Nitric Oxide Modulates Superoxide Release and Peroxynitrite Formation in Human Blood Vessels

Tomasz J. Guzik, Nick E.J. West, Ravi Pillai, David P. Taggart, Keith M. Channon

Abstract—Nitric oxide and superoxide have important roles as vascular signaling molecules. Nitric oxide (NO) reacts rapidly with superoxide, producing peroxynitrite. The relative balance between these radicals has important implications for vascular pathophysiology in hypertension and other vascular disease states. However, the relationships between superoxide, NO, and peroxynitrite formation in human blood vessels remain unclear. Accordingly, we systematically measured NO, superoxide, and peroxynitrite production from human internal mammary arteries, radial arteries, and saphenous veins from 78 patients undergoing coronary bypass surgery. Basal superoxide release was detected in all vessels at similar levels. However, endothelial removal or nitric oxide synthase inhibition increased mean superoxide release, with a corresponding reduction in peroxynitrite formation. Conversely, NO donors and superoxide scavengers both reduced superoxide release, whereas only NO donors increased peroxynitrite formation. These changes were much larger in arteries that in veins, but there were striking correlations between superoxide production, NO bioavailability, and peroxynitrite formation between the vessel types. Our findings provide direct evidence for coordinated vascular signaling mediated by interactions between NO, superoxide, and peroxynitrite and have important implications for studies of the functional effects of these radicals in human blood vessels. (Hypertension. 2002;39:1088-1094.)

Key Words: endothelium ■ oxidative stress ■ vascular diseases ■ nitric oxide ■ human

The superoxide anion (O2−) is a reactive oxygen species produced by cellular oxidases in the vascular wall, which reacts rapidly with nitric oxide (NO), producing peroxynitrite.1 The reaction between NO and superoxide occurs at almost diffusion-limited rate, 6 times greater than the removal of superoxide by copper-zinc superoxide dismutase (Cu/Zn SOD). The interaction between NO and superoxide depletes NO bioactivity and is functionally important because NO is a pivotal modulator of key vascular functions, including regulation of smooth muscle tone and blood pressure, platelet activation, and vascular cell signaling.2 Consequently, the loss of NO bioactivity associated with increased vascular superoxide plays a potentially important role in the pathogenesis of endothelial dysfunction associated with conditions such as hypertension3 and atherosclerosis.4–6 In addition to the effects mediated by NO scavenging, increasing evidence suggests additional roles for superoxide–nitric oxide interactions in the modulation of vascular signaling through redox-sensitive mechanisms.7 Furthermore, peroxynitrite (ONOO−), formed by the NO-superoxide reaction, has additional detrimental effects on vascular function, by oxidation of cellular proteins and lipids, low-density lipoprotein (LDL) particles,8 or direct cell toxicity.9 However, the formation of peroxynitrite may also generate nitrosylated thiols that function as endogenous NO donors that can induce vasorelaxation10 and inhibit platelet aggregation.11

Taken together, these observations from in vitro studies and animal models suggest that the relative balance between NO and superoxide production and the formation of peroxynitrite determines the roles of the individual radicals in the regulation of vascular function in hypertension and vascular disease states. In human vessels, recent studies have shown that reduced NO-mediated vasorelaxations in arteries and veins from patients with vascular disease risk factors is associated with increased superoxide production.12,13 However, whether this association is due to direct NO-superoxide scavenging with peroxynitrite formation and how these interactions modulate the respective bioactivities of the individual radicals in human blood vessels is unclear. Accordingly, we sought to systematically evaluate the relationships and interactions between superoxide production, NO bioavailability, and peroxynitrite formation in human arteries and veins from patients with vascular disease.

Methods

Patients and Blood Vessels

Undistended segments of human saphenous vein (HSV), internal mammary artery (IMA) and radial artery (RA) were obtained from a
Clinical and Demographic Characteristics of Patients

