Low-Density Lipoprotein Cholesterol Determines Oxidative Stress and Endothelial Dysfunction in Saphenous Veins From Patients With Coronary Artery Disease


Objective—There is evidence for a relationship between endothelial dysfunction and cardiovascular disease, but a causative role for oxidative stress remains to be determined.

Methods and Results—We studied 188 patients with severe coronary artery disease (CAD), of whom 51 were age and sex matched with 51 healthy controls undergoing varicose vein surgery. Relaxation of saphenous vein to calcium ionophore, apocynin, and allopurinol was studied together with the markers of oxidative stress, total antioxidant capacity and reduced/oxidized glutathione ratio. Vascular superoxide levels were measured using lucigenin chemiluminescence and hydroethidine. Relaxation to calcium ionophore was decreased in CAD compared with control patients (maximum relaxation 26±2% versus 60±1%; \( P<0.001 \)). Total superoxide production was increased (0.89±0.09 versus 0.56±0.06 nmol/mg per min; \( P=0.008 \)), whereas superoxide inhibition with apocynin or allopurinol had a greater effect on vasorelaxation in CAD patients. Low-density lipoprotein (LDL) cholesterol predicted relaxation to calcium ionophore (\( P<0.001 \)) and oxidative stress markers (\( P<0.001 \)) in CAD patients.

Conclusions—Endothelial dysfunction is associated with raised levels of superoxide and biomarkers of oxidative stress in saphenous veins from CAD patients. LDL cholesterol is a major determinant of endothelial dysfunction and oxidative stress in these patients. These results support intensive LDL cholesterol-lowering therapy as suggested by recent clinical trials. (Arterioscler Thromb Vasc Biol. 2006;26:218-223.)

Key Words: endothelium ▪ nitric oxide ▪ free radicals ▪ lipids ▪ atherosclerosis

Endothelial dysfunction occurs in conjunction with coronary artery disease (CAD). It is observed both in the coronary and peripheral vasculature. Moreover, risk factors for CAD have been almost universally associated with a degree of endothelial dysfunction in humans. Increased production of reactive oxygen species, in particular, superoxide and radicals derived from superoxide, has been associated with endothelial dysfunction in animal models of disease, and there is increasing evidence of a link between oxidative stress and endothelial dysfunction in humans. It has been reported that endothelial dysfunction and increased oxidative stress may predict future events in patients with CAD. However, comparative data on endothelial function, direct measures of superoxide in human vessels, and biomarkers of oxidative stress are not available in patients with CAD nor in control subjects with no documented cardiovascular disease. Circulating biomarkers of oxidative stress have been investigated in patients with essential hypertension and in control subjects, but the relationship between these markers and endothelial function has not been examined. In addition, although the degree of endothelial function has been consistently linked to the number of risk factors present in patients with CAD, the relative importance of individual risk factors in determining levels of oxidative stress and endothelial function remains uncertain.

To address these questions, we have studied endothelial function and levels of oxidative stress in a group of patients with severe CAD undergoing coronary artery bypass graft surgery. In addition, endothelial function and levels of oxidative stress markers were compared in a subgroup of 51 of these patients and 51 age- and sex-matched control patients with no documented cardiovascular disease who were undergoing surgery for removal of varicose veins.
Methods

Subjects
We recruited 188 patients undergoing elective coronary artery bypass graft surgery. A subgroup of 51 patients were age (within 2 years) and sex matched with 51 patients undergoing elective surgery for removal of varicose veins. Patients undergoing coronary artery bypass graft surgery had obstructive CAD demonstrated by coronary angiography; all of the control patients had a normal ECG and no history of arterial hypertension, angina, CAD, or peripheral artery disease. The study was approved by the local ethics committee, and all of the patients gave written informed consent. Venous samples were collected at the time of surgery, stored in HEPES buffer overnight, and assayed the following day. Blood samples were collected 5 to 10 days before coronary artery bypass graft surgery or within 1 month of varicose vein surgery. Patients fasted and rested supine for 30 minutes before donating blood.

