Abstract—In vitro and animal studies suggest that oxidative stress is associated with endothelial dysfunction. We tested whether local oxidative stress and nitric oxide (NO) bioavailability in the coronary circulation is associated with coronary endothelial dysfunction in humans. Blood samples were obtained simultaneously from the left main coronary artery and the coronary sinus for measurement of F2-isoprostanes, myeloperoxidase, nitrotyrosine, and superoxide dismutase in 20 patients without significant coronary disease. Afterward, coronary blood flow and the vascular response to intracoronary acetylcholine and N\(^\text{G}\)-monomethyl-L-arginine (L-NMMA) were assessed. The gradient of isoprostanes between the arterial levels and coronary sinus correlated with the change in coronary artery diameter in response to acetylcholine \((r = -0.79, P < 0.0001)\). Isoprostanes net production across the left anterior descending artery territory correlated with a decrease in superoxide dismutase activity \((r = 0.66, P = 0.002)\) and decrease in coronary artery diameter in response to L-NMMA \((r = 0.48, P < 0.05)\). Myeloperoxidase and nitrotyrosine gradients were similar in patients with endothelial dysfunction and controls. The effect of L-NMMA was similar in both groups. We conclude that coronary endothelial dysfunction in humans is characterized by local enhancement of oxidative stress without a decrease in basal NO release. This study supports the hypothesis that local oxidative stress has a role in reduction of NO bioavailability in humans with coronary endothelial dysfunction. (Hypertension. 2008;51:127-133.)

Key Words: endothelial function ■ oxidative stress ■ nitric oxide ■ acetylcholine

The endothelium, separating the vascular wall from the blood components, has an essential function in the regulation of vascular tone.\(^1\)\(^-\)\(^3\) Both systemic\(^4\) and coronary\(^5\) endothelial dysfunction have been demonstrated to be independent predictors of cardiovascular events. Endothelial dysfunction is manifested by reduced activity of endothelium dependent vasodilators, in particular nitric oxide (NO), by increased activity of vasoconstrictors, and by altered antiinflammatory and anticoagulant functions of the endothelium.\(^2\)\(^-\)\(^3\) The reduction in NO dependent vasodilatation may be secondary to a decrease in NO production or alternatively increase in the degradation of NO by a mechanism such as oxidative stress.\(^6\)

Enhanced oxidative stress may occur locally in the vessel wall or systemically and represents an imbalance between oxidants and antioxidants.\(^7\) Although both endothelial dysfunction and oxidative stress may serve as markers for atherosclerotic risk, neither is directly correlated with the presence of atherosclerosis.\(^2\) Previously we have shown in animal models of coronary artery disease risk factors and early atherosclerosis that coronary endothelial dysfunction is associated with increase local and systemic oxidative stress and decrease in NO activity.\(^8\)\(^-\)\(^9\) Moreover, this abnormality was restored with antioxidant vitamins that reduced the endogenous oxidative stress.\(^10\) Although cardiovascular risk factors are associated with endothelial dysfunction,\(^11\) the observation that patients with different degrees of endothelial function might have similar traditional risk factors profile\(^2\) supports the concept that this relationship is complex and may be affected by additional mediators, such as oxidative stress.

F\(_{2}\)-isoprostanes are prostaglandin (PG) isomers that are derived from free radical–catalyzed peroxidation of arachidonic acid.\(^12\)\(^,\)\(^13\) As products of lipid peroxidation, they are considered to be reliable markers of enhanced systemic oxidative stress in vivo.\(^11\)\(^-\)\(^15\) Elevated isoprostanes levels are present in various disease states related to atherosclerosis, including cigarette smoking, hypertension, hyperlipidemia, obesity, and diabetes mellitus, as well as in established coronary artery disease and acute coronary syndromes, un-
dorscoring their role as potential markers or participants in atherosclerosis and its complications.16–20

