Vascular Superoxide Production by NAD(P)H Oxidase Association With Endothelial Dysfunction and Clinical Risk Factors

Tomasz J. Guzik, Nick E.J. West, Edward Black, Denise McDonald, Chandi Ratnatunga, Ravi Pillai, Keith M. Channon

Abstract—Superoxide anion plays important roles in vascular disease states. Increased superoxide production contributes to reduced nitric oxide (NO) bioactivity and endothelial dysfunction in experimental models of vascular disease. We measured superoxide production by NAD(P)H oxidase in human blood vessels and examined the relationships between NAD(P)H oxidase activity, NO-mediated endothelial function, and clinical risk factors for atherosclerosis. Endothelium-dependent vasorelaxations and direct measurements of vascular superoxide production were determined in human saphenous veins obtained from 133 patients with coronary artery disease and identified risk factors. The predominant source of vascular superoxide production was an NAD(P)H-dependent oxidase. Increased vascular NAD(P)H oxidase activity was associated with reduced NO-mediated vasorelaxation. Furthermore, reduced endothelial vasorelaxations and increased vascular NAD(P)H oxidase activity were both associated with increased clinical risk factors for atherosclerosis. Diabetes and hypercholesterolemia were independently associated with increased NADH-dependent superoxide production. The association of increased vascular NAD(P)H oxidase activity with endothelial dysfunction and with clinical risk factors suggests an important role for NAD(P)H oxidase–mediated superoxide production in human atherosclerosis. The full text of this article is available at http://www.circresaha.org.

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

Superoxide production by vascular tissues and its interaction with nitric oxide may play important roles in vascular disease pathophysiology.1–2 Superoxide reacts rapidly with nitric oxide (NO), reducing NO bioactivity and producing peroxynitrite,3,4 a strong oxidant that can nitrosylate cellular proteins and lipoproteins.5 Recent evidence suggests that increased superoxide production accounts for a significant proportion of the NO deficit in several animal models of vascular disease, including hypercholesterolemia,6,7 hypertension,8–10 and heart failure.11 In addition to effects mediated by scavenging NO, superoxide directly stimulates mitogenesis in vascular smooth muscle cells12,13 and reduces endothelial NO synthase expression and activity in endothelial cells.14

Potential sources of vascular superoxide production include NAD(P)H-dependent oxidases,8,15 xanthine oxidase,5,16 lipoxygenase, mitochondrial oxidases, and NO synthases.17 The NAD(P)H oxidase, originally characterized in neutrophils, is present in vascular smooth muscle cells15,18 and endothelial cells19–21 and is the principal source of superoxide production in some animal models of vascular disease.7,8 Furthermore, p22phox, one of the components of the NAD(P)H oxidase, is expressed in atherosclerotic human coronary arteries.22 Genetic polymorphisms in the CYBA gene, encoding p22phox, have recently been associated with coronary artery disease in case-control studies and in a prospective study of coronary artery disease progression or regression.23 Despite the potential importance of superoxide production by the NAD(P)H oxidase in atherosclerosis, functional studies of human vascular superoxide production have been limited and have not investigated NAD(P)H oxidase activity.24,25 Consequently, the relationships between superoxide production by NAD(P)H oxidase, atherosclerotic risk factors, and endothelial dysfunction in human blood vessels remain unclear.

Accordingly, we sought to investigate the importance of NAD(P)H oxidase in human vascular superoxide production, with the use of saphenous veins from patients with systemic risk factors for atherosclerosis. We find that an NAD(P)H oxidase is the principal source of superoxide production in human saphenous veins and that increased vascular NAD(P)H oxidase activity is associated with impaired NO-mediated endothelial function and with increased atherosclerotic risk factor profile.

Materials and Methods

Patients

Segments of human saphenous veins were obtained from patients undergoing routine coronary artery bypass surgery at the John
Radcliffe Hospital, Oxford, UK. The collection of tissue specimens was approved by the local research ethics committee. Clinical risk factors were categorized as follows: hypercholesterolemia (total plasma cholesterol level $\geq 4.8 \text{ mmol/L}$); smoking (current or within last 6 months); diabetes (fasting glucose level $> 5.5 \text{ mmol/L}$ or current treatment with insulin or oral hypoglycemic agents); and hypertension (current treatment with antihypertensive agents).