<table>
<thead>
<tr>
<th></th>
<th>HSV</th>
<th>IMA</th>
<th>RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (mean ± SEM)</td>
<td>62 ± 1.0</td>
<td>62 ± 1.5</td>
<td>57 ± 2.9</td>
</tr>
<tr>
<td>Sex, M:F</td>
<td>50:11</td>
<td>45:10</td>
<td>12:4</td>
</tr>
<tr>
<td>Smoking</td>
<td>23 (38)</td>
<td>20 (37)</td>
<td>5 (31)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>44 (79)</td>
<td>39 (70)</td>
<td>12 (75)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>12 (19)</td>
<td>10 (18)</td>
<td>2 (12)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>38 (63)</td>
<td>33 (60)</td>
<td>10 (62)</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-blockers</td>
<td>33 (54)</td>
<td>30 (55)</td>
<td>7 (44)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>49 (80)</td>
<td>45 (82)</td>
<td>14 (87)</td>
</tr>
<tr>
<td>Nitrates</td>
<td>40 (65)</td>
<td>37 (67)</td>
<td>11 (69)</td>
</tr>
<tr>
<td>Lipid-lowering agents</td>
<td>50 (82)</td>
<td>44 (81)</td>
<td>12 (75)</td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>33 (54)</td>
<td>29 (53)</td>
<td>8 (50)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>27 (44)</td>
<td>25 (43)</td>
<td>7 (44)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM or n (%). None of the differences between vessel types were statistically significant.

total of 78 patients undergoing coronary artery bypass graft surgery. This approach, which has been used in several previous studies, provides a readily available source of fresh human vessels, with “paired” artery and vein specimens from the same patient. Vessels were harvested using a no-touch technique before surgical dissection. Each segment was immediately transferred to ice cold Krebs-Henseleit buffer, delicately flushed, and carefully dissected with microsurgical instruments to remove excess adventitial tissue. Measurements were started within 30 minutes of harvesting. All vessels were collected before topical administration of drugs such as papaverine. The integrity of endothelium after harvesting was checked by immunohistochemistry for the endothelial cell marker CD31 in vessel segments from 25 patients, which revealed complete luminal staining with no endothelial loss. Patient characteristics are presented in the Table. Collection of specimens was approved by the Research Ethics Committee of John Radcliffe Hospital (Oxford, UK) and informed consent was obtained.

Isometric Tension Studies
NO bioavailability was assessed using isometric tension studies as described previously. Briefly, in each experiment arterial and venous rings (~4 mm in length) were precontracted to a stable plateau with phenylephrine (PE, typically using 3 μmol/L), titrated to produce 60% of the maximal constriction of PE, as determined in preconstricted vessels in response to the NOS inhibitor L-NAME (100 μmol/L). After washouts and further precontraction with PE, tonic NO bioavailability was assessed by the additional contraction in response to the NOS inhibitor N^G^-nitro-L-arginine methyl ester (L-NNAME, 100 μmol/L). Finally, relaxations to the NO donor, sodium nitroprusside (SNP, 1 mmol/L to 1 μmol/L), were determined. All experiments were performed in the presence of indomethacin (10 μmol/L) to inhibit vascular prostaglandin synthesis.

Vascular Superoxide and Peroxynitrite Production
Superoxide production was measured from human blood vessels by lucigenin-enhanced chemiluminescence, using previously described and validated methods. Peroxynitrite production was quantified using luminol-enhanced chemiluminescence. Luminol reacts preferentially with peroxynitrite in bicarbonate-based buffer, yielding an unstable luminol endoperoxide that generates luminescence. Vessel segments were opened longitudinally to expose the endothelial surface and then equilibrated for 30 minutes in oxygenated Krebs-HEPES buffer (in mmol/L: NaCl 99, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1, CaCl₂ 1.9, NaHCO₃ 25, Glucose 11, and HEPES 20, pH 7.4) in the presence or absence of either superoxide dismutase (SOD, 350 U/mL) or NOS inhibitors (L-NMMA, 100 μmol/L, or L-NAME, 100 μmol/L). Different NO synthase inhibitors were used to ensure that the effects observed were not dependent on the properties of one specific inhibitor. In some experiments vessels were denuded of endothelium by gentle rubbing of the luminal surface. Chemiluminescence was measured using a Berthold FB12 luminometer (Berthold Detection Systems GmbH) modified to maintain a temperature of 37°C. After dark adaptation, vascular segments (~40 mg wet weight) were added to scintillation vials containing 2 mL Krebs-HEPES buffer with 5 or 25 μmol/L lucigenin (for superoxide) or 500 μmol/L luminol (for peroxynitrite). Chemiluminescence was recorded continuously for 15 minutes, and then the response to the superoxide scavenger Tiron (10 mmol/L) or the NO donor SNP (10 μmol/L) was measured for an additional 5 to 10 minutes. To validate specificity of luminol-enhanced chemiluminescence for peroxynitrite, additional control measurements were performed in the presence of the peroxynitrite scavenger selenite (50 μmol/L) or catalase (1000 U/mL) to remove hydrogen peroxide that can generate luminol chemiluminescence under some conditions. Data were reported as relative light units per second per milligram (RLU/sec/mg) of dry weight of tissue after subtraction of background luminescence.

