Mechanisms of Increased Vascular Superoxide Production in Human Diabetes Mellitus
Role of NAD(P)H Oxidase and Endothelial Nitric Oxide Synthase

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Background—Increased superoxide production contributes to reduced vascular nitric oxide (NO) bioactivity and endothelial dysfunction in experimental models of diabetes. We characterized the sources and mechanisms underlying vascular superoxide production in human blood vessels from diabetic patients with coronary artery disease compared with nondiabetic patients.

Methods and Results—Vascular superoxide production was quantified in both saphenous veins and internal mammary arteries from 45 diabetic and 45 matched nondiabetic patients undergoing coronary artery bypass surgery. NAD(P)H-dependent oxidases were important sources of vascular superoxide in both diabetic and nondiabetic patients, but both the activity of this enzyme system and the levels of NAD(P)H oxidase protein subunits (p22phox, p67phox, and p47phox) were significantly increased in diabetic veins and arteries. In nondiabetic vessels, endothelial NO synthase produced NO that scavenged superoxide. However, in diabetic vessels, the endothelium was an additional net source of superoxide production because of dysfunctional endothelial NO synthase that was corrected by intracellular tetrahydrobiopterin supplementation. Furthermore, increased superoxide production in diabetes was abrogated by the protein kinase C inhibitor chelerythrine.

Conclusions—These observations suggest important roles for NAD(P)H oxidases, endothelial NO synthase uncoupling, and protein kinase C signaling in mediating increased vascular superoxide production and endothelial dysfunction in human diabetes mellitus. (Circulation. 2002;105:1656-1662.)

Key Words: diabetes mellitus ■ atherosclerosis ■ endothelium ■ superoxide ■ nitric oxide

Superoxide production by vascular tissues and its interaction with nitric oxide (NO) play important roles in vascular pathophysiology.1-2 Superoxide reacts rapidly with NO, reducing NO bioactivity and producing the oxidative peroxynitrite radical.3-4 Abnormal vascular endothelial function and atherosclerosis are prominent features of diabetes mellitus,5 and evidence from experimental studies suggests that increased superoxide production accounts for a significant proportion of the NO deficit in diabetic vessels. In addition to NO scavenging, superoxide may alter the activity and regulation of endothelial NO synthase activity in endothelial cells6 and has other potentially proatherogenic actions on smooth muscle cell proliferation, inflammatory cell recruitment, and redox-sensitive gene expression.7

Potential sources of vascular superoxide production include NAD(P)H-dependent oxidases,8,9 xanthine oxidase,10 lipoygenase, mitochondrial oxidases, and NO synthases.11 NAD(P)H oxidases appear to be the principal source of superoxide production in several animal models of vascular disease, including diabetes.1,8,12 Furthermore, NAD(P)H oxidase proteins and activity are present in human blood vessels, including atherosclerotic coronary arteries,13 and in saphenous veins and mammary arteries from patients with coronary artery disease,1,14 which suggests that this oxidase system plays an important role in vascular disease states.15

Endothelial NO synthase (eNOS), present in the vascular endothelium, produces NO by oxidation of l-arginine to l-citrulline. NO has diverse antiatherogenic actions on the vessel wall, including antioxidant effects by direct scavenging of superoxide. However, eNOS may be a source of superoxide production under certain conditions because of enzymatic “uncoupling” of l-arginine oxidation and oxygen reduction by the oxygenase and reductase domains of eNOS, respectively. Recent studies suggest that reduced availability of the cofactor tetrahydrobiopterin (BH4) may result in eNOS uncoupling and that this may be an important contributor to...
the imbalance between production of NO and superoxide production in vascular disease. Hyperglycemia increases NOS-dependent superoxide production in human endothelial cells, and recent data from animal studies suggest a possible role for BH4 in mediating the eNOS dysfunction observed in diabetic vessels and endothelial cells.

