Coronary Artery Superoxide Production and Nox Isoform Expression in Human Coronary Artery Disease

Tomasz J. Guzik, Jerzy Sadowski, Bartłomiej Guzik, Andrew Jopek, Boguslaw Kapelak, Piotr Przybyłowski, Karol Wierzbicki, Ryszard Korbut, David G. Harrison, Keith M. Channon

Background—Oxidative stress plays important role in the pathogenesis of atherosclerosis and coronary artery disease (CAD). We aimed to determine the sources and selected molecular mechanisms of oxidative stress in CAD.

Methods and Results—We examined basal and NAD(P)H oxidase-mediated superoxide (O$_2^-$) production using lucigenin chemiluminescence, ferricytochrome c and dihydroethidium fluorescence in human coronary arteries from 19 CAD and 17 non-CAD patients undergoing heart transplantation. NAD(P)H oxidase subunits and xanthine oxidase expression were measured. Superoxide production was greater in coronary arteries from patients with CAD, even in vessels without overt atherosclerotic plaques, and was doubled within branching points of coronary arteries. Studies using pharmacological inhibitors and specific substrates showed that NAD(P)H oxidases (60%) and xanthine oxidase (25%) are primary sources of O$_2^-$ in CAD. Losartan significantly inhibited superoxide production in coronary arteries. NAD(P)H oxidase activity and protein levels of the NAPDH oxidase subunits p22phox, p67phox, and p47phox were significantly increased in CAD, as were mRNA levels for p22phox and nox2, and no NAD(P)H oxidase subunit mRNA levels correlated with NAD(P)H oxidase activity in vessels from individual patients. Activity and protein expression of xanthine oxidase were increased in CAD, whereas xanthine dehydrogenase levels were not changed.

Conclusions—Increased expression and activity of NAD(P)H oxidase subunits and xanthine oxidase, in part mediated through angiotensin II and PKC-dependent pathways, are important mechanisms underlying increased oxidative stress in human coronary artery disease.

Key Words: endothelium ■ NAD(P)H oxidase ■ nitric oxide ■ oxidant stress ■ reactive oxygen species

Increased vascular production of reactive oxygen species is a characteristic feature of vascular disease states, including coronary artery disease (CAD). In particular, superoxide (O$_2^-$) and products of O$_2^-$ promote atherosclerosis by quenching nitric oxide (NO) and activating redox-sensitive signaling pathways that modulate vessel remodeling and plaque stability. Accordingly, endothelial dysfunction associated with overproduction of O$_2^-$ has been shown to provide prognostic information in patients with coronary artery disease. Potential sources of vascular O$_2^-$ include the NAD(P)H oxidases, xanthine oxidase, cyclooxygenases, nitric oxide synthases, or mitochondrial oxidases. Recent studies have shown that the NAD(P)H oxidases and xanthine oxidase have important roles in human vessels. Azumi et al first showed that NAD(P)H oxidase is present in human coronary arteries. Several molecular homologues of the NAD(P)H oxidase large membrane subunit, (gp91phox; nox2) are present in vascular cells, and could contribute to ROS production during development of CAD. More recent studies have shown that NAD(P)H oxidase subunit expression is correlated with both severity of atherosclerosis and with features of plaque stability in human coronary arteries. Despite these important findings, a systematic analysis of the sources of O$_2^-$ production in human coronary arteries is lacking. Furthermore, it is not clear how coronary O$_2^-$ production is regulated in the presence of CAD and how it is affected by traditional risk factors for CAD.

Accordingly, we aimed to determine the sources and molecular mechanisms of O$_2^-$ production in freshly isolated human coronary arteries from patients with and without CAD. We sought to evaluate how the variation in superoxide production is related to the molecular composition of coronary artery NAD(P)H oxidases and the presence of CAD.

Materials and Methods

Patients and Blood Vessels

Segments of human coronary arteries were obtained from explanted hearts of 36 patients undergoing heart transplantation; 19 patients presented with coronary artery disease (CAD) and 17 patients were without CAD (dilated [n=14]) or hypertrophic cardiomyopathy.
CAD diagnosis was based on a history of myocardial infarction and on coronary angiography. Collection of tissues was approved by the Local Research Ethics Committee and informed consent was obtained. Immediately after harvesting, hearts were placed in ice-cold Krebs buffer. Segments of left anterior descending artery were isolated from all patients and divided into segments for cryosections; RNA extraction (in 28 patients; 15 CAD and 13 non-CAD) and protein homogenates. One segment was placed in ice cold Krebs-Henseleit buffer and transported to the laboratory for intact ring studies. In a subset of patients without CAD, vascular rings from different locations of the coronary tree were obtained, including continuous segments and bifurcations.