Oxidative Fluorescent Microtopography
The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate in situ production of superoxide from 30-μm frozen tissue sections as previously described. Cytosolic DHE exhibits blue fluorescence, but once it is oxidized by superoxide to ethidium bromide, it intercalates within the cell’s DNA, staining its nucleus a fluorescent red (excitation at 488 nm, emission 610 nm). Serial sections (30 μm) were equilibrated under identical conditions for 30 minutes at 37°C in Krebs-HEPES buffer. Fresh buffer containing DHE (2 μmol/L) was applied topically onto each tissue section, coverslipped and incubated for 30 minutes in a light-protected, humidified chamber at 37°C, and then viewed by fluorescent confocal microscopy (Bio-Rad MRC-1024), using the same imaging settings in each case. In each experiment, parallel sections were incubated in the presence of polyethylene glycol–conjugated SOD (PEG-SOD, 500 U/mL).

Statistical Analysis
Results are expressed as mean ± SEM; n indicates the number of patients. Statistical comparisons between the 2 groups were made using the Student t test for independent or dependent samples. Comparisons between multiple groups were made using ANOVA followed by post hoc tests. P<0.05 was considered statistically significant.

Results
Bioassay of NO Bioavailability in Human Arteries and Veins
First, we compared NO bioavailability in mammary arteries, radial arteries, and saphenous veins by measurement of ACh-induced vasorelaxations and by additional contraction responses in precontracted vessels in response to the NOS inhibitor L-NNAME. The latter approach provides a bioassay of NO bioavailability in response to isometric tension, rather than stimulation by pharmacologic agonists. ACh-induced vasorelaxations were significantly greater in radial artery than in mammary artery, and both were greater than in saphenous vein (Figure 1), whereas all vessels relaxed equally to the endothelium-independent direct NO donor SNP (1 μmol/L). Similarly, the additional contractions induced by NOS inhibition were significantly higher in radial artery (Figure 1). Functional blockade of endothelial NOS (eNOS) was con-
firmed by complete loss of vasorelaxations to ACh following L-NAME treatment, and the endothelial-dependence of the contraction response was confirmed in vessel rings with endothelium removed, which did not contract in response to L-NAME. These findings indicate that NO production, stimulated either by a receptor-mediated agonists or by isometric tension, is greater in human radial and internal mammary arteries than in saphenous veins.

Superoxide Release From Human Veins and Arteries
Basal superoxide release from intact vessel rings measured using low concentration lucigenin chemiluminescence tended to be higher in radial arteries (22.3±3.5 RLU/sec/mg, n=8) than in internal mammary arteries (16.4±1.5 RLU/sec/mg, n=25) or human saphenous veins (15.7±1.5 RLU/sec/mg, n=25, paired versus mammary arteries), but these differences were not statistically significant. SOD reduced chemiluminescence by 70% and Tiron by >80% in both arteries and veins, confirming specificity for superoxide. We also used the intracellular fluorescent dye dihydroethidium to visualize superoxide production in vessel cryosections. Both arteries and veins showed superoxide generation throughout the vessel wall, in endothelial and medial layers as well as in the adventitia (Figure 2). PEG-SOD virtually abolished dihydroethidium fluorescence. Although DHE staining revealed some superoxide production in endothelium, removal of endothelium from HSV or IMA segments typically increased net superoxide release (RLU/sec/mg, mean±SEM: from 20.9±2.0 to 30.1±2.8 [n=5 IMA, P<0.01] and from 17.4±1.9 to 24.3±1.8 [n=6 HSV, P<0.05]), suggesting that NO production from the vascular endothelium has a direct bearing on net vascular superoxide release.

Effects of NOS Inhibitors and NO Donors on Vascular Superoxide Release
To further investigate differences between NO-superoxide interactions in human arteries and veins, we systematically studied the effects of a nitric oxide donor (SNP, 10 μmol/L) and a NOS inhibitor (L-NMMA, 100 μmol/L) on basal superoxide release in a series of paired segments of internal mammary artery and saphenous vein (Figure 3). As expected, addition of the superoxide scavenger Tiron (10 mmol/L)
reduced superoxide release by approximately 90% from both L-NMMA–treated and control vessel rings. Blockade of NOS by L-NMMA increased superoxide release (in 9/10 vessels) in both saphenous veins and mammary arteries (n=1100 pairs, P<0.05). This mean increase was much more pronounced in mammary arteries than in saphenous veins (54% versus 22% increase; P<0.05), suggesting a greater contribution of basal NO production to limiting superoxide release in arteries than in veins. Finally, addition of SNP (100 μmol/L) to vascular rings resulted in rapid quenching of superoxide by chemical NO production (Figure 3). The degree of inhibition was similar in L-NMMA–treated and control rings, showing that this effect was independent of endogenous NO production.