Despite the importance of increased superoxide production in endothelial dysfunction and vascular disease in diabetes, the characteristics and mechanisms of vascular superoxide production in human diabetes remain poorly defined. Accordingly, we evaluated the sources and relative magnitude of superoxide production in both arteries and veins taken from patients with type II diabetes compared with vessels from matched nondiabetic patients. In particular, we sought to investigate both the NAD(P)H oxidase system and the potential role of eNOS dysfunction in contributing to vascular superoxide production.

Methods

Patients and Blood Vessels
Segments of internal mammary arteries and human saphenous veins were obtained from patients undergoing routine coronary artery bypass surgery at the John Radcliffe Hospital, Oxford, UK. The Local Research Ethics Committee approved collection of tissue specimens, and all patients gave written informed consent. Patients with type II diabetes mellitus had fasting glucose >5.5 mmol/L and/or current treatment with insulin or oral hypoglycemic agents. An equal number of nondiabetic subjects were matched for other major demographic and clinical risk factors: hypercholesterolemia (total plasma cholesterol >4.8 mmol/L), smoking (current or within last 6 months), and hypertension (current treatment with antihypertensive agents). Vessels were collected immediately after surgical harvesting and transported to the laboratory in ice-cold Krebs-HEPES buffer.

Vascular Superoxide Production
Superoxide production was measured by both lucigenin-enhanced chemiluminescence and ferricytochrome c reduction by previously described and validated methods. Briefly, intact vessel segments were equilibrated in Krebs-HEPES gassed with 95% O2/5% CO2 for 30 minutes at 37°C. Lucigenin-enhanced chemiluminescence from intact vessels was measured in buffer (2 mL) containing low-concentration lucigenin (5 μmol/L). In some experiments, superoxide generation was measured in the presence of various oxidase inhibitors with 20 μmol/L lucigenin. Superoxide production was expressed as relative light units per second per milligram of vessel dry weight.

Vascular superoxide production was also measured by superoxide dismutase (SOD)–inhibitable ferricytochrome c reduction assays, as described previously. Briefly, equal portions of vascular homogenate were incubated in 1 mL of buffer containing ferricytochrome c (80 μmol/L) in the presence of NAD(P)H or NADH (100 μmol/L) at 37°C for 45 minutes, then absorbance was measured at 550 nm. All experiments were performed with and without SOD (400 U/mL). Superoxide production was calculated as the portion of ferricytochrome c reduction inhibited by SOD.

Oxidative Fluorescent Microtopography
In situ superoxide generation was evaluated in vascular cryosections with the oxidative fluorescent dye dihydroethidium (DHE). Cryosections (30 μm) were incubated with DHE (2 μmol/L) in PBS, with or without polyethylene glycol (PEG)-conjugated SOD, or oxidase inhibitors. Fluorescence images were obtained with a BioRad MRC 1024 scanning confocal microscope. In each case, paired segments of diabetic and nondiabetic vessels were analyzed in parallel with identical imaging parameters.

Results

Patient Characteristics
Vessels were obtained from a total of 90 patients (76 men, 14 women; 45 diabetics and 45 nondiabetics) undergoing coronary artery bypass grafting. Demographic and clinical characteristics, shown in Table 1, revealed that diabetic and nondiabetic patients were closely matched for these factors except for specific therapies for diabetes.

Vascular Superoxide Generation Is Increased in Human Arteries and Veins in Diabetes
Basal superoxide production from both saphenous veins and internal mammary arteries was determined by lucigenin-enhanced chemiluminescence from intact vessel rings from diabetic and nondiabetic patients (Figure 1). Specificity for

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BP indicates blood pressure.

Western Immunoblotting
Portions of vascular homogenate, equalized for protein content, were separated by SDS-PAGE (12% gels) and transferred to nitrocellulose membranes. NAD(P)H oxidase components were detected with mouse monoclonal antibodies against p67phox or p47phox (Transduction Laboratories) or by rabbit polyclonal antibodies against a p22phox C-terminus peptide (generously provided by Dr Imajoh-Ohmi, Tokyo, Japan). Bands were detected by horseradish peroxidase–conjugated secondary antibodies and visualized by chemiluminescence.