Vascular Reactivity of Veins
Two- to 3-mm rings of vein were studied in organ chambers in a buffer containing indomethacin (0.02 mmol/L) to inhibit prostanoid-mediated effects as described previously.13 Only nonvaricosed portions of vein were studied. Vessels were constricted with phenylephrine (3 μmol/L), and relaxations to calcium ionophore A23187 (0.01 to 10 μmol/L; endothelium dependent vasodilator), sodium nitroprusside (0.001 to 10 μmol/L; endothelium independent vasodilator), apocynin [0.01 to 0.3 mmol/L; reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase inhibitor], and allopurinol (0.01 to 0.3 mmol/L; inhibitor of xanthine oxidase) were examined. From previous experience, we know that the vasodilator effect of apocynin is endothelium mediated, because it can be reversed by the nitric oxide synthase inhibitor Nω-nitro-L-arginine-methyl ester.4 Pilot experiments before the current work have shown that apocynin and allopurinol at doses ≥1 mmol/L exert unspecific effects on vascular tone; we have, therefore, used maximum doses of 0.3 mmol/L. Additional studies have been carried out to investigate the combined effects of allopurinol and apocynin on vasorelaxation. Relaxation was expressed as a percentage of the constriction to phenylephrine.

Superoxide Production
Superoxide levels were measured in blood vessels from a subset of age- and sex-matched CAD and control patients (n=30). Superoxide was measured using lucigenin chemiluminescence as described previously5 and oxidative fluorescent microtopography with hydroethidine. For the latter, frozen sections were prepared and incubated with the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI; 0.5 μg/mL for 2 minutes) followed by hydroethidine (2 μmol/L for 20 minutes). Fluorescence was detected using a Bio-Rad laser scanning confocal microscope with an optical band pass filter of 470 to 520 nm and a band pass filter of 580 to 610 nm. Vessels from CAD and control patients were analyzed in parallel under identical laser settings and fluorescence quantified using techniques similar to those described by Chamseuddine and Miller.11 Changes in superoxide production after 60 minutes of incubation with allopurinol (0.1 mmol/L) and apocynin (0.1 mmol/L) were assessed by lucigenin chemiluminescence in the 15 patients with CAD.

Markers of Oxidative Stress
Total antioxidant capacity was measured in plasma using a commercially available kit (AOP-490; Oxis International Inc) based on the reduction of Cu2+ to Cu+. The reduced/oxidized glutathione (GSH/GSSG) ratio was measured in whole blood using a commercially available kit (GSH/GSSG-412, Oxis International Inc). GSH reacts with Ellman’s reagent to form a spectrophotometrically detectable product at 412 nm. In a separate reaction, GSSG was reduced to GSH, which was then determined in the same manner.

Statistical Analysis
All of the comparisons of markers of oxidative stress and superoxide production between CAD and control patients were made in age- and sex-matched samples. This was done to create similar age/sex profiles for the 2 groups, because CAD patients tended to be older and have a higher male/female ratio than patients undergoing varicose vein surgery. Normal distribution of data was examined by the Kolmogorov-Smirnov test. Where appropriate, unpaired Student t tests were used for the comparisons of relaxation at different concentrations to calcium ionophore, sodium nitroprusside, apocynin, and allopurinol between the 2 groups. The Mann-Whitney U test was used for comparison of non-normally distributed data between the groups.

To investigate which risk factors were independently related to vasorelaxation, multiple linear regression analysis was performed using data from the 188 CAD patients, and estimated effects, 95% CIs, and P values were tabulated. Multiple correlation coefficients were calculated as an overall measure of the relationship between risk factors and vasorelaxation. Data not following normal distributions (total antioxidant capacity and reduced/oxidized glutathione ratio) were log transformed for this analysis. Coefficients and their confidence intervals for these 2 analyses given in Table II (available online at http://www.atvb.ahajournals.org) have been back transformed from the log scale, and they indicate the estimated proportional increase in the response associated with a unit increase in the predictor. Where appropriate, Pearson correlation coefficients and Spearman correlation coefficients are displayed.