Effects of NOS Inhibitors and NO Donors on Vascular Peroxynitrite Formation
To provide complementary data on peroxynitrite formation in response to NO donors and NOS inhibitors, parallel experiments were performed using luminol-enhanced chemiluminescence to determine peroxynitrite formation from paired saphenous vein and mammary artery segments treated with either the NO donor SNP, the NOS inhibitor L-NMMA, or the superoxide scavenger Tiron (Figure 4). Basal peroxynitrite formation was significantly higher in mammary arteries than in saphenous veins, reflecting the higher production of both superoxide and NO observed in our earlier experiments (Figures 1 and 3). Luminol-enhanced chemiluminescence was approximately 95% inhibited by the addition of the superoxide scavenger Tiron in both saphenous veins and mammary arteries. The peroxynitrite scavenger ebselen also abolished luminol chemiluminescence (45.4 versus 3.1 RLU/sec/mg, n=4 mammary arteries, P<0.01), whereas treatment with catalase to remove possible confounding effects of hydrogen peroxide did not significantly alter chemiluminescence (45.4 versus 51.8 RLU/sec/mg, P=NS).

NO donors and NOS inhibitors produced changes in peroxynitrite production that were the converse of the
changes observed in superoxide production (Figure 4). Addition of the NO donor SNP significantly increased peroxynitrite formation to greater levels in mammary arteries than in saphenous veins. Conversely, inhibition of endogenous NO production by incubation of vessel rings with L-NMMA resulted in significant inhibition of peroxynitrite formation, which could be overcome by addition of SNP to provide exogenous NO (Figure 4).

Relative Magnitude of Nitric Oxide-Superoxide Interactions and Peroxynitrite Formation in Arteries and Veins

Although net superoxide release in the basal state did not differ between mammary arteries, radial arteries, and saphenous veins, superoxide production measured under conditions of NOS inhibition revealed significantly higher total superoxide generation in radial arteries ($n=8$, 51.5±6.4 RLU/sec/mg) and internal mammary arteries ($n=16$, 38.7±7.2 RLU/sec/mg) than in saphenous veins ($n=16$, 19.1±2.9 RLU/sec/mg, $P<0.01$ versus radial and mammary arteries). Analysis of the relative increases in superoxide production during NOS inhibition in comparison with bioassays of NO bioactivity and with peroxynitrite production revealed that these parameters were consistently related with each other in saphenous veins and mammary and radial arteries in a graded fashion across the 3 vessel types (Figure 5). These findings suggest that the release of superoxide from human blood vessels is modulated not only by total superoxide production, but also by NO production. Matched increases in superoxide and NO production result in increased peroxynitrite formation, and these relationships account for the similar degree of net superoxide release from arteries and veins.

Discussion

This study provides new insights into the functional interaction between superoxide and NO in human blood vessels from patients with vascular disease. We show that, in both human arteries and veins, superoxide release is significantly modulated by interaction with endothelial-derived NO, which reduces the bioavailabilities of both radicals and produces peroxynitrite. Under basal conditions, greater superoxide production in arteries than in veins is balanced by increased NO production resulting in higher arterial peroxynitrite formation. These findings have important implications for the interpretation of studies investigating vascular NO and superoxide bioavailabilities and show that their roles in blood vessel pathophysiology need to be considered together rather than in isolation.