**Vasomotor Studies**

Vasomotor responses to phenylephrine, acetylcholine (ACh), calcium ionophore, and sodium nitroprusside (SNP) were determined as previously described.26 All experiments were performed in the presence of indomethacin (10 $\mu$mol/L). Responses were expressed as a percentage of the maximal contraction or precontracted tension.

**Vascular Superoxide Production**

Superoxide production was measured by lucigenin-enhanced chemiluminescence, according to previously described and validated methods.6,8,27,28 Vessel segments were equilibrated in oxygenated Krebs-HEPES solution for 30 minutes at 37°C. Lucigenin-enhanced chemiluminescence from intact vessels was measured in buffer (2 mL) containing lucigenin (5 $\mu$mol/L),27,28 then NADH (100 $\mu$mol/L) or other substrates were added. In other experiments, chemiluminescence was measured in vascular homogenates, in buffer containing lucigenin (250 $\mu$mol/L), as previously described.26 Vascular homogenates were separated into soluble (cytosolic) and particulate (membrane-associated) fractions by ultracentrifugation, as previously described.26 Superoxide production was expressed as relative light units (RLU) $\cdot$ s$^{-1}$ $\cdot$ mg vessel dry weight$^{-1}$ or RLU $\cdot$ s$^{-1}$ $\cdot$ mg protein$^{-1}$.

We measured NADH-stimulated superoxide production in both vascular homogenates and intact rings prepared from the same patients (n=30) and found a significant correlation ($r=0.7, P<0.001$), suggesting that either approach provides an equivalent measure of NAD(P)H oxidase activity. The lucigenin-based assay was validated against an independent measure of superoxide production using superoxide dismutase (SOD)-inhibitable ferricytochrome c reduction, as previously described.30 Equilibrated vessel rings or portions of vascular homogenate were incubated in 1 mL of buffer containing ferricytochrome c (80 $\mu$mol/L) at 37°C for 45 minutes, then absorbance was measured at 550 nm. Similar experiments were performed in the presence of NADH (100 $\mu$mol/L). All experiments were performed in parallel with and without SOD (400 U/mL). NADH-dependent ferricytochrome c reduction was calculated as the portion of ferricytochrome c reduction inhibited by SOD. NADH-stimulated superoxide production measured by ferricytochrome c reduction from both intact vessels and vascular homogenates was closely correlated with measurements determined in parallel by lucigenin-enhanced chemiluminescence (intact vessels: n=9; $r=0.91, P<0.0001$; homogenates: $r=0.94, P<0.001$).

**Statistical Analysis**

Data are expressed as mean±SEM. Vasomotor responses are the average of a mean 3.4 rings for each patient; in all cases n refers to numbers of patients. Statistical significance of differences between superoxide production in response to different substrates or inhibitors was assessed by Student’s t tests. Correlation between vasorelaxations and superoxide production was assessed by Spearman’s rank correlation coefficient and between superoxide production and individual risk factors using multiple ANOVA (type III sums of squares). A value of $P<0.05$ was considered significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**Patient Characteristics**

Saphenous vein was obtained from 133 patients (107 men, 26 women) undergoing coronary artery bypass grafting. Demographic and clinical characteristics are shown in Table 1. All patients had at least one clinical risk factor for atherosclerosis, and all patients had been administered multiple drug therapy.

**Superoxide Generation From Human Saphenous Veins**

Basal superoxide production was determined by lucigenin-enhanced chemiluminescence from intact vessel rings. Specificity for superoxide was demonstrated by coincubation with either SOD (350 U/mL) or tiron (10 mmol/L). Superoxide production was greatly reduced by diphenyleneiodonium, an inhibitor of flavin-containing oxidases (Table 2). In contrast, neither oxypurinol, rotenone, nor N-methyl-L-arginine significantly inhibited superoxide production. The addition of NADH (100 $\mu$mol/L) stimulated superoxide release >10-fold; NADH-stimulated superoxide release was inhibited also by diphenyleneiodonium but not by oxypurinol, rotenone, or N-methyl-L-arginine (Table 2). Because NADH has been reported to stimulate activity of extracellular xanthine oxidase, we measured superoxide release from intact vessel rings in response to hypoxanthine. There was no significant increase in superoxide release in the presence of 1 mmol/L hypoxanthine (n=8; basal $8.2 \pm 0.9$ versus hypoxanthine $9.3 \pm 2.3$ RLU$\cdot$s$^{-1}\cdot$mg$^{-1}$; $P=NS$). We investigated also superoxide release by vascular homogenates in the presence of substrates for specific oxidases (Figure 1A). Again, the greatest stimulation of superoxide production was generated by NADH. NADPH produced $\approx25\%$ of the stimulation seen with NADH. In contrast, neither succinate nor hypoxanthine stimulated superoxide production by vascular homogenates.