The purpose of the present study was to investigate whether the relationship between local oxidative stress and endothelial dysfunction, as shown in basic science studies and animal models, can be translated into a clinical setting. Our first aim was to assess whether the local decrease in NO bioavailability in patients with coronary endothelial dysfunction is associated with a decrease in basal NO production or increase in NO consumption. Our secondary aim was to explore different mechanisms by which local coronary oxidative stress may cause decrease in NO availability. To address our aims, we assessed: coronary endothelial function, the response to intracoronary administration of N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA), and the net production in the coronary circulation of F\textsubscript{2}-isoprostane 8-iso-PGF\textsubscript{2a}, myeloperoxidase and nitrotyrosine, and superoxide dismutase (SOD) activity, in patients with minimal coronary artery disease.

Methods

Patient Population
The study was approved by the Mayo Clinic Institutional Review Board and informed consent was obtained. The study group consisted of 20 patients that underwent diagnostic coronary angiography and were referred for evaluation of coronary artery disease because of chest pain. Exclusion criteria included: significant coronary artery stenosis (>30%),
21 ejection fraction <45%, unstable angina, previous myocardial infarction, use of radiographic contrast agents within 12 hours, significant systemic disease, and pregnancy. Medications that may affect cardiovascular hemodynamics were discontinued for at least 48 hours before the study.

Study Protocol
A 6- or 7-Fr guiding catheter was placed into the left main coronary artery, and a 5-F multipurpose or amplatz left catheter was placed in the coronary sinus.1

Blood samples for PG\textsubscript{F\textsubscript{2a}} isoprostanes, SOD, myeloperoxidase, and nitrotyrosine were obtained simultaneously from the coronary sinus and left main coronary artery before endothelial function assessment and were stored at –80°C until assay. After obtaining blood samples, 5000 U of heparin were given systemically. Coronary blood flow, coronary flow reserve (CFR), and the response to acetylcholine and nitroglycerine were assessed as previously described using a Doppler guide wire (Flowire, SciMed Life System) in the midportion of the left anterior descending coronary artery.21,22

Then, L-NMMA, a specific inhibitor of nitric oxide synthesis was infused intracoronary, at a rate of 32 μmol/min for 5 minutes and then at 64 μmol/min for another 5 minutes, to assess tonic basal release of NO from the coronary arteries.23 Endothelium-dependent coronary flow response was calculated as the percent change in coronary blood flow (CBF) in response to acetylcholine. According to our previous studies, we defined microvascular endothelial dysfunction as ≤50% increase in CBF in response to the maximal dose of acetylcholine compared with baseline CBF. A decrease in diameter >20% in response to the maximum dose of acetylcholine is considered abnormal epicardial endothelial function.21,22 Endothelial dysfunction was defined as being either microvascular or epicardial,21 NO dependent coronary artery diameter (CAD) and CBF response were calculated as the percent change in CAD and CBF in response to L-NMMA. Coronary vascular resistance (CVR) was estimated as mean arterial blood pressure/CFB.24

Isoprostanes levels were measured with an enzyme immunoassay kit (Cayman) for 8-iso-PGF\textsubscript{2a}.25 Superoxide dismutase activity was measured using the Cayman Chemical SOD Assay Kit (Cayman Chemical Inc.).26 Plasma myeloperoxidase was measured by a 2-site “sandwich” enzyme-linked immunosorbent assay (Immunodiagnostik Bensheim).27 Plasma nitrotyrosine was analyzed using the Nitrotyrosine ELISA Test Kit (Cell Sciences).

The gradient of each of the above biological substances was calculated as coronary sinus concentration (or activity)–aortic concentration (or activity). We defined net production of each substance in the left anterior descending artery territory as the gradient×CBF.

Data Analysis
Continuous variables are presented as median and interquartile range (IQR) and dichotomous variables as numbers and percentages. All comparisons were analyzed by nonparametric methods. The baseline characteristics of groups were compared by use of 2-sided Wilcoxon signed rank test for continuous variables and by the Pearson chi-square statistic for categorical variables. The level selected for statistical significance was set at probability value <0.05. Pearson correlation coefficient and the nonparametric Spearman correlation method were used for correlation analysis.

