Superoxide Production, Risk Factors, and Endothelium-Dependent Relaxations in Human Internal Mammary Arteries

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Background—In a variety of disease states, endothelium-dependent vasodilation is abnormal. Reduced nitric oxide (NO) production, increased destruction of NO by superoxide, diminished cellular levels of L-arginine or tetrahydrobiopterin, and alterations in membrane signaling have been implicated. We examined these potential mechanisms in human vessels.

Methods and Results—Relaxations to acetylcholine, the calcium ionophore A23187, and nitroglycerin, as well as superoxide production and NO synthase expression, were examined in vascular segments from patients with identified cardiovascular risk factors. Endothelium-dependent relaxations were also studied after incubation with L-arginine, L-sepiapterin, and liposome-entrapped superoxide dismutase (SOD) and after organoid culture with cis-vaccenic acid. Relaxations to acetylcholine and to a lesser extent the calcium ionophore A23187 were highly variable and correlated with the number of risk factors present among the subjects studied. Treatment of vessels with L-arginine, L-sepiapterin, liposome-entrapped SOD, or cis-vaccenic acid did not augment endothelium-dependent relaxations. Hypercholesterolemia was the only risk factor associated with high levels of superoxide; however, there was no correlation between superoxide production and the response to either endothelium-dependent vasodilator used.

Conclusions—In human internal mammary arteries, depressed endothelium-dependent relaxations could not be attributed to increases in vascular superoxide production, deficiencies in either L-arginine or tetrahydrobiopterin, or reduced membrane fluidity. Variability in signaling mechanisms may contribute to the differences in responses to acetylcholine and the calcium ionophore A23187. (Circulation. 1999;99:53-59.)

Key Words: arteries ■ atherosclerosis ■ endothelium ■ nitric oxide ■ risk factors

In several disease states, endothelium-dependent vascular relaxation is abnormal. The mechanisms underlying this abnormality of vascular function remain controversial. In both experimental animal and human vessels, atherosclerosis has been shown to reduce relaxation to acetylcholine more impaired than relaxation to a calcium ionophore. This suggests an alteration in membrane signaling, perhaps because of alterations in G-protein function. This concept has been controversial because others have reported that endothelium-dependent vascular relaxation to the calcium ionophore A23187 is also abnormal in vessels from hypercholesterolemic animals and humans. Another mechanism underlying altered endothelium-dependent vascular relaxation relates to increased oxidative degradation of nitric oxide (NO•). The aortas of cholesterol-fed rabbits produce large amounts of superoxide anions (O2•−), which are probably responsible for inactivation of NO•. Furthermore, peroxynitrite anion, the product of the reaction between NO• and O2•−, can oxidize lipoproteins, and oxidized lipoproteins can in turn inhibit endothelial G-protein expression and function. Thus, alterations in signaling might be related to increases in the production of oxygen radicals in the vessel. A final mechanism thought to be responsible for alterations in NO• release relates to an insufficient supply of the substrate L-arginine or the cofactor of NO synthase tetrahydrobiopterin.

Studies of vascular function in humans are largely performed in vivo. In this situation, it is difficult to control factors such as the baseline tone of the vessel or neurohumoral influences. In vivo studies are also limited because of time constraints and safety issues regarding interventional drugs in humans. Finally, it is impossible to directly assess O2•− production of vessels in vivo.

Given these considerations, we studied internal mammary artery (IMA) segments, a vascular segment relatively free of...
atherosclerosis, obtained from humans undergoing coronary artery bypass surgery. A striking preliminary finding was the marked variability of responses to acetylcholine among the various subjects. By using in vitro techniques and the organoid culture approach, we were able to examine potential mechanisms that may diminish endothelium-dependent vasodilation.

**Methods**

**Patient Characteristics and Risk Factors**
Age >60 years and plasma cholesterol levels >200 mg/dL were considered risk factors. Cigarette smoking was excluded as a risk factor if patients stopped smoking >6 months before surgery. In addition, a history of either hypertension or diabetes was considered a risk factor. Drugs used by each patient were determined by chart review.

**Vessel Preparation**
Immediately after removal, distal segments of the left IMA, not used for surgical implantation, were placed in a container with modified Krebs-HEPES buffer and maintained at 4°C. After transfer to the laboratory, connective tissue was removed, and the IMA was cut into ring segments for subsequent study.