All of the analyses were carried out using Minitab version 13.1 (Minitab Inc). Unless otherwise indicated, results are shown as mean±SE, including 95% CIs where appropriate, and P<0.05 was considered significant.

Results
Patient Characteristics
Patient demographics are shown in the Table. The subpopulation selected for comparison with control patients was similar to the whole CAD patient group with the exception of a higher proportion of males. Control patients had significantly lower blood pressure, cholesterol levels, and body mass index than CAD patients, and none were receiving any treatment for cardiovascular disease.

Vascular Reactivity in Age- and Sex-Matched CAD and Control Patients
Endothelium-dependent relaxations to calcium ionophore were attenuated in vessels from CAD compared with control patients across the full concentration response curve (Figure 1) with maximal relaxation (calcium ionophore 10 μmol/L) being 26±2% and 60±1%, respectively, in CAD and control patients (P<0.001; 95% CI, 29% to 39%). In contrast, endothelium-independent relaxation to sodium nitroprusside was not attenuated in the CAD patients; in fact, at the highest dose (10 μmol/L), relaxation was greater in CAD than in control patients. At the highest concentrations examined, both the xanthine oxidase inhibitor, allopurinol (0.3 mmol/L), and the NADPH oxidase inhibitor, apocynin (0.3 mmol/L), caused greater relaxations in vessels from CAD compared with control patients. In the presence of allopurinol, relaxations were 25±2% and 16±1% (P<0.001; 95% CI, −14% to −4%), whereas in the presence of apocynin, relaxations were 23±2% and 10±1% (P<0.001; 95% CI, −18% to −8%), respectively, in CAD and control tissues. In the total group of CAD patients (n=188), there was no correlation...
between maximum relaxation to allopurinol and maximum relaxation to apocynin ($r=0.021; P=0.777$).

### Direct Measurement of Vascular Superoxide
Lucigenin chemiluminescence measurements of superoxide were significantly greater in blood vessels from CAD than from control patients ($0.89\pm0.10$ versus $0.55\pm0.06$ nmol/mg per min; $P=0.008$; 95% CI, 10.1 to 59.5 nmol/mg per min; Figure 2A). Apocynin ($P=0.021$) and allopurinol ($P=0.038$) reduced superoxide production significantly by $0.12\pm0.05$ nmol/mg per min (95% CI, 0.11 to 0.16 nmol/mg per min) and $0.16\pm0.09$ nmol/mg per min (95% CI, 0.14 to 0.25 nmol/mg per min) in patients with

### Figure 1
Vasorelaxation in rings from age- and sex-matched CAD and control patients. Vessels were constricted with phenylephrine (3 μmol/L) and relaxation to (A) calcium ionophore, (B) sodium nitroprusside, (C) allopurinol, and (D) apocynin examined. Results are shown as mean±SE. n=51 for all groups. Differences in relaxation between CAD and control patients were examined by unpaired t tests. *$P<0.01$; **$P<0.001$. Insets in C and D display original tracings showing the additive effect of allopurinol and apocynin induced vasorelaxation. In C, allopurinol (Allo) was given in increasing doses followed by 0.3 mmol/L apocynin (Apo). Reverse order in D. Doses in the insets are given in mmol/L. PE denotes phenylephrine. In these experiments (n=8), maximum relaxation to allopurinol was $47\pm4\%$, to apocynin $36\pm5\%$, and to allopurinol plus apocynin $76\pm8\%$, which did not differ significantly from the theoretical maximum of $83\pm8\%$ ($P=0.448$).
CAD (Figure 2B). The higher levels of superoxide production in patients with CAD were confirmed using hydroethidine (Figure 2D, 2E, 2G, and 2H) and were observed throughout the vessel wall. In contrast to hydroethidine the intensity of DAPI staining was similar in vessel sections from CAD and control patients (Figure 2C and 2F).