Results
The clinical characteristics of the patient population are outlined in Table 1. Patients were divided into 2 groups according to their endothelial function as determined by their response to intracoronary acetylcholine: controls (normal endothelial function, n=12) and endothelial dysfunction (n=8). There were no significant differences in the clinical characteristics or the degree of coronary artery disease between the 2 groups, although there was a trend toward higher prevalence of risk factors and higher Framingham risk score in patients with endothelial dysfunction.

Hemodynamic data are presented in Table 2. Baseline CBF and CFR to adenosine were similar in both groups (Table 2). Abnormal endothelial function was observed in both the epicardial and microvascular circulation. There was a good correlation between the degree of CBF response and CAD changes during acetylcholine infusion: r\textsubscript{e}=0.62, P=0.003.
Table 2. Hemodynamic Data

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normal Endothelial Function (n=12)</th>
<th>Abnormal Endothelial Function (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean blood pressure, mm Hg</td>
<td>94 (88, 109)</td>
<td>98 (95, 102)</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>65 (58, 84)</td>
<td>66 (59, 79)</td>
</tr>
<tr>
<td>Baseline CBF, mL/min</td>
<td>34 (25, 45)</td>
<td>53 (30, 60)</td>
</tr>
<tr>
<td>CFR to adenosine</td>
<td>2.7 (2.35, 3.2)</td>
<td>2.8 (1.95, 3.1)</td>
</tr>
<tr>
<td>Baseline CVR, mm Hg mL⁻¹</td>
<td>2.7 (2.4, 3.7)</td>
<td>1.9 (1.7, 2.8)</td>
</tr>
<tr>
<td>Δ % CBF to Ach [10⁻⁴]</td>
<td>-8 (-19, 2)</td>
<td>-1 (-22, 25)</td>
</tr>
<tr>
<td>Δ % CAD to Ach [10⁻⁴]</td>
<td>-2.5 (-10, 0)</td>
<td>-6 (-8, 3)</td>
</tr>
<tr>
<td>Δ % CBF to Ach [10⁻⁵]</td>
<td>-3 (-8, 65)</td>
<td>20 (-14, 68)</td>
</tr>
<tr>
<td>Δ % CAD to Ach [10⁻⁵]</td>
<td>-9 (-13, 5)</td>
<td>-11 (-29, -2)</td>
</tr>
<tr>
<td>Δ % CBF to Ach [10⁻⁴]</td>
<td>73 (62, 161)</td>
<td>2 (-28, 36)*</td>
</tr>
<tr>
<td>Δ % CAD to Ach [10⁻⁴]</td>
<td>0 (-12, 7)</td>
<td>-24 (-40, -18)*</td>
</tr>
<tr>
<td>Δ % CAD to nitroglycerine</td>
<td>7 (1, 23)</td>
<td>14 (5, 34)</td>
</tr>
<tr>
<td>Δ % CBF to L-NMMA (32 μmol/min)</td>
<td>-23 (-33, 7)</td>
<td>-14 (-29, -7)</td>
</tr>
<tr>
<td>Δ % CAD to L-NMMA (32 μmol/min)</td>
<td>0 (-20, 5)</td>
<td>-15 (-24, 6)</td>
</tr>
<tr>
<td>Δ % CBF to L-NMMA (64 μmol/min)</td>
<td>-24 (-35, 3)</td>
<td>-37 (-42, 4)</td>
</tr>
<tr>
<td>Δ % CAD to L-NMMA (64 μmol/min)</td>
<td>-5 (-20, 0)</td>
<td>-17 (-25, -12)</td>
</tr>
<tr>
<td>Δ % CVR to L-NMMA (64 μmol/min)</td>
<td>39 (22, 74)</td>
<td>60 (23, 91)</td>
</tr>
</tbody>
</table>

CBF indicates coronary blood flow; CFR, coronary flow reserve; CVR, coronary vascular resistance; CAD, coronary artery diameter; Ach, acetylcholine. *P<0.05.

