 1 month demonstrated no effect on resistance vessel NO-mediated arterial relaxation (Gilligan *et al.*, 1994).

Reports on antioxidants in NO-mediated arterial relaxation of conduit arteries have been more positive. Postmenopausal women treated with estrogen demonstrated improvement in NO-dependent flow-mediated dilation of the brachial artery (McCrohon *et al.*, 1996) and a similar effect has been observed with α-tocopherol (Koh *et al.*, 1998). Abnormally high remnant lipoprotein levels are associated with impaired NO bioactivity in the brachial artery that is improved with α-tocopherol supplementation (Motoyama *et al.*, 1998a). In patients with coronary spastic angina, NO-mediated arterial relaxation in the coronary arteries appears to be normalized by treatment with α-tocopherol (Motoyama *et al.*, 1998b). In contrast, older individuals treated with vitamin E did not demonstrate any improvement in endothelial NO bioactivity (Simons *et al.*, 1999). Thus, many studies of human conduit arteries indicate a salutary effect of α-tocopherol by normalizing NO bioactivity.

Recent data also indicate that lipid-soluble antioxidants may act synergistically with cholesterol reduction therapy. Treatment of hypercholesterolemic patients with a combination of α-tocopherol in a cholesterol-lowering drug (simvastatin) over 4 weeks provided an additional benefit over and above that of simvastatin alone (Neunteufl *et al.*, 1998). In the coronary circulation, probucol improved NO-mediated arterial relaxation in patients treated with the lipid-lowering drug lovastatin compared with a combination of cholestyramine and lovastatin (Anderson *et al.*, 1995). These observations suggest that the effect of lipid-soluble antioxidants in NO bioactivity in patients may depend, in part, on the cholesterol status of these patients.

Water-soluble antioxidants

Lipid-soluble compounds represent only part of the cellular antioxidant defenses, the remainder of nonenzymatic cellular antioxidant protection is provided by water-soluble antioxidants, such as glutathione (GSH) and ascorbic acid. Vitamin C is more abundant in the plasma than is GSH, but both are present within the cell cytosol in millimolar concentrations (Wendel and Cikryt, 1980; Bray and Taylor, 1993). There is considerable evidence that both vitamin C and GSH are strictly required for normal cellular function. For example, all cells appear to demonstrate the active transport and/or synthesis of both GSH and vitamin C. Animals depleted of either vitamin C or GSH become moribund and die (Murphy *et al.*, 1991; Martensson *et al.*, 1993). Thus, it is not surprising these compounds may have some effect on NO bioactivity.

Glutathione: The role of endogenous GSH in the action and metabolism of NO elaborated from endothelial cells is controversial. Early studies with the inducible isoform of NOS suggested that GSH was required as a cofactor for NO production. Similar studies in endothelial cells produce mixed results (Murphy *et al.*, 1991; Hecker *et al.*, 1992). Bradykinin-induced release of NO from cultured bovine endothelial cells was impaired by alkylating thiols with *N*-ethylmaleimide (NEM) or oxidizing intracellular thiols with 2,2′-dithiodipyridine (DTDP) (Hecker *et al.*, 1992). Treatment with DTDP was not associated with a demonstrable reduction in the endothelial cell GSH, suggesting that the level of intracellular thiols is not important in the release of NO (Hecker *et al.*, 1992). One proposed alternative factor responsible for the effects of NEM and DTDP was the oxidation state of critical thiols involved in calcium homeostasis (Hecker *et al.*, 1992). Other studies with cultured porcine (Murphy *et al.*, 1991) or bovine (Mugge *et al.*, 1991a) endothelial cells also failed to demonstrate a correlation between thiol depletion and NO production.

In contrast, studies in human cells have been more consistent with a role for GSH in NO production. Human umbilical vein endothelial cells (HUVEC) were studied to examine the effect of thiol manipulation on endothelial cell NO production. Reduction of intracellular GSH levels with 1-chloro-2,4-dinitrobenzene (CDNB), a compound that forms a covalent complex with GSH through glutathione-*S*-transferase activity, was associated with a dose-dependent reduction of both cellular GSH and NO production (Ghigo *et al.*, 1993). Conversely, increasing cellular GSH with GSH ester enhanced NO production and was strongly correlated with intracellular GSH (Ghigo *et al.*, 1993).