Statistical Analysis
Data are expressed as mean±SEM. In all cases, n refers to numbers of patients. Statistical significance of differences was assessed by Student’s t tests. A value of P<0.05 was considered statistically significant.
superoxide was demonstrated by coincubation with SOD (500 U/mL). In both veins and arteries, basal superoxide release was significantly elevated in vessels from patients with diabetes; in mammary arteries, total superoxide release was almost doubled.

Role of the Endothelium in Superoxide Production in Human Blood Vessels

To investigate the importance of the endothelium in vascular superoxide production, we studied internal mammary artery segments denuded of endothelium (Figure 2). In arteries from nondiabetic patients, endothelium removal resulted in a significant increase in superoxide release, which suggests that in these vessels the net contribution of the endothelium is to reduce vascular superoxide release by production of NO. In marked contrast, endothelium removal in artery segments from diabetic patients significantly reduced superoxide release, which suggests that in diabetic vessels, the endothelium is a net contributor to total vascular superoxide production.

To further investigate these regional differences in superoxide production in the vessel wall, we visualized vessel cryosections using the intracellular fluorescent dye DHE (Figure 2B). Oxidative fluorescent microtopography revealed superoxide generation in endothelial and medial layers and to a lesser extent in the adventitia of both veins and arteries. Medial and adventitial fluorescence was modestly increased in diabetic vessels. However, in vessel sections from patients...
with diabetes, endothelial cells showed strikingly increased fluorescence compared with other regions of the vessel wall when visualized with identical imaging parameters. As expected, PEG-conjugated SOD (PEG-SOD) abolished DHE fluorescence.

Sources of Vascular Superoxide in Human Diabetes

To investigate the enzymatic sources of superoxide production in diabetic and nondiabetic vessels, we measured superoxide production in response to a range of potential oxidase inhibitors and substrates (Table 2). In both diabetic and nondiabetic vessels, superoxide production was inhibited by diphenylene iodonium, an inhibitor of flavin-containing oxidases such as NAD(P)H oxidases. However, the response to inhibition of NOS with \(N\)-methyl-L-arginine was strikingly different between diabetic and nondiabetic vessels. In both veins and arteries from nondiabetic patients, NOS inhibition significantly increased superoxide release because of the loss of superoxide scavenging by NO. In contrast, NOS inhibition in diabetic vessels decreased superoxide release, which suggests that the net effect of NOS activity in these vessels is superoxide production rather than NO production. Oxypurinol, indomethacin, and rotenone had minimal or more modest effects on superoxide production. Indomethacin resulted in statistically significant inhibition in saphenous veins from nondiabetics and mammary arteries from both groups of patients. Oxypurinol inhibited superoxide release in some diabetic mammary arteries, but these effects were not consistently different between diabetic and nondiabetic vessels in all patients.

Increased NAD(P)H Oxidase Activity and Protein Subunits in Diabetic Vessels

To investigate the potential importance of vascular NAD(P)H oxidases in mediating increased vascular superoxide production in diabetes, we compared NADH and NAD(P)H-dependent superoxide production in diabetic and nondiabetic arteries and veins. We quantified superoxide using 2 different methods: lucigenin-enhanced chemiluminescence in intact vessel rings and SOD-inhibitable ferricytochrome c reduction in vessel homogenates (Figure 3). Addition of NADH or NAD(P)H (100 \(\mu\)mol/L) stimulated superoxide release more than 10-fold; NADH-stimulated superoxide release was inhibited by diphenylene iodonium but not by oxypurinol, rotenone, or \(N\)-methyl-L-arginine (data not shown). NADH/NAD(P)H-stimulated superoxide production from both saphenous veins and internal mammary arteries was signifi-

![Figure 3. NAD(P)H oxidase activity in human diabetes. NAD(P)H oxidase activity in diabetic and nondiabetic saphenous veins (HSV, n=30) and mammary arteries (IMA, n=10), was measured in response to NAD(P)H or NADH in intact vessels with 5 \(\mu\)mol/L lucigenin-enhanced chemiluminescence (A) and in vascular homogenates by SOD-inhibitable ferricytochrome c reduction assay (B). \(\Delta P<0.05\) vs non-DM. RLU indicates relative light units.](image-url)
cantly greater in vessels from diabetic patients than from nondiabetic patients.