Relation Between Isoprostanes and Endothelial Dysfunction

Isoprostanes concentrations in the coronary sinus were significantly higher in patients with endothelial dysfunction 212 (IQR: 178, 315) pmol/L versus controls; 135 (IQR: 117, 180) pmol/L, P=0.01. In comparison to the coronary artery, isoprostane levels in the coronary sinus increased by 29% (IQR: 13, 59) in patients with endothelial dysfunction and decreased by 28% (IQR: 11, 48) in controls, P<0.01 for both. Both the gradients across the coronary circulation and isoprostanes net production were significantly higher in patients with endothelial dysfunction (Figure 1). There was a significant inverse correlation between CAD change in response to acetylcholine and isoprostanes gradients (r=-0.79, P<0.0001; r_s=-0.73, P=0.0002) and net production (r=-0.79, P<0.0001; r_s=-0.8, P<0.0001; Figure 2); and a weaker correlation between acetylcholine induced changes in CBF and isoprostanes gradient (r=-0.37, P=0.1; r_s=-0.5, P=0.025) or net production (r=-0.37, P=0.11; r_s=-0.57, P=0.008). Because statin therapy may potentially affect local oxidative stress, we also analyzed the results according to the status of this therapy. Patients on statins did not have significant differences in isoprostanes levels compared with patients not receiving statins (P=0.6), and had similar relationship between endothelial dysfunction and isoprostanes levels (Figure 2). Isoprostanes net production was nonsignificantly higher in patients with hypertension 1.13 (–1.4; 2.14) pmol/min compared with patients without hypertension [–1.56 (–2.7; 0.69) pmol/min, P=0.06].

Relation Between Superoxide Dismutase and Endothelial Dysfunction

SOD activity levels in the arterial circulation were 1.7 (IQR: 1.13, 1.78) versus 1.17 (IQR: 1.02, 1.35) U/mL, P=0.2 in patients with endothelial dysfunction and controls, respectively. Coronary sinus SOD activity in patients with endothelial dysfunction decreased by 34% (IQR: 13, 40%), P<0.01 compared with arterial activity and increased nonsignificantly by 33% (IQR: –17, 64%) in controls, resulting in a significant difference in the relative changes between patients with endothelial dysfunction and controls, P=0.005. Relative changes in SOD activity in the coronary circulation were significantly correlated with changes in both coronary artery diameter (r=0.69, P<0.001; r_s=0.68, P=0.001) and CBF (r=0.65, P=0.002; r_s=0.65, P=0.004) in response to acetylcholine. There was a significant inverse correlation (r=-0.66, P=0.002; r_s=-0.7, P=0.0005) between the calculated total SOD activity in the left anterior descending artery territory (SOD gradient×CBF) and isoprostanes net production (Figure 3A). The status of statin therapy had no effect on this correlation.
Effect of L-NMMA on Resting Coronary Vascular Tone

There was a 2% increase in mean arterial pressure with no change in heart rate during L-NMMA infusion. The effect of L-NMMA on CAD, CBF, and CVR (Table 2) indicate similar blockade of tonic basal release of NO from the coronary epicardial arteries in both groups. Isoprostanes net production was inversely correlated with CAD change in response to L-NMMA \((r=-0.51, P=0.03; r=-0.48, P<0.05, \text{Figure 3B})\). There was a positive correlation between the CAD response and CBF response to L-NMMA \((r=0.68, P=0.002)\).

Association of Endothelial Dysfunction With Myeloperoxidase and Nitrotyrosine

Systemic arterial myeloperoxidase levels appeared to be higher in patients with endothelial dysfunction compared with controls 131 ng/mL (60, 186) versus 81 ng/mL (26, 117), but these differences did not reach statistical significance levels. Myeloperoxidase levels in the coronary circulation were similar in both groups without significant difference in gradient or net production (Figure 3C). Arterial nitrotyrosine levels were also similar in patients with endothelial dysfunction and controls: 107 nmol/L (74, 123) versus 108 (55, 118), respectively, without significant difference in gradient between groups (Figure 3D).