**Isolated Vascular Ring Experiments**
Studies of isometric tension development were performed on 4 to 6 IMA ring segments from each subject by use of methods previously reported. A resting tension of 2.5 g was progressively applied. All experiments were performed in the presence of indomethacin (10⁻⁵ mol/L) to prevent the synthesis of vascular prostaglandins. After 30 minutes of equilibration, IMA segments were precontracted with the thromboxane A₂ analog (U46619, 10⁻⁵ mol/L) to prevent the synthesis of vascular prostaglandins. After 30 minutes of equilibration, IMA segments were precontracted with the thromboxane A₂ analog (U46619, 10⁻⁵ mol/L) to prevent the synthesis of vascular prostaglandins.

**Liposomal-Entrapped Superoxide Dismutase.**
Liposomal-entrapped superoxide dismutase (SOD) was prepared as described previously. IMA segments were incubated for 45 minutes at 37°C in Krebs/HEPES buffer containing 1500 U/mL of SOD. After incubation, the rings were washed of the liposomal-entrapped SOD and allowed to equilibrate for 30 minutes at 37°C. Superoxide production was estimated with lucigenin-enhanced chemiluminescence as previously described. The primary antibody was applied in 1.0% BSA in PBS and incubated in a humidified chamber for 60 minutes at room temperature. The sections were washed in PBS and then incubated with a biotinylated secondary antibody (horse antimouse IgG at a 1:400 dilution; Vector Laboratories) with the Vectastain ABC alkaline phosphatase system and Vector substrate kit 1 (red reaction product).

**Immunocytochemistry for endothelial NO synthase immunostaining (eNOS) was performed with antibody H-32 (1/100 dilution of tissue culture supernatant).** The primary antibody was applied in 1.0% BSA in PBS and incubated in a humidified chamber for 60 minutes at room temperature. This was followed by washing in PBS and incubation with the avidin-biotin enzyme complex and chromogenic substrate as described by the manufacturer. NOS proteins were visualized with the Vectastain Elite ABC peroxidase system (Vector Laboratory). Staining was not present in sections treated with secondary antibodies only or with nonimmune IgG.

**Measurements of Vascular Superoxide Production**
IMA segments were placed in a modified Krebs-HEPES buffer and allowed to equilibrate for 30 minutes at 37°C. Superoxide production was estimated with lucigenin-enhanced chemiluminescence as previously described. Counts were obtained at 2-minute intervals at room temperature for 20 minutes, and the respective background counts were later subtracted. The vessels were then dried by placing them in a 90°C oven for 24 hours for determination of dry weight. For all estimates of superoxide production, 2 vessel segments with and without endothelium were studied. In each case, the results from the 2 segments were averaged, and this average value was used for subsequent analysis.

**Liposomal-Entrapped Superoxide Dismutase.**
Liposomal-entrapped superoxide dismutase (SOD) was prepared as described previously. IMA segments were incubated for 45 minutes at 37°C in Krebs-HEPES buffer containing 1500 U/mL of SOD. After incubation, the rings were washed of the liposomal-entrapped SOD and studied in organ chambers as described above. Liposomes without SOD were used as controls for these experiments. In other studies, vessels were exposed to liposomal-entrapped SOD for 24 hours by use of organoid culture conditions as described below.

**Vessel Organoid Culture**
We used organoid culture to permit prolonged exposure of vessels, liposome-entrapped SOD, sepiapterin, or cis-vaccenic acid. Briefly, IMAs were harvested and maintained in sterile conditions. Segments were then placed in M-199 supplemented with amino acids, 1-glutamine (2 mmol/L), penicillin-streptomycin (100U/mL), and vitamins in 35-mm tissue culture plates. These were maintained in a humidified incubator at 37°C and 5% CO₂ for 1 day with or without the various interventions. Preliminary studies showed that vessels maintained under these conditions had similar responses to various vasoactive agents before and after the culture period (Table 1).

**Immunocytochemistry**
Serial sections of IMAs from 26 randomly selected patients were examined for endothelial cell integrity. Briefly, frozen paraformaldehyde-fixed tissues sections were thawed and fixed in acetone for 5 minutes, dried, and rehydrated in PBS. Endothelial cells were identified by use of Ulex lectin (Vector Laboratories) with the Vectastain Elite ABC peroxidase system and Vector substrate kit 1 (red reaction product).