Markers of Oxidative Stress
The levels of oxidized glutathione were significantly higher in blood from CAD compared with control patients (18±3 versus 4±1 μmol/L; P<0.001), and the ratio of reduced/oxidized glutathione was lower (129±31 versus 641±115; P<0.001). In contrast, levels of reduced glutathione did not differ between CAD and control patients (1127±81 versus 1133±49 μmol/L; P=0.556). Consistent with these findings, the total antioxidant capacity was significantly reduced in plasma from the same group of CAD patients (1703±57 versus 2012±67 Cu²⁺-reducing equivalents; P<0.001).

Risk Factor Analysis
Multiple regression analysis using risk factors that could be related to endothelial dysfunction and oxidative stress was conducted on the total CAD population studied (188 patients). This larger group of CAD patients showed a similar degree of oxidative stress and endothelial dysfunction to the subgroup matched to control patients (Table I, available online at http://www.atvb.ahajournals.org). Control patients had been selected to have contrasting clinical characteristics to the CAD patients and were, therefore, not included in the regression analysis.

The analysis identified LDL cholesterol as a highly significant predictor of relaxation to calcium ionophore in the CAD patients (β coefficient, −4.7 per mmol/L; P=0.001). There was no evidence that age, sex, body mass index, blood pressure, diabetes status, statin, and angiotensin-converting enzyme inhibitor therapy contributed to endothelium-dependent relaxation (Table II, available online at http://atvb.ahajournals.org). LDL cholesterol level was also a significant determinant of biomarkers of oxidative stress (GSH/GSSG ratio: β coefficient, −53.1 per mmol/L, <0.005; total antioxidant capacity: β coefficient, −12.3 per mmol/L, <0.0005), but, here, other risk factors, including diastolic blood pressure, age, and smoking, also played a role (Table II). Thus, significant negative correlations between LDL cholesterol and markers of endothelial function and oxidative stress were observed (Figure 3). However, the markers of

![Figure 2. Superoxide levels in veins from age- and sex-matched CAD (n=15) and control (n=15) patients. Magnification, ×20. A, Superoxide levels measured by lucigenin chemiluminescence. B, Superoxide levels in patients with CAD at baseline and after incubation (60 minutes) with allopurinol (0.1 mmol/L) and apocynin (0.1 mmol/L). C, DAPI staining in a vein from a CAD patient. D, Hydroethidine fluorescence in the same vein. E, Merged images of C and D. F, DAPI staining in a vein from a control patient. G, Hydroethidine fluorescence in the same vein. H, Merged images of F and G. Bar in C represents 100 μm. E indicates endothelium. Numbers in the right bottom corner of the vessels in C, D, F, and G represent average fluorescence on a scale of 0 to 256 over the area of the vessel whereas the numbers in E and H show the ratio of DAPI/hydroethidine fluorescence. **P<0.01 when superoxide production in vessels from CAD and control patients compared by unpaired t test. *P<0.05 when superoxide production after incubation with allopurinol or apocynin is compared with baseline values.

![Figure 3. Correlation between LDL cholesterol and endothelial function and oxidative stress in CAD patients. Scatter plots of LDL cholesterol concentration and (A) maximum response to calcium ionophore A23187 (CaI; 10 μmol/L), (B) log reduced/oxidized glutathione ratio (GSH/GSSG) and (C) log total antioxidant capacity (TAC). Pearson correlation coefficients (r) and Spearman correlation coefficients (ρ) are shown in the figures.](http://atvb.ahajournals.org/)
oxidative stress did not correlate directly with relaxation to calcium ionophore (log total antioxidant capacity versus calcium ionophore: \( P = 0.072 \); log ratio of reduced to oxidized glutathione versus calcium ionophore: \( P = 0.651 \)).

LDL cholesterol was also a significant predictor of relaxation to the NADPH oxidase inhibitor apocynin (\( \beta \) coefficient, \(-4.42 \) per mmol/L, \( P = 0.019 \); Table II). In contrast to the other phenotypes examined, relaxation to the xanthine oxidase inhibitor allopurinol was not determined by LDL cholesterol. In diabetic subjects, xanthine oxidase inhibition with allopurinol had a greater effect on the percentage of vasorelaxation than in nondiabetic subjects (35\( \pm \)3 versus 21\( \pm \)1%; \( P < 0.001 \); 95% CI, \(-20\%\) to \(-7\%\); Table II).