**Vascular Superoxide Production**
Superoxide production was measured from intact vessel rings and vascular homogenates using lucigenin-enhanced chemiluminescence (5 μmol/L), and ferricytochrome c reduction (80 μmol/L), using previously described and validated methods. The maximal activity of NAD(P)H oxidase was measured by adding NADPH (100 μmol/L) to vascular homogenates, and O$_2^-$ was detected using 5 μmol/L lucigenin.

**Oxidative Fluorescent Microtopography**
In situ superoxide generation was visualized in vascular cryosections (30 μm) with the SOD-inhibitable dihydroethidium (DHE) (2 mmol/L) fluorescence, as described previously. Images were obtained with a BioRad MRC 1024 scanning confocal microscope using the excitation/emission wavelengths 488 nm/610 nm. In each case, paired segments of CAD and non-CAD vessels that had been collected and stored under identical conditions were analyzed in parallel with identical imaging parameters.

**Western Immunoblotting**
Portions of vascular homogenates were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. NAD(P)H oxidase components were detected using mouse monoclonal antibodies against p67phox or p47phox, (BD Phamrines) or by rabbit polyclonal antibodies against p22phox (kindly provided by Dr Imajoh-Ohmi, Japan). Xanthine dehydrogenase (150 kDa)/Xanthine oxidase (130 kDa) was detected using rabbit polyclonal antibody (kindly provided by Dr Hoidal, Utah), as described. Bands were detected by horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies (Promega, UK), visualized using a chemiluminesence detection system (Supersignal; Pierce) and analyzed using National Institutes of Health Image software.

**Reverse-Transcription Polymerase Chain Reaction and Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction**
RNA was isolated from snap-frozen coronary artery segments using TRI-reagent and re-purified using RNA easy kit with DNase digestion. The cDNA synthesized using ImProm RT System (Promega) from 20 ng total RNA was subjected to quantitative polymerase chain reaction (PCR) using SYBR Green PCR Quantitiec (Qiagen) and Rotorgene 3000 fluorescent real-time PCR system (Corbett Research, Australia) as described before, and detailed in an online supplement (see http://atvb.ahajournals.org). The housekeeping gene MLN-51 was used.

**Statistical Analysis**
All data are expressed as means±SEM with n equal to the number of patients. Comparisons between groups of patients or treatments were made using Student t test or 1-way ANOVA, followed by the Student-Newman-Keuls post-hoc test. Correlation between oxidation activity and nox expression was assessed by simple linear regression. Values of P<0.05 were considered statistically significant.

**TABLE 1. Clinical Characteristics of Patients From Whom Coronary Artery Specimens Were Obtained**

<table>
<thead>
<tr>
<th></th>
<th>No CAD</th>
<th>CAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>Age, mean±SEM</td>
<td>48.9±3.7</td>
<td>51.4±2.0</td>
</tr>
<tr>
<td>Sex, M:F</td>
<td>13:4</td>
<td>18:1</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>4 (23%)</td>
<td>6 (32%)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>2 (11%)</td>
<td>16 (85%)*</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>1 (6%)</td>
<td>3 (16%)</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>4 (24%)</td>
<td>11 (58%)*</td>
</tr>
<tr>
<td>BMI</td>
<td>23.0±0.8</td>
<td>26.9±0.6*</td>
</tr>
<tr>
<td>Cholesterol, mmol/L(mean±SEM)</td>
<td>5.1±0.5</td>
<td>5.3±0.4</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L(mean±SEM)</td>
<td>5.0±0.3</td>
<td>5.6±0.2*</td>
</tr>
<tr>
<td>MI/CABG, n (%)</td>
<td>0 (0%)</td>
<td>12 (63%)*</td>
</tr>
<tr>
<td>PAD,TIA, n (%)</td>
<td>0 (0%)</td>
<td>16 (85%)*</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>17.8±6.7</td>
<td>18.0±1.6</td>
</tr>
</tbody>
</table>

**Results**

**Patient Characteristics**
Fresh coronary artery specimens were obtained from 36 patients undergoing heart transplantation. As expected demographic and clinical characteristics revealed that there were significant differences in vascular disease profile between the CAD and non-CAD patients (Table 1). As expected, the presence of CAD was associated with greater incidences of previous myocardial infarction, transient ischemic attack, peripheral arterial disease, and typical CAD risk factors. Matching degree of heart failure was present in both CAD and non-CAD patients.