Next, we investigated the relative abundance of NAD(P)H oxidase protein subunits in vessels from diabetic and nondiabetic patients using Western immunoblotting. We found increased levels of the p22phox membrane-bound subunit and the p67phox and p47phox cytosolic subunits in both saphenous veins and mammary arteries from diabetic patients (Figure 4). Relative quantification of protein bands, normalized to smooth muscle α-actin, revealed that levels of these NAD(P)H oxidase protein subunits were almost 3-fold higher in diabetic arteries and veins than in vessels from nondiabetic patients.

**eNOS Dysfunction in Human Diabetes Mellitus**

We next sought to further investigate the potential role of eNOS dysfunction in contributing to vascular superoxide production in diabetic patients. We measured superoxide production in internal mammary arteries in response to NOS inhibition and in response to BH4 supplementation. We incubated vessels with the synthetic pterin sepiapterin (10 μmol/L), which is converted to BH4 intracellularly via the pterin salvage pathway, then washed extensively to avoid potential confounding by nonspecific antioxidant effects of high extracellular BH4 concentrations. In vessels from patients with diabetes, sepiapterin significantly reduced vascular superoxide production. As observed previously with NG-nitro-L-arginine methyl ester (L-NAME), NOS inhibition with Nω-nitro-L-arginine methyl ester (L-NAME) in this experiment again increased superoxide release from nondiabetic vessels but reduced superoxide release from diabetic vessels. Similar reductions in superoxide production after either sepiapterin or L-NAME suggest that sepiapterin was effective in abolishing the proportion of superoxide release mediated by eNOS dysfunction in diabetic vessels.

To investigate the potential importance of PKC signaling in human diabetic vessels, we preincubated vessels with the PKC inhibitor chelerythrine chloride. Chelerythrine (3 μmol/L) modestly reduced superoxide production (by ~25%) in nondiabetic internal mammary artery segments (Figure 5). However, this reduction was significantly greater in vessels from diabetic patients (>60%), reducing superoxide production to levels observed in nondiabetics, which suggests that PKC inhibition abrogates the increased vascular superoxide production in diabetic patients.

To visualize changes in eNOS-mediated endothelial superoxide production, we used oxidative fluorescent microtopog-
raphy with DHE (Figure 6). Endothelial DHE fluorescence in diabetic internal mammary artery and saphenous vein tissue sections was virtually abolished by incubation with sepiapterin and by incubation with L-NAME. Importantly, DHE fluorescence in other regions of the vessel wall was unaffected in each case, providing a within-section control and demonstrating the endothelium-specific nature of the effect of NOS inhibition or cofactor supplementation. Incubation with PEG-conjugated SOD abolished all cellular fluorescence, leaving only autofluorescence from the elastic lamina.

Discussion

We have used mammary arteries and saphenous veins as model systems to investigate the characteristics, sources, and mechanisms of vascular superoxide production in human diabetes mellitus. Vessels from patients with diabetes generate significantly more superoxide, from 2 principal sources:

first, activity and protein levels of the vascular NAD(P)H oxidase system are increased; second, in diabetic vessels, the endothelium is a net contributor to total vascular superoxide release, rather than to scavenging of superoxide by NO production. This increased endothelial superoxide production appears to be caused by dysfunctional eNOS, mediated by availability of the cofactor BH4. Finally, these changes appear to be mediated, at least in part, by PKC signaling.