**Materials**
Acetylcholine, the calcium ionophore A23187, nitroglycerin, indomethacin, l-arginine, l-sepiapterin, cis-vaccenic acid (cis-11-octadecenoic acid), and lucigenin (bis-N-methylacridinium nitrate) were obtained from Sigma Chemical Co. The thromboxane A₂ analog (U46619) was obtained from the Upjohn Co. The calcium ionophore A23187 was dissolved in ethanol (final concentration, 0.1%). cis-Vaccenic acid was diluted in ethanol (500 mmol/L). Indomethacin was dissolved in a 50 mmol/L solution of sodium carbonate. All dilutions were prepared in distilled water and stored in ice.

**Calculations and Statistical Analysis**
Data are expressed as mean±SEM. Vascular relaxations are expressed as percent of thromboxane A₂ analog−induced vasoconstriction. Responses to each agent were obtained in 3 to 4 vascular
segments and averaged for each patient. The numbers provided refer to the number of patients. Statistical analysis was performed by a t test for unpaired observations or by a Mann-Whitney U test when the distribution of variances was heterogeneous, as determined by a z test for proportionals. To determine the influence of individual risk factors on vascular superoxide production and endothelium-dependent vascular relaxation, stepwise multiple linear regression was used. To compare the influence of varying number of risk factors on endothelium-dependent vascular relaxation, ANOVA followed by Scheffe’s test was used. Simple linear regression analysis was used for determining the relation between 2 variables. A value of \( P < 0.05 \) was considered statistically significant.

### Results

#### Patient Characteristics

For initial studies examining vascular relaxations to acetylcholine, the calcium ionophore A23187, and nitroglycerin, IMAs from 97 patients undergoing coronary artery bypass surgery were studied. Of these, 63 were men and 34 were women. Only 3 women were receiving estrogen replacement. Male and female populations differed by the frequency of cigarette smoking and hypercholesterolemia (Table 2). The frequency of therapy with long-acting nitrates was greater in women compared with men. Almost all patients were on multiple drug therapy.

#### Vascular Relaxations

Endothelium-dependent relaxations to acetylcholine were quite variable in both men and women, with values ranging from 0% to 89%. In men, the maximal response to acetylcholine was 38±3.3% (Figure 1). Responses to the calcium ionophore A23187 were greater than those to acetylcholine, with a maximal relaxation of 64±3% (range, 18% to 100%). These responses followed a similar pattern in women, although relaxations to acetylcholine were significantly less than those observed in men (\( P=0.0025 \) by Mann-Whitney U test). Nitroglycerin produced potent relaxations of all vessels of both sexes. There was no influence of drug therapy on the degree of endothelium-dependent vascular relaxation to either acetylcholine or the calcium ionophore (data not shown).

### Risk Factors and Endothelial Dysfunction

Although 4 risk factors were studied, we had only 4 men and 6 women with 4 risk factors and therefore analyzed only subjects with 1 to 3 risk factors. For both acetylcholine and the calcium ionophore A23187, responses were significantly diminished in subjects with 3 compared with 1 risk factor (Figure 2). Thus, some of the variability observed in endothelial dysfunction may be explained by the number of risk factors present in the patient.

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### Table 2. Demographic Characteristics

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Men (n=63)</th>
<th>Women (n=34)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>63±1</td>
<td>64±2</td>
<td>0.24</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>15 (24)</td>
<td>4 (0.1)</td>
<td>0.035</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>38 (60)</td>
<td>22 (65)</td>
<td>0.336</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>19 (30)</td>
<td>20 (59)</td>
<td>0.138</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>29 (46)</td>
<td>21 (62)</td>
<td>0.001</td>
</tr>
<tr>
<td>Plasma cholesterol level, mg/dL</td>
<td>201±7</td>
<td>219±11</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent percent of total male or female population. Data are mean±SEM.
The average lucigenin signal was 4589 ± 6554 counts per minute per 1 mg (range, 669 to 12309 counts per minute per 1 mg). Endothelium removal resulted in a significant decrease in lucigenin counts (3135 ± 450 counts per minute per 1 mg; range, 88 to 9959 counts per minute per 1 mg; P = 0.04). This finding suggests that in human IMAs, the endothelium is an important source of \( \text{O}_2^- \) production. Importantly, there was no correlation between levels of \( \text{O}_2^- \) production and the maximal response to either acetylcholine or the calcium ionophore (Figure 3).