**Vascular Superoxide Production in CAD**
Basal O$_2^-$ production from freshly-isolated human coronary arteries, determined by lucigenin-enhanced chemiluminescence, was greater in patients with CAD (Figure 1A). We have also measured O$_2^-$ production from segments of coronary arteries obtained from autopsy of non-CAD subjects without heart failure (n=8). We found that O$_2^-$ production was lower in those specimens than in non CAD subjects with heart failure although this difference did not reach statistical significance (data not shown). This observation was confirmed by in situ dihydroethidium staining, which showed that O$_2^-$ production was increased in all vascular layers of arteries from CAD patients, even in vessels without overt histological features of atherosclerotic plaques (Figure 1B).
Spatial Differences in Superoxide Production in Human Coronary Tree

We compared $\mathrm{O}_2^-$ production in a bifurcation segment of the left anterior descending artery (incorporating the origin of the principal diagonal branch) with a segment of left anterior descending artery taken immediately before the bifurcation. Basal $\mathrm{O}_2^-$ production measured by lucigenin (5 $\mu$M) enhanced chemiluminescence was doubled in bifurcation segments when compared with pre-bifurcation segments (20.6 ± 3.2 versus 11.5 ± 2.8; $P < 0.001$; $n = 5$). Similarly, maximally-stimulated NAD(P)H oxidase activity measured at these sites was also significantly increased within the bifurcation segment (2418 ± 359 versus 1422 ± 415 RLU/sec per mg; $P = 0.003$).

Sources of Superoxide Production in Human Coronary Arteries

We investigated the enzymatic sources of $\mathrm{O}_2^-$ production in intact segments of human coronary arteries using a range of oxidase inhibitors (Figure 2A). The greatest inhibition of $\mathrm{O}_2^-$ production in coronary arteries from both CAD and non-CAD patients was caused by NAD(P)H oxidase inhibitors (diphenylethionium and apocynin). Oxyxpurinol, a xanthine oxidase inhibitor, decreased $\mathrm{O}_2^-$ production by $\approx 25\%$, but only in vessels from CAD patients. Importantly, both apocynin and oxyxpurinol abolished the difference between CAD and non-CAD vessels. An additional 15% inhibition of $\mathrm{O}_2^-$ production in CAD coronary arteries was observed in response to indomethacin, although this effect was of only borderline significance.

Corresponding changes in $\mathrm{O}_2^-$ production were observed when oxidase substrates were added to vascular homogenates using ferricytochrome c reduction assays (Figure 2). NADPH-stimulated $\mathrm{O}_2^-$ production was doubled in CAD vessels and xanthine oxidase activity was also significantly higher in vessels from CAD patients.

To further characterize NAD(P)H oxidase regulation in human coronary arteries, we studied the roles of angiotensin II and protein kinase C (PKC) in modulating vascular $\mathrm{O}_2^-$ production. Inhibition of PKC by chelerythrine significantly decreased both basal $\mathrm{O}_2^-$ production and NADPH-stimulated oxidase activity in human coronary arteries (Table 2A). Angiotensin II increased both basal $\mathrm{O}_2^-$ production and NADPH-stimulated oxidase activity. This effect was inhibited by the AT1 receptor antagonist losartan (Table 2B). Interestingly, losartan inhibited NADPH oxidase activity in the basal state, not only in the presence of angiotensin II (Table 2B).

Taken together, these findings suggest that NAD(P)H oxidase(s) are the major source of superoxide in human
Xanthine Oxidase in Human Coronary Arteries

In agreement with previous data our results, suggest that increased xanthine oxidase activity contributes in part to vascular O$_2^-$ production in CAD. We therefore examined xanthine oxidoreductase (XOR) protein expression in vascular homogenates. XOR is initially synthesized as the 150 kDa xanthine dehydrogenase (XDH), and is proteolytically cleaved to xanthine oxidase (XO). XDH uses NAD as a substrate to form superoxide. We found that although XOR is initially synthesized as the 150 kDa and nox2 mRNA levels were statistically significant. Nox1 mRNA was detectable at very low levels in only 7 of 28 samples.