To further characterize this vascular NADH-dependent oxidase activity, we determined the specific activity of the enzyme in subcellular fractions of saphenous vein homogenates generated from intact vessels and from vessels denuded of endothelium (Figure 1B). NADH-dependent superoxide production was reduced by $\approx40\%$ in vascular homogenates prepared after endothelial denudation, suggesting that endo-

<table>
<thead>
<tr>
<th>Age (year; mean±SEM)</th>
<th>64±0.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M:F)</td>
<td>107:26</td>
</tr>
<tr>
<td>Risk Factors</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>50 (38)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>97 (73)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>32 (24)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>88 (66)</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
</tr>
<tr>
<td>$\beta$-blockers</td>
<td>80 (60)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>115 (86)</td>
</tr>
<tr>
<td>Nitrates</td>
<td>70 (65)</td>
</tr>
<tr>
<td>Lipid-lowering agents</td>
<td>106 (80)</td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>69 (52)</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitors</td>
<td>61 (46)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are percentages of total number.
TABLE 2. Characteristics of Vascular Superoxide Release

<table>
<thead>
<tr>
<th>Superoxide Production, RLU · s⁻¹ · mg⁻¹</th>
<th>Basal</th>
<th>+ NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel alone</td>
<td>22.4±3.4</td>
<td>350±31.8</td>
</tr>
<tr>
<td>+ Diphenyleneiodonium</td>
<td>6.6±1.7*</td>
<td>94.3±13.4*</td>
</tr>
<tr>
<td>+ Oxypurinol</td>
<td>19.1±4.3</td>
<td>371±39.3</td>
</tr>
<tr>
<td>+ Rotenone</td>
<td>20.8±2.7</td>
<td>341±35.5</td>
</tr>
<tr>
<td>+ SOD</td>
<td>7.2±2.8*</td>
<td>165±24.9*</td>
</tr>
<tr>
<td>+ Tiron</td>
<td>5.9±1.0*</td>
<td>80.5±8.9*</td>
</tr>
<tr>
<td>+ N-methyl-L-arginine</td>
<td>26.2±3.9</td>
<td>448±70.6</td>
</tr>
</tbody>
</table>

Data are mean±SEM.

Superoxide production was determined in multiple saphenous vein rings from 13 patients by lucigenin-enhanced chemiluminescence (5 μmol/L lucigenin) in the presence or absence of various inhibitors of oxidase systems (diphenyleneiodonium 100 μmol/L, oxypurinol 100 μmol/L, rotenone 100 μmol/L, or N-methyl-L-arginine 1 mmol/L) or scavengers of superoxide (SOD 350 U/mL or tiron 10 mmol/L). Vessel rings were incubated with these agents for 30 minutes before and during superoxide determination. Other rings were stimulated with NADH (100 μmol/L) with or without incubation with inhibitors. *P<0.05 vs vessel ring alone.

NADH-stimulated oxidase activity contains a substantial proportion of NADH-stimulated oxidase activity. Subcellular fractionation of homogenates by ultracentrifugation into soluble (cytosolic) and particulate (membrane) fractions revealed that >99% of the NADH-stimulated oxidase activity was localized in the particulate fraction.

Taken together, these data suggest that a membrane-associated NAD(P)H-dependent oxidase, rather than xanthine oxidase, mitochondrial oxidases, or NO synthase, is the predominant source of superoxide production in human saphenous veins from patients with atherosclerosis.