Discussion

The current study demonstrates that coronary endothelial dysfunction in humans is associated with preserved basal NO production, enhanced local release of isoprostanes, and a decrease in SOD activity in the coronary circulation. These results emphasize the potential role of endogenous oxidative stress in modulating coronary endothelial function in humans.

Basal NO Release in Endothelial Dysfunction

The similar response to L-NMMA in both groups supports previous findings that imply that decreased basal NO activity is not the main mechanism of endothelial dysfunction.\(^{28}\) Several lines of evidence from in vitro and human studies support these findings. In vitro data suggest decreased bioavailability of NO in spite of upregulation of eNOS.\(^ {29} \) Furthermore, in patients with systemic vasculitis and endothelial dysfunction there is similar forearm blood flow response to nitroprusside and L-NMMA despite different response to acetylcholine.\(^ {30} \)

Effect of Oxidative Stress on Endothelial Dysfunction

The correlation between isoprostanes production and coronary artery diameter change in response to both acetylcholine and L-NMMA suggests that inactivation of NO by oxidative stress may be an important mechanism for endothelial dysfunction.\(^ {28} \) Previously, we demonstrated in animal studies the association between oxidative stress and endothelial dysfunction.\(^ {9,31} \) Experimental diet-induced hypercholesterolemia in pigs resulted in blunted renal endothelium-dependent responses to acetylcholine which were restored by both acute and chronic antioxidant interventions.\(^ {9,31} \) In the coronary circulation in hypercholesterolemic pigs, endogenous NO bioavailability was decreased and chronic administration of antioxidants also preserved coronary endothelial function.\(^ {32} \) The current study is in accord with these previous observations by demonstrating a concurrent decrease in the antioxi-
dant defense mechanism, SOD, and increase in the endogenous oxidative stress.

Interaction Between Oxidative Stress, SOD, and Endothelial Dysfunction

One may expect induction of SOD in response to oxidative stress. Interestingly, all major antioxidant enzymes exhibit temporal variation with the progression of atherosclerosis and initially show a significant upregulation at the early stage of atherosclerosis, before lesion formation, but are markedly downregulated thereafter.33 Thus, antioxidant defenses seem to weaken significantly once atherosclerosis becomes more established, which may accelerate atherogenesis.34 The mechanism by which antioxidant enzymes are downregulated is not fully understood but may involve a feed-forward mechanism of oxidative stress, as loss of nitric oxide may lead to a decline in SOD expression.35 In turn, the decreased SOD activity may be a contributor to endothelial dysfunction and subsequently atherosclerosis. Even though we measured SOD activity, this may reflect changes in protein levels. Indeed, enzymatic activity is more likely to change rapidly than protein levels. On the other hand, extracellular SOD is made predominantly by vascular smooth muscle, but binds to the heparan sulfates on the endothelial cell surface and can be internalized by adjacent endothelial cells. Speculatively, endothelial cells deficient in extracellular SOD might take up circulating extracellular SOD and thereby decrease its levels in the coronary sinus. Although we assume that the plasma SOD is an indicator of vessel wall SOD, such data are not available in humans. Therefore, we cannot rule out the possibility that the plasma SOD that we measured does not fully reflect the expression of SOD in the vascular wall.

Pathways for NO Consumption

One of the pathways for NO consumption in tissues is its reaction with superoxide to yield peroxynitrite. We assessed this pathway by measurement of nitrotyrosine levels, an indicator of peroxynitrite formation. However, we did not find an association between nitrotyrosine and isoprostanes production, or a correlation between nitrotyrosine formation and the degree of endothelial dysfunction, indicating that alternative pathways may be more important for depletion of NO in patients with endothelial dysfunction.