These findings are important because they reveal the mechanisms underlying increased vascular superoxide production in human diabetes, and they suggest clear associations with the endothelial dysfunction characteristic of diabetic vessels even in the absence of macroscopic atherosclerosis. The present study supports previous in vivo and in vitro data indicating that vessels from diabetic patients show marked abnormalities in endothelial function characterized by reduced NO bioactivity, and they support data from experimental models of diabetes that reveal increased superoxide production. Our findings suggest 2 important and potentially related mechanisms that underlie these functional deficits. First, increased superoxide production by NAD(P)H oxidases, in common with atherosclerosis and other preatherosclerotic states, likely reduces NO bioactivity by direct scavenging. NAD(P)H oxidases are expressed in vascular cells and macrophages in atherosclerotic coronary arteries, although the lack of overt atherosclerosis in saphenous veins and mammary arteries suggests that macrophages are less likely a major source of NAD(P)H oxidase activity in the vessels in the present study. The increased levels of the NAD(P)H oxidase protein subunits in diabetic vessels, in association with increased enzymatic activity, suggest that upregulated gene expression or posttranscriptional upregulation of protein levels is important in mediating increased NAD(P)H oxidase activity in human vascular disease. Second, in diabetic vessels, the endothelium is a significant net source of superoxide because of a profound loss of normal eNOS function, characterized by a transition from NO production to superoxide production. This observation suggests that diabetes appears to result in specific and marked defects in endothelial biology compared with other systemic risk factors for vascular disease. Indeed, the patient groups in the present study were closely matched for other risk factors and medication to reduce the confounding effects of factors that are associated with increased vascular superoxide generation. Furthermore, we observed similar abnormalities in endothelial function in both saphenous veins and mammary arteries, which suggests that the effects of diabetes on endothelial function are systemic and are not restricted to arteries that develop overt atherosclerosis.

Recent studies have highlighted the potential importance of dysfunctional eNOS regulation in vascular disease states. Our data now provide the first direct evidence for enzymatic uncoupling of dysfunctional eNOS in human endothelium, leading to increased superoxide production. Furthermore, our observation that eNOS-mediated superoxide production can be normalized by incubation with sepiapterin adds further evidence to previous in vitro and animal studies suggesting that this effect is mediated by BH4 availability. Our use of sepiapterin, followed by extensive...
washing, rather than high-concentration BH4 makes nonspecific superoxide scavenging by this redox-active molecule unlikely. Our findings in human vessels support a potential mechanistic relationship between increased NAD(P)H oxidase activity and eNOS dysfunction, proposed on the basis of similar findings in experimental diabetes in rats and in atherosclerotic apolipoprotein E knockout mice, in which both increased NAD(P)H oxidase activity and eNOS dysfunction contributed to increased total vascular superoxide production and reduced NO bioactivity. Peroxynitrite, generated from NO and superoxide, directly oxidizes BH4 to BH2 (dihydrobiopterin), a biopterin that does not support eNOS oxidation.29 Indeed, some data suggest that competition between BH2 and BH4 for eNOS binding may increase eNOS uncoupling. Therefore, upregulation of vascular superoxide production by NAD(P)H oxidases may in turn lead to eNOS uncoupling through oxidation of BH4, which reduces NO production and further increases endothelial superoxide production. Furthermore, our data from human blood vessels add to findings in experimental models of diabetes and nitrate tolerance and recent studies of flow-mediated vasodilatation in hyperglycemia that suggest an important role for PKC in mediating increased NAD(P)H oxidase activity and eNOS dysfunction in human diabetes.

In conclusion, we find that significantly increased superoxide production in human blood vessels from patients with diabetes is mediated by upregulated NAD(P)H oxidase activity and by a striking increase in endothelial superoxide production mediated by eNOS. This suggests important and potentially related roles for the NAD(P)H oxidase system and BH4-dependent eNOS uncoupling, possibly mediated by PKC signaling, in the pathophysiology of endothelial dysfunction in human diabetes mellitus.

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