Reconciliation of these discrepant findings is difficult. A variety of thiol-modifying agents have been employed and not all changes in NO bioactivity have been correlated with changes in GSH. One factor that has not been carefully characterized in these experiments is the level of oxidative stress. Cells cultured under ambient oxygen tension tend to be hyperoxic and, thus, under an increased level of oxidative stress. Moreover, the media concentrations of ascorbic acid were not particularly well controlled in the above studies, suggesting that intracellular antioxidant protection may have varied considerably among studies. More careful attention to potential antioxidant interactions will be necessary to reconcile these differences. One potential indirect action of GSH worth considering is its interaction with tetrahydrobiopterin. The NOS cofactor, tetrahydrobiopterin, is readily oxidized, and thiol compounds have been shown to protect tetrahydrobiopterin from oxidation. Moreover, endothelial cell L-arginine transport is sensitive to adequate intracellular GSH levels (Patel *et al.*, 1996). The potential effects of intracellular GSH on cellular NOS cofactor levels has important implications for NO production as a relative lack of either tetrahydrobiopterin or L-arginine reduces enzymatic efficiency and stimulates NOS-mediated oxidant production, particularly superoxide (Pou *et al.*, 1992; Xia *et al.*, 1996, 1998; Vasquez-Vivar *et al.*, 1998).

Another consideration for the role of GSH in NO bioactivity relates to the formation of *S*-nitrosothiols. Once formed, NO can combine with oxygen to form oxides of nitrogen that react with biologic thiols to form *S*-nitrosothiols (Wink *et al.*, 1993b), compounds that possess bioactivity reminiscent of authentic NO (Keaney, Jr. *et al.*, 1993b). The relative abundance of GSH among intracellular thiols species available for *S*-nitrosothiol formation renders the formation of *S*-nitroso-glutathione (GSNO) kinetically feasible, and GSNO formation has been implicated in cellular functions such as neutrophil glucose metabolism and oxidant production (Clancy *et al.*, 1994). To the extent that GSNO formation contributes to NO bioactivity, intracellular GSH levels may modulate the extent of GSNO formation. Thus, there are many potential mechanisms through which GSH has the potential to modulate NO bioavailability.

Recent human studies support a role for GSH in the bioactivity of endothelium-derived NO. Patients with coronary artery disease are known to demonstrate impaired NO-mediated arterial relaxation in the conduit arteries of the coronary and brachial circulation. Manipulation of intracellular GSH levels in such patients appears to alter NO-mediated arterial relaxation. Specifically, coronary artery disease pa-

tients treated with L-2-oxo-4-thiazolidine, a compound that selectively increases intracellular GSH, produced improved endothelium-dependent NO-mediated arterial relaxation in the brachial artery (Vita *et al.*, 1998). This effect was specific for the endothelium, as nitroglycerin-mediated arterial relaxation was unaffected. In vasospastic angina, intracoronary GSH improved acetylcholine vasomotor response (Kugiyama *et al.*, 1998a). Whether this observation relates to the extracellular metabolism of GSH and the cellular uptake of cysteine is not clear, since infusion of *N*-acetylcysteine did not result in improvement of NO-mediated dilation in normal subjects (Creager *et al.*, 1997).

Ascorbic acid: Ascorbic acid is among the most effective water-soluble antioxidants in human tissues and fluids (Frei *et al.*, 1988). Ascorbic acid is an extremely versatile antioxidant that effectively scavenges a wide variety of radical species and oxidant, including superoxide, H_2O_2, obvious peroxyl radicals, hydroxyl radicals, hypochlorous acid (HOCl), and singlet oxygen.

There is data to suggest that physiologically relevant concentrations of ascorbic acid have important implications for NO bioactivity. Oral ascorbic acid administration improves NO-mediated arterial relaxation in patients with documented coronary artery disease (Levine *et al.*, 1996). Chronic treatment with ascorbic acid (500 mg/day) over 30 days also enhances NO-mediated arterial relaxation in patients with coronary artery disease (Gokce *et al.*, 1999). In a wide range of disease states, the acute infusion of ascorbic acid at pharmacologic concentrations (~10 m*M*) has been shown to improve NO-mediated arterial relaxation (Table 1).

Given that ascorbic acid is a potent scavenger of superoxide and superoxide is a major mechanism of impaired NO bioactivity, many have speculated that ascorbic acid improves NO bioactivity due to superoxide scavenging. Recent studies suggest an alternative explanation. In isolated arterial segments, superoxide from a variety of sources will readily impair NO-mediated arterial relaxation, and this effect is reversed by physiologic concentrations of SOD (~1–3 μM) (Rubanyi and Vanhoutte, 1986; Ignarro *et al.*, 1987b). In contrast, physiologic concentrations of ascorbic acid (<10 m*M*) fail to inhibit the impairment of NO bioactivity by superoxide in isolated arterial segments (Jackson *et al.*, 1998). Competing reactions with disparate kinetics provide a plausible explanation for these observations. Because the reaction between NO and superoxide is 10^5-fold faster than the reaction between superoxide and ascorbic acid, the concentration of ascorbic acid must exceed that of NO by factor 10^5 to compete effectively with NO for superoxide. Assuming relevant NO concentrations of 0.1–1.0 μM adjacent to endothelial cells (Malinski *et al.*, 1993), a concentration of 10–100 m*M* ascorbic acid is required to prevent the interaction of NO and superoxide. This value is in excellent agreement with experimental data (Jackson *et al.*, 1998) and exceeds what would be considered physiologically relevant. Thus, although ascorbic acid is an efficient scavenger of superoxide, the rate of this reaction is insufficient to compete effectively with NO.