The interaction between NO and superoxide in vascular tissues has been well described in vitro and in animal models of hypertension and atherosclerosis, but its importance in human vessels has been less clear. In agreement with the recent observations of Shapira and colleagues, we observed greater NO bioactivity in radial arteries than in internal mammary arteries, and both were greater than in saphenous veins. These differences can be attributed more to intrinsic differences in total eNOS activity than to differential activation by signaling pathways because the differences were similar when measured by both ACh-induced vasorelaxation (receptor-mediated eNOS activation) and by the contractile response to NOS inhibition mediated by eNOS activation in response to isometric tension. In agreement with the findings of Berry et al and Schmalfuss et al, we also observed significantly higher total vascular superoxide generation in arteries than in veins. However, our findings now clarify the results of these studies by demonstrating that total superoxide release is only measured after NOS blockade because of superoxide scavenging by endothelial-derived NO. Our further experiments confirmed this interaction in human blood vessels by using superoxide scavengers to inhibit the NO-superoxide interaction or by enhancing it with the use of NO donors. Corresponding measurements of peroxynitrite formation revealed the converse changes in response to NOS antagonists or NO donors. Furthermore, our experiments showed that NO-superoxide interactions are more pronounced in arteries than in veins.
The cellular sources of vascular superoxide may vary in different vessel types from different species. Using DHE fluorescence in human blood vessels revealed marked superoxide production from the media and adventitia and a modest proportion directly from the endothelium. In hypertensive angiotensin II–infused rat or mouse aortas, the adventitia is the major source of superoxide, whereas the endothelium appears to be a more important contributor in the diabetes mellitus or deoxycorticosterone acetate (DOCA) salt–hypertensive animals.

Our studies provide a rationale for systematically estimating the balance between superoxide and NO bioavailability in vascular segments. An increase in superoxide release after endothelial removal suggests that the endothelium produces a net excess of NO, rather than superoxide. However, inhibition of NOS, rather than removal of endothelium, resulted in even greater increase in superoxide release, showing that the endothelium itself is a significant source of superoxide in these human blood vessels, as suggested by DHE fluorescence. Although the endothelium appears to produce substantially greater NO than superoxide, when the whole blood vessel is taken into account, the total vascular generation of superoxide seems to exceed the capacity of endothelium to generate NO in these ex vivo conditions, because we found that an NO donor added to vascular rings increased peroxynitrite formation. However, the NO-superoxide balance may be different in vessels in vivo because of tonic activation of endothelial NO production by shear stress and because of other sources of NO such as nitrosothiols. It is also likely that the relative balance between NO and superoxide varies not only with blood vessel type, as we observed, but also under different biological conditions. Indeed, variations in NO production in the ex vivo assay conditions used may account for the apparent differences in net superoxide release from human vessels found in previous studies. Differences in clinical characteristics and vascular disease risk factors also have substantial influences on both NO and superoxide production in human vessels. Furthermore, medications such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or ACE inhibitors could potentially influence both oxidase activities and NO production. However, in this study neither medication nor clinical risk factors differed significantly among the groups of patients from whom different vessels were obtained. However, the blood vessels analyzed in this study were obtained from patients with hypertension (>70%) and other atherosclerosis risk factors. It is likely that the vessels used in our study had higher superoxide production and lower NO bioavailability than healthy vessels, and the interaction between superoxide and NO may be different, as is the case in normocholesterolemic rabbit aortas or nonhypertensive rat compared with aortas from animals with hypercholesterolemia or after angiotensin II infusion. Indeed, we observed some variability in responses to L-NAME or L-NMMA in vessels from different subjects, suggesting that variability in NO bioavailability could be related to individual clinical risk-factor profiles, as we have observed previously in relation to endothelium-dependent relaxations.

The finding of significantly greater NO-superoxide interaction in human arteries than in veins may have important implications. Interestingly, despite greater superoxide and peroxynitrite formation in arteries, endothelium-dependent vasorelaxations are also greater in arteries than in veins. It may be that superoxide or peroxynitrite, in addition to NO, could act as a mediator of endothelium-dependent relaxations in these vessels. Peroxynitrite is a vasorelaxant in rabbit carotid and bovine pulmonary arteries. Higher peroxynitrite formation in arteries may also favor the development of disorders more specific for arteries, such as hypertension or atherosclerosis, whereas veins are not susceptible to classical atherosclerosis despite exposure to all systemic risk factors except arterial blood pressure.

**Perspectives**

We find that superoxide is released from the endothelium and from cells throughout the vessel wall in human arteries and veins. Endothelium-derived NO scavenges superoxide to produce peroxynitrite, and this mutual scavenging between superoxide and NO is more pronounced in human arteries than in veins. Endothelial NO has profound effects on net superoxide release, leading to a corresponding increase in peroxynitrite formation in arteries compared with veins, where NO bioactivity is greater. These findings suggest that NO-mediated vascular signaling needs to be considered as one part of the more complex pathways that are also mediated by superoxide and peroxynitrite radicals and their respective targets. Furthermore, this has important implications for functional studies of vascular NO and superoxide signaling in hypertension and other vascular disease states. Determination of vascular superoxide release needs to take into account the effects of mutual scavenging between superoxide and NO.

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