**Discussion**

We have demonstrated contrasting levels of oxidative stress and endothelial function between patients with severe CAD and age- and sex-matched individuals with no documented cardiovascular disease. This is the first study to assess endothelial function, superoxide production, and oxidative stress, not only in vessels used for revascularization in coronary artery bypass graft surgery, but also in the equivalent vessels from healthy control subjects. Moreover, we demonstrated the predominant role of LDL cholesterol in determining oxidative stress and endothelial dysfunction in CAD patients.

Our studies showed severe depression of relaxation to calcium ionophore in veins from CAD patients despite normal responses to sodium nitroprusside, consistent with a specific defect in endothelium-dependent nitric oxide pathways. Superoxide levels, measured directly within the vessel wall using 2 methods, were elevated in blood vessels from CAD compared with control patients. In addition, in the organ bath studies, inhibition of xanthine oxidase and NADPH oxidase with allopurinol and apocynin, respectively, caused significantly greater relaxation in vessels from CAD compared with control patients. These findings indicate excess superoxide production as an important cause of the attenuation of endothelium-dependent relaxations in CAD patients and suggest that both xanthine oxidase and NADPH oxidase contribute to superoxide production in these patients. Consistent with these observations, increased NADPH oxidase and xanthine oxidase activity has been suggested in patients with CAD, and inhibitors of these enzymes have been shown to inhibit superoxide production in the saphenous vein from CAD patients. Precise quantitative measures of the relative contribution of NAD(P)H oxidase and xanthine oxidase to vascular superoxide production have not been readily obtained in the present experiments. Apocynin acts by blocking the assembly of the NADPH oxidase complex. NOX4 has been shown to be the predominant homologue in vascular smooth muscle from conduit vessels. NOX4 is constitutively active; thus, apocynin may have low potency in the saphenous vein and only inhibit a proportion of NAD(P)H oxidase activity.

The degree of endothelial dysfunction has been reported to be related to the number of risk factors present in CAD patients. However, Huraux et al failed to show any relationship between superoxide levels and endothelial dys-
purinol on endothelial function in patients with diabetes.\textsuperscript{32} Xanthine oxidase inhibition has been suggested to have therapeutic potential in patients with heart failure.\textsuperscript{33}

There has been much interest recently in the inhibition of vascular NADPH oxidase as a means to reduce oxidative stress and progression of cardiovascular disease.\textsuperscript{34,35} Our data indicate that there is a significant relationship between LDL cholesterol and vascular oxidative stress across the entire range of LDL cholesterol concentrations. This provides a mechanistic explanation in support of intensive LDL cholesterol-lowering therapy as suggested by recent clinical trials.

Acknowledgments

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References

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### TABLE I. Comparison of vasorelaxation and oxidative stress markers between total CAD patient group and CAD patient subgroup.

<table>
<thead>
<tr>
<th></th>
<th>Total CAD Group</th>
<th>CAD Subgroup</th>
<th>p</th>
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<tbody>
<tr>
<td>Number of patients</td>
<td>188</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>% Relaxation to calcium ionophore (10 µM)</td>
<td>26.3 ± 13.4</td>
<td>26.2 ± 13.7</td>
<td>0.980</td>
</tr>
<tr>
<td>% Relaxation to sodium nitroprusside (10 µM)</td>
<td>73.5 ± 24.4</td>
<td>76.8 ± 23.2</td>
<td>0.414</td>
</tr>
<tr>
<td>% Relaxation to apocynin (0.3 mM)</td>
<td>23.4 ± 16.4</td>
<td>23.3 ± 16.3</td>
<td>0.978</td>
</tr>
<tr>
<td>% Relaxation to allopurinol (0.3 mM)</td>
<td>24.4 ± 14.6</td>
<td>25.0 ± 12.7</td>
<td>0.816</td>
</tr>
<tr>
<td>TAC (Cu$^{2+}$ reducing equivalents)</td>
<td>1625 (1575;1677)</td>
<td>1662 (1575;1774)</td>
<td>0.521</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>91 (77;108)</td>
<td>83 (63;109)</td>
<td>0.587</td>
</tr>
</tbody>
</table>

TAC, total antioxidant capacity; GSH/GSSG, reduced to oxidised glutathione ratio. Student's t-test was used to compare between the groups. TAC and GSH/GSSG were log transformed before Student's t-test was applied. Note the absence of any significant difference between the groups.