Recent data indicate that ascorbic acid has direct effects on endothelial NO production. Cultured endothelial cells incubated with physiologic ascorbic acid produce more NO than vehicle-treated cells (Heller *et al.*, 1999; Huang *et al.*, 2000). We have found this effect of ascorbic acid to be tetrahydrobiopterin-dependent, suggesting an interaction between these two intracellular antioxidant species (Huang *et al.*, 2000). Consistent with this observation, the effect of tetrahydrobiopterin to improve endothelial NO bioactivity in smokers is abrogated by pretreatment with ascorbic acid (Heitzer *et al.*, 2000).

SUMMARY

NO is an important component of vascular homeostasis and abnormal NO bioactivity has been implicated in number of disease states with important public health implications. One clear mechanism of impaired NO bioactivity and vascular disease is excess vascular oxidative stress. There is now a wealth of developing data that manipulation of vascular antioxidant stress is the considerable influence of the biologic activity of endothelium-derived NO. It remains to be seen if this influence can be exploited in a manner that truly alters the course of human disease.

TABLE 1. CLINICAL STUDIES OF ASCORBATE AND NO BIOACTIVITY

Study	*Condition/risk factor*	*Vascular bed*	*Number of participants*	*Dose (mg)*	*Study design*	*Effect*
Gilligan *et al.* (1994)	Hypercholesterolemia	Forearm (resistance)	19	1 g/day	Uncontrolled	Negative[a]
Ting *et al.* (1996)	Diabetes mellitus (II)	Forearm (resistance)	20	24 mg/min	Controlled	Positive
Levine *et al.* (1996)	CAD	Forearm (FMD)	46	2 g orally	Randomized	Positive
Heitzer *et al.* (1996)	Smoking	Forearm (resistance)	20	18 mg/min	Controlled	Positive
Motoyama *et al.* (1997)	Smoking	Forearm (FMD)	40	10 mg/min	Controlled	Positive
Ting *et al.* (1997)	Hypercholesterolemia	Forearm (resistance)	23	24 mg/min	Controlled	Positive
Solzbach U *et al.* (1997)	Hypertension	Coronary (epicardial)	22	3 g i.v.	Controlled	Positive[b]
Plotnick *et al.* (1997)	High-fat meal	Forearm (FMD)	20	1 g orally	Randomized	Positive
Hornig *et al.* (1998)	Heart failure	Forearm (FMD)	23	25 mg/min	Randomized	Positive
Timimi *et al.* (1998)	Diabetes mellitus (I)	Forearm (resistance)	20	24 mg/min	Controlled	Positive
Taddei *et al.* (1998)	Hypertension	Forearm (resistance)	82	24 mg/min	Controlled	Positive
Kugiyama *et al.* (1998b)	Vasospastic angina	Coronary (epicardial)	66	10 mg/min	Controlled	Positive
Ito *et al.* (1998)	Heart failure and CAD	Forearm (FMD)	22	1 g i.v.	Uncontrolled	Positive
Chambers *et al.* (1999)	Induced hyper-homo-cysteinemia	Forearm (FMD)	17	1 g/day	Randomized	Positive[c]
Gokce *et al.* (1999)	CAD	Forearm (FMD)	46	500 mg/day	Randomized	Positive
Heitzer *et al.* (2000)	Smoking	Forearm (resistance)	45	18 mg/min	Controlled	Positive

Abbreviations: CAD, coronary artery disease; FMD, brachial artery flow-mediated dilation; I or II indicates type I or II diabetes mellitus.
[a]This study also employed α-tocopherol and β-carotene.
[b]No improvement in coronary resistance vessel function.
[c]Only for the patients with CAD, not with heart failure.

ABBREVIATIONS

CDNB, 1-Chloro-2,4 dinitrobenzene; DTDP, 2,2′-dithiodipyridine; EDRF, endothelial-derived relaxing factor; GSH, glutathione; GSNO, *S*-nitroso-glutathione; HOCl, hypochlorous acid; HUVEC, human umbilical vein endothelial cells; LDL, low-density lipoprotein; NEM, *N*-ethylmaleimide; NO, nitric oxide; NOS, nitric oxide synthase; PKC, protein kinase C; SOD, superoxide dismutase.

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