Endothelial Vasomotor Function in Human Saphenous Veins

We used isometric vasomotor studies to investigate NO bioactivity in saphenous vein segments. Maximal endothelium-dependent, receptor-mediated relaxation to ACh was 24.7±1.3% (n=118), whereas endothelium-dependent, receptor-independent relaxations to calcium ionophore (A23187) were greater in all patients (39.6±1.2%, P<0.0001 versus ACh). These responses were inhibited by incubation with nitro-L-arginine or by endothelial removal (data not shown). A large variability between patients in relaxations to these endothelium-dependent agonists was demonstrated (ACh: range 1% to 67%; A23187: range 8% to 78%), but maximal relaxations to ACh and calcium ionophore were significantly correlated within patients (r=0.4, P=0.005). In contrast to ACh, SNP produced complete relaxation in all vessels, indicating that reduced ACh relaxations were not due to inability of medial smooth muscle to relax in response to NO.

Relationship Between NADH-Stimulated Superoxide Generation, Endothelial Function, and Clinical Risk Factors

To investigate the potential importance of vascular NAD(P)H oxidase activity in patients with systemic risk factors for atherosclerosis, we compared NADH-dependent superoxide production by vessel rings and NO-mediated, endothelium-dependent vasorelaxations in patients with an increasing number of clinical risk factors. NADH-dependent superoxide production varied by >10-fold between patients, from 79 to 827 RLU · min⁻¹ · mg⁻¹ (n=116). However, NADH-dependent superoxide production correlated inversely with maximal relaxation to ACh in individual patients (Figure 2;
vascular superoxide production in human blood vessels. We have used saphenous veins as a model system to study superoxide production and maximal relaxation to the direct NO donor SNP (Figure 2; \( r = 0.40, P = 0.002 \)). In contrast, there was no relationship between superoxide production and maximal relaxation to the direct NO donor SNP (Figure 2; \( r = 0.003, P = 0.95 \)).

Furthermore, an increased number of atherosclerotic risk factors was associated with both reduced ACh-dependent vasorelaxations (Figure 3; \( r = -0.53, P < 0.0001 \)) and increased NADH-dependent superoxide production (Figure 3; \( r = 0.49, P < 0.0001 \)). Analysis of individual risk factors revealed that diabetes and hypercholesterolemia were independently associated with increased NADH-dependent superoxide production (Table 3). These data indicate that in human saphenous veins, impaired endothelial NO bioactivity is associated with increased NADH-dependent vascular superoxide production and that both of these factors are more marked in patients with increased atherosclerotic risk factors.

**Discussion**

We have used saphenous veins as a model system to study vascular superoxide production in human blood vessels. Human saphenous veins from patients with atherosclerosis generate superoxide, predominantly by an NAD(P)H-dependent oxidase. Furthermore, we demonstrate associations between NAD(P)H-dependent superoxide release, reduced NO-mediated vasorelaxations, and clinical risk factors for atherosclerosis.

These findings are important because they suggest an association between endothelial dysfunction and increased vascular superoxide production in human atherosclerosis. Our study is in agreement with previous in vivo31 and in vitro24 data showing that ACh-mediated vasorelaxations are inversely related to the number of atherosclerotic risk factors present. Huraux et al.24 found a large variability in both NO-mediated vascular relaxations and basal superoxide production in internal mammary arteries, as we did in saphenous veins, but did not find consistent associations between these two parameters or clinical risk factors. However, we determined NADH-stimulated superoxide release, given that we found this oxidase system to be the predominant source of vascular superoxide production in human saphenous vein and that the activity of this enzyme system is associated with endothelial dysfunction and with clinical risk factors.

Superoxide production is increased in animal models of vascular disease such as hypercholesterolemia,6,7 hypertension,8–10 heart failure,11 and diabetes, and, in most cases, the NAD(P)H oxidase system appears to be the predominant source of superoxide.6–8 Our identification of the NAD(P)H oxidase system as the principal source of superoxide production in human vessels from patients with atherosclerotic risk factors highlights the potential importance of this oxidase in human atherosclerosis.32 The NAD(P)H oxidase system is present in human vascular smooth muscle cells and endothelial cells in culture19,20 and comprises a multisubunit enzyme complex including the p47phox, p67phox, gp91phox, p22phox, and Rac proteins. The p22phox subunit is required for oxidase activity in smooth muscle cells,15 and activity and expression of the enzyme in animal models and cell culture are stimulated by angiotensin II16,18. Of particular relevance to the findings of our study are recent data indicating that the p22phox subunit is expressed in the endothelium, media, and adventitia of human coronary arteries and that p22phox protein is increased in atherosclerotic arteries.22 Our functional data are in agreement with this model, showing that NAD(P)H oxidase activity is present in both the endothelium and smooth muscle cells of human coronary arteries.