We further examined the types of risk factor present and how they affected both endothelium-dependent vasodilation and \( \text{O}_2^- \) production. Patients were then divided into those with and those without each risk factor, and the lucigenin signal and peak response to acetylcholine were averaged (Figure 4). In this analysis, absence of a risk factor did not imply that other risk factors were not present. Only in the case of hypercholesterolemia was steady-state \( \text{O}_2^- \) production increased. In subjects with diabetes, hypertension, or cigarette smoking, \( \text{O}_2^- \) production was similar to those without these respective risk factors. Of note, even though the vascular \( \text{O}_2^- \) production was highest in individuals with hypercholesterolemia, responses to acetylcholine and the calcium ionophore A23187 were similar to those without hypercholesterolemia. In contrast, smokers tended to have lower levels of \( \text{O}_2^- \) production yet had the worst impairment in endothelium-dependent vascular relaxation (Figure 4).

We further considered the possibility that \( \text{O}_2^- \) produced within the medial layer, which might escape detection by lucigenin-enhanced chemiluminescence, could reduce endothelium-dependent vascular relaxation. To address this issue, we incubated vessels for either 45 minutes (in HEPES buffer) or 24 hours in the organoid culture in liposome-entrapped SOD (750 U/mL). As shown in Figure 6, treatment with liposome-entrapped SOD for 45 minutes and 24 hours did not affect endothelium-dependent vascular relaxations.

### Effects of l-Arginine and l-Sepiapterin

Vessels were treated with either l-arginine (10\(^{-3}\) mol/L) or sepiapterin (10\(^{-2}\) mol/L) for 30 minutes before and during administration of either acetylcholine or the calcium ionophore A23187. Neither intervention affected relaxations to either vasodilator (Figure 7). Of note, responses to the calcium ionophore were slightly worsened by sepiapterin.
In 6 additional studies, we also treated vessels for 24 hours with sepiapterin (10^{-2} mol/L) in the organoid culture before study. This likewise did not improve vascular relaxations (data not shown).

**Correlation of Responses Between Acetylcholine, Histamine, and Bradykinin**

As shown in Figure 8, there was an excellent correlation between peak relaxations to acetylcholine and histamine (Figure 8A), whereas the relaxations caused by acetylcholine and bradykinin were poorly correlated (Figure 8B). Thus, it would appear that alterations in endothelium-dependent vascular relaxation to acetylcholine are mirrored by impaired responses to histamine but not bradykinin.

**Alterations in Membrane Fluidity**

To enhance membrane fluidity, vessels were exposed to cis-vaccenic acid (5 mmol/L) for 24 hours in the organoid culture. Responses to acetylcholine and calcium ionophore A23187 were similar in presence or absence of liposome entrapped SOD in both experiments (n=6).

**Immunohistochemistry**

IMA segments were free of atherosclerosis as determined by immunohistochemistry. Ulex staining in vessels from 26 patients showed that the intimal surface was almost uniformly covered with endothelium.

We compared eNOS in IMA segments from 3 patients with reasonably intact relaxations to acetylcholine (>60%) to...
immunostaining in 3 patients with markedly diminished acetylcholine-induced relaxations. Despite the marked differences in acetylcholine responses, immunostaining for eNOS was quite similar among these various segments (Figure 10).

**Discussion**

In the present study, the range of relaxations to acetylcholine varied from 0% to 70% and for the calcium ionophore A23187 from 18% to 100%. It would appear, therefore, that some patients exhibited extremely reduced endothelium-dependent vascular relaxations to these agents, particularly acetylcholine. We performed an extensive number of additional studies to attempt to ascertain the mechanism responsible for these seemingly impaired responses. Using an organoid culture approach, we were able to expose vessels to various interventions for ≤24 hours. We were unable to augment relaxations to both endothelium-dependent agents using either l-arginine, l-sepiapterin, liposome-entrapped SOD, or cis-vaccenic acid.

The variability of vascular relaxations observed in these studies was probably not due to inadvertent endothelial denudation in some segments. Histological examination revealed an intact endothelial layer in all but a rare segment studied. Even in those, Ulex staining demonstrated >80% endothelial coverage of the internal elastic lamina.

Our present findings agree with those of Vita and coworkers, who showed that responses of coronary arteries to acetylcholine correlate with the number of risk factors present. Although many of our subjects had multiple risk factors, smoking was notably associated with impaired endothelium-dependent relaxations. In contrast, it was impossible to demonstrate that the presence of any 1 other risk factor worsened these responses. This is likely because most patients had >1 risk factor, and the absence of any 1 risk factor did not exclude the possibility that other risk factors might contribute to alterations in endothelial function.