Data following normal distribution are expressed as mean ± standard deviation, otherwise geometric mean (95% CI of geometric mean) is given.
TABLE II. Analysis of the relationship between risk factors and endothelial dysfunction in the presence of NAD(P)H oxidase (apocynin) or xanthine oxidase inhibition (allopurinol) in CAD patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Calcium ionophore (n=134)</th>
<th>Apocynin (n=142)</th>
<th>Allopurinol (n=142)</th>
<th>GSH/GSSG (n= 108)</th>
<th>TAC (n = 142)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>β coef (CI)</td>
<td>p</td>
<td>β coef (CI)</td>
<td>p</td>
</tr>
<tr>
<td>Age (10 years)</td>
<td>0.807</td>
<td>-0.26 (-2.4, 1.9)</td>
<td>0.751</td>
<td>-0.43 (-3.2, 2.3)</td>
<td>0.386</td>
</tr>
<tr>
<td>Sex (0=F, 1=M)</td>
<td>0.876</td>
<td>-0.38 (-5.1, 4.4)</td>
<td>0.461</td>
<td>2.3 (-3.8, 8.4)</td>
<td>0.190</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.427</td>
<td>-0.19 (-0.66, 0.28)</td>
<td>0.234</td>
<td>0.35 (-0.24, 0.94)</td>
<td>0.186</td>
</tr>
<tr>
<td>Smoking (0=no, 1=yes)</td>
<td>0.789</td>
<td>0.68 (-4.3, 5.7)</td>
<td>0.357</td>
<td>-3 (-9.3, 3.3)</td>
<td>0.356</td>
</tr>
<tr>
<td>Diabetic (0=no, 1=yes)</td>
<td>0.436</td>
<td>-2.2 (-7.7, 3.3)</td>
<td>0.377</td>
<td>3.2 (-4.1, 11)</td>
<td>0.000</td>
</tr>
<tr>
<td>DBP (10mmMg)</td>
<td>0.188</td>
<td>-1.3 (-3.3, 0.65)</td>
<td>0.546</td>
<td>-0.77 (-3.3, 1.7)</td>
<td>0.947</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>0.001</td>
<td>-4.7 (-7.6, -1.9)</td>
<td>0.019</td>
<td>4.4 (0.64, 8.2)</td>
<td>0.326</td>
</tr>
<tr>
<td>ACE I (0=no, 1=yes)</td>
<td>0.401</td>
<td>-1.9 (-6.3, 2.5)</td>
<td>0.433</td>
<td>2.2 (-3.3, 7.7)</td>
<td>0.020</td>
</tr>
<tr>
<td>Statin (0=no, 1=yes)</td>
<td>0.937</td>
<td>-0.21 (-5.6, 5.1)</td>
<td>0.321</td>
<td>3.5 (-3.4, 10)</td>
<td>0.374</td>
</tr>
</tbody>
</table>

Analysis of variance p-values for the null hypothesis of no relationship between risk factors and endothelium dependent vasodilatation (calcium ionophore), relaxation in the presence of NAD(P)H oxidase or xanthine oxidase inhibition, and reduced to oxidised glutathione ratio (GSH/GSSG) ratio and total antioxidant capacity (TAC) are displayed. Regression coefficients (β coef) and their 95% Confidence Intervals (CI) for the risk factors are also given. The coefficients of determination ($R^2$) for these analyses are 12.1%, 8.0%, 27.6%, 36.0% and 36.6% respectively.

BMI, Body mass index; DBP, diastolic blood pressure; LDL, LDL-cholesterol; ACE I, angiotensin-converting enzyme inhibitors. Smoking refers to current active smoking.