**Table 3. Risk Factors Associated With Vascular NADH-Dependent Superoxide Release**

<table>
<thead>
<tr>
<th>Risk Factor (RF)</th>
<th>With RF (n)</th>
<th>Without RF (n)</th>
<th>With RF</th>
<th>Without RF</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>83</td>
<td>33</td>
<td>292±18</td>
<td>275±24</td>
<td>0.4</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>76</td>
<td>40</td>
<td>324±19</td>
<td>226±17</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Smoking</td>
<td>45</td>
<td>71</td>
<td>335±25</td>
<td>258±16</td>
<td>0.08</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>31</td>
<td>85</td>
<td>360±31</td>
<td>261±14</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Data are mean±SEM.

Type III sums-of-squares ANOVA was performed using clinical risk factors as categorical covariates and NADH-dependent superoxide production, expressed in RLU ⋅ min⁻¹ ⋅ mg⁻¹ vessel, as the dependent variable.
and media/adventitia. Recent reports suggest a possible association between atherosclerosis and genetic polymorphisms in the CYBA gene encoding p22phox. A larger prospective study, based on the Lipoprotein and Coronary Artery Study (LCAS), identified a strong association between CYBA genotype and the likelihood of coronary artery disease progression or regression over 2.5 years. Our study contributes important functional data to this emerging association between NAD(P)H oxidases and atherosclerosis, by showing that increased atherosclerotic risk factor profile and endothelial dysfunction are associated with increased NAD(P)H oxidase enzyme activity. However, the exact identity of this oxidase, or oxidases, in the cell types present in human saphenous vein remains to be determined. Recent studies revealed NAD(P)H oxidase components other than those found in the neutrophil enzyme, such as the gp91 homolog Mox-1 in vascular smooth muscle cells. Furthermore, knockout mouse models suggest functional redundancy in the NAD(P)H oxidase components p47phox and gp91phox. These findings raise the possibility that the vascular NAD(P)H oxidases may have significant differences from the neutrophil enzyme. Future functional studies of NAD(P)H oxidase activity need to identify the NAD(P)H oxidase components present in different cell types in human vessels, address mechanisms of activation and transcriptional regulation of the oxidase components by factors such as angiotensin II and atherosclerotic risk factors, and assess the functional importance of genetic polymorphisms in CYBA and related genes.

We and others have found that vascular NAD(P)H oxidase activity can be stimulated by extracellular NADH applied to intact vessel segments, or intact vascular cells, as well as in homogenates. This could be due to transport of reducing equivalents into cells, thus indirectly increasing intracellular NADH levels. Alternatively, the membrane-associated NAD(P)H oxidase subunits in vascular cells may have different transmembrane orientations from neutrophil NADPH oxidase, as has been observed in fibroblasts. Importantly, our finding of a close correlation between NADH-stimulated superoxide release from intact vessels and vascular homogenates from the same patient suggests that this hypothesis and with observations in human internal mammary arteries. However, the significant correlation between ACh- and A23187-induced relaxations and the association of NADH-dependent superoxide production with both ACh- and A231287-stimulated vasorelaxations suggest that a change in G protein–coupled receptor signaling is unlikely to be the sole mechanism underlying reduced NO-mediated vasorelaxations, given that A23187 activates endothelial NO synthase independently of any receptor-mediated pathway. Importantly, increased NADH-dependent superoxide production does appear to be specifically associated with reduced endothelial NO bioactivity rather than the ability of medial smooth muscle to relax to exogenous NO, because maximal endothelium-independent relaxations to SNP were not related to superoxide production or to endothelium-dependent relaxations.

In conclusion, we find that increased vascular NAD(P)H oxidase activity is associated with reduced NO-mediated vasomotor function and with increased atherosclerotic risk factor profile. This suggests a potentially important role for the NAD(P)H oxidase system in the pathophysiology of human atherosclerosis.

Acknowledgments

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References


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