In previous work, hypercholesterolemia and atherosclerosis in experimental animals have been associated with an increase in vascular superoxide production. Superoxide reacts rapidly with NO, leading to the formation of less vasoactive molecules; indeed, in previous studies, the use of SOD or inhibitors of -O2 production improved endothelium-dependent vascular relaxations dramatically. We were unable to show that a similar phenomenon occurred in vessels from human subjects. Notably, there was no association between -O2 production and the response to either endothelium-dependent vasodilator used, and responses were not improved by treatment with liposome-entrapped SOD. In keeping with previous studies in animals, hypercholesterolemia was associated with an increase in -O2 production as assessed by lucigenin-enhanced chemiluminescence. Importantly, among the 12 vessels treated with liposome-entrapped SOD, 5 were from patients with hypercholesterolemia, and even among this subgroup in which -O2 production was increased, liposome-entrapped SOD did not improve endothelium-dependent vasodilation. It is unlikely that oxygen radical production produced long-term changes in vascular function, such as nitrination of G proteins by peroxynitrite, because a 24-hour treatment with liposome-entrapped SOD did not improve endothelium-dependent vascular relaxation.

An interesting finding in the present study is that there was an inverse correlation between age and vascular -O2 production. The mechanisms responsible for this remain unclear; however, this may reflect a replacement of vascular cells with collagen, which occurs with aging.

Treatment with both l-arginine and tetrahydrobiopterin has been reported to improve responses to acetylcholine in the human brachial artery. In the present study, neither was effective. Our findings are partially in agreement with studies by Bossaller et al, Flavahan, and Shimokawa et al, who have suggested that alterations in endothelium-dependent vascular relaxation in hypercholesterolemia and after endothelial regeneration are due to abnormalities of G-protein function. These investigators have consistently observed markedly abnormal responses to the acetylcholine and other G-protein–mediated agents, whereas responses to the calcium ionophore A23187, which bypasses G-protein signaling, are relatively normal. One explanation for alterations in G-protein signaling is related to changes in membrane fluidity, preventing interactions of the G proteins with the receptors responsible for eNOS activation. We attempted to address this using cis-vaccenic acid. This agent has been shown to enhance G-protein function in turkey erythrocyte.
membranes; however, it had no effect on responses in the present experiments. An alternative explanation is that Gl protein expression is impaired by the various risk factors present in this population. This would not likely be improved by exposure to cis-vaccenic acid. Such a concept is in accord with studies by Tsutsui et al., who showed that Gl expression was impaired by age, hypertension, and hypercholesterolemia. Others have shown that both native and minimized oxidized LDL interferes with receptor activation of Gl proteins and in some cases alters Gl-protein expression. Of note, an excellent correlation between peak responses to histamine and acetylcholine was found. Both acetylcholine and histamine use Gl and bradykinin use Gq as the principal signaling G protein. In keeping with this, responses to acetylcholine and bradykinin were not well correlated.

The present studies, while providing insight into how risk factors might affect endothelial function, may not be applicable to overt atherosclerosis. The vessel studied, the IMA, is relatively free of atherosclerosis, and in the present experiments, histological examination revealed a virtual absence of intimal thickening in the IMA segments studied. Likewise, the present studies may not reflect conditions soon after the onset of exposure to risk factors. It is conceivable that the acute effect of conditions such as hypercholesterolemia, hypertension, and cigarette smoking might be quite different than the effects of these many years after their onset. In keeping with this, short-term animal models have supported a role for many of the factors that seem to have been excluded in these studies, such as increases in O2 production, alterations in tetrahydrobiopterin supply, or alterations in l-arginine use in conditions such as hypercholesterolemia.

In summary, the present studies demonstrate a marked variability in endothelium-dependent vascular relaxations to both acetylcholine and the calcium ionophore A23187, which may in part be due to differences in risk factors present among the individuals studied. We were unable to attribute reduced responses to increases in vascular O2 production, deficits in l-arginine or tetrahydrobiopterin, or changes in membrane fluidity. Variability in signaling mechanisms may contribute to the differences in responses to acetylcholine and the calcium ionophore A23187. In the future, efforts toward enhancing endothelial G protein expression and receptor coupling may be of benefit in improving endothelial function.

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