Alternative pathway for depletion of NO may involve myeloperoxidase,36 which modulates endothelial-mediated vasomotor function by direct interaction with NO.37 In the present study we did not find a correlation between myeloperoxidase net production in the coronary circulation and endothelial function, which may imply that myeloperoxidase have a more important role in acute coronary syndrome.38

Role of Isoprostanes in Coronary Endothelial Dysfunction

Potential mechanism for the coronary endothelial dysfunction observed in this study may be a direct vasoconstrictive effect of isoprostanes. Isoprostanes have been shown to enhance vasoconstriction in hypercholesterolemia both in vitro25 and in vivo.15 The potential role of isoprostanes as participants in atherosclerosis is further supported by their presence in human atherosclerotic coronary lesions.39 The precise mechanism that could account for their decreased levels across the coronary circulation in patients with normal endothelial function remains to be determined. This finding is in accord with our recent observation of net extraction of lipoprotein associated phospholipase A2 and lysophosphatidylcholine in patients without atherosclerosis.40 We speculate that one of the functions of the normal vessel wall and normal endothelium is extraction or breakdown of inflammatory mediators and reactive oxidative species. Isoprostanes are excreted into the urine and therefore the gradient across the coronary circulation can be maintained.41

The measurement of F2-isoprostanes is considered to be a reliable method to assess lipid peroxidation and oxidative stress in vivo in humans.42 Both systemic and urinary isoprostane levels may represent total lipid peroxidation but not necessarily local isoprostanes production, because of the large variability between peripheral and local levels of isoprostanes in different organs.43 The current study extends these previous observations and suggests that measurement of F2-isoprostanes may serve as a marker for regional endothelial dysfunction in humans. Interestingly, we found a strong correlation between the changes in CAD in response to acetylcholine and the gradients of isoprostanes and a weaker correlation between changes in CBF and isoprostanes gradients. This observation may suggest that isoprostanes may play a more important role in epicardial than in microcircularly endothelial dysfunction.

Hence, the current study demonstrates for the first time the association between the endogenous oxidative stress and coronary endothelial dysfunction in humans. This study underscores the significance of local imbalance in oxidative stress in association with regional endothelial dysfunction.

Limitations and Clinical Perspectives

Although there were no significant differences in baseline characteristics between the groups, we cannot exclude the presence of confounding factors. A potential limitation is the use of ELISA for isoprostanes analysis. However, ELISA measurements have been shown to correlate well with gas chromatography/mass spectrometry and may bias the results toward the null hypothesis. Thus, a better assay is likely to show a stronger association. Furthermore, the calculations of gradients and net production are based on simultaneous measurements of arterial and coronary sinus samples, and this is likely to reduce the noise. The measured SOD activity may represent different forms of SOD. It is likely to reflect extracellular SOD, although some degree of spillover from red blood cells may occur.

In the current study we observed increased levels of isoprostanes in the coronary sinus but not in the arterial circulation in patients with endothelial dysfunction. This may represent a more regional contribution and activation of the oxidative stress pathway. Local oxidative stress in early stages of coronary atherosclerosis may be detected earlier than systemic oxidative stress. Although clinical trials have failed to demonstrate a beneficial effect of antioxidant supplements such as vitamin C on cardiovascular morbidity and mortality, the level of oxidative stress was not measured in...
those studies. It may be postulated that antioxidant therapy may be directed at the reduction of oxidative stress in patients with evidence of increased local coronary oxidative stress. This hypothesis is underscored by recent data from our laboratory which demonstrated that the chronic administration of antioxidants to normal pigs resulted in enhanced oxidative stress and endothelial dysfunction. It should be noted that measurement of local coronary oxidative stress is an invasive research tool and unlikely to be used clinically, but systemic levels can be used. The effect of antioxidants and statins on local coronary oxidative stress should be explored in future studies.

**Perspectives**

Patients with atherosclerosis often have evidence of both endothelial dysfunction and oxidative stress. However, the precise mechanism for an association between the two in humans is unknown. The present study demonstrates for the first time that endothelial dysfunction in humans with early coronary atherosclerosis is characterized by local coronary production of isoprostanes without reduction of the basal NO release. This study supports the role of local oxidative stress in coronary endothelial dysfunction in humans.

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**Disclosures**

None.

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