Macrophage colony stimulating factor receptor (MfCSFR) expression, as a measure of inflammatory cell content, was significantly higher in CAD vessels than non-CAD vessels (0.37±0.04 vs 0.98±0.1; P<0.001), whereas there was no significant difference in α-smooth muscle actin expression.

NAD(P)H Oxidase Subunits in Human Coronary Arteries

Because NAD(P)H oxidases appear to play the principal role in superoxide production by human coronary arteries, we next sought to evaluate the NAD(P)H oxidase protein subunits in vessel extracts. Western blotting revealed both membrane (p22phox) and cytoplasmic (p67phox and p47phox) subunits in coronary artery protein homogenates. In keeping with measurements of NAD(P)H oxidase activity, p22$^{phox}$ protein was more abundant in coronary arteries from CAD patients (Figure 3A). Protein levels of the cytoplasmic subunits were also significantly increased between CAD and non-CAD patients (Figure 3A). We also analyzed the mRNA expression of the NAD(P)H oxidase membrane subunits (nox1, nox2, nox4 and p22phox) using quantitative real-time fluorescent RT-PCR. We found that p22$^{phox}$ and nox2 mRNA levels were greatly increased in CAD (Figure 4). Nox4 levels tended to be higher in CAD, although this difference did not reach statistical significance. Nox1 mRNA was detectable at very low levels in only 7 of 28 samples.
(3.5±0.41 versus 4.4±0.54; P=0.3). Nox4 expression correlated with α-smooth muscle actin mRNA levels (data not shown; R=0.5; P<0.007), whereas nox2 expression was very highly correlated to McfSFR mRNA levels (data not shown; R=0.8; P<0.0001).

To determine how molecular regulation of NAD(P)H oxidases is related to vascular superoxide production, we quantified the relationships between NAD(P)H oxidase subunit mRNA levels and NADPH-stimulated oxidase activity measured from individual coronary arteries. Nox4 mRNA levels were most significantly correlated with NADPH oxidase activity measured from homogenates (Figure 5A), and this relationship was even more pronounced when nox4 mRNA was expressed per α-smooth muscle actin mRNA levels. Interestingly, p22phox and nox2 mRNA levels did not correlate with oxidase activity in the whole group. However, we observed that McfSFR mRNA levels in coronary arteries varied in a bimodal distribution. Of 28 vessel segments, 6 contained strikingly higher McfSFR mRNA than the remaining vessels, suggesting that these vessels had a much higher inflammatory cell content because of advanced CAD (Figure 5B). In the 22 vessels with lower inflammatory cell content, expression of p22phox and nox2 mRNA were significantly correlated with oxidase activity (Figure 5C). In contrast, oxidase activity in the vessels with advanced CAD lesions and very high McfSFR expression was not related to p22phox or nox2 expression, (Figure 5C) but was instead correlated with McfSFR expression (data not shown; R=0.5; P=0.02).

Discussion
In this study we show that human coronary artery disease is associated with increased coronary artery $O_2^{-}$ production. Studies using pharmacological inhibitors and substrate stimulation indicated that the NAD(P)H oxidases are the major sources of superoxide production, and that xanthine oxidase also contributes modestly to $O_2^{-}$ production in CAD. We also found that the NAD(P)H oxidase is activated by angiotensin II in vessels with CAD and that this enzyme is regulated by protein kinase C. CAD is associated with increased NAD(P)H oxidase subunit expression, mainly p22phox and nox2, related in part to higher monocyte/macrophage infiltration. However, the level of nox4 expression is most strongly correlated with increased NAD(P)H oxidase activity in human coronary arteries, independent of the presence of CAD.

Importantly, our study shows that net basal $O_2^{-}$ production is greater in human coronary arteries in the setting of CAD and associated risk factors. Although increased vascular oxidative stress is a feature of several vascular diseases, no previous study has quantified $O_2^{-}$ production from intact, freshly isolated human coronary arteries. Studies of histological sections and frozen tissue homogenates have shown that $O_2^{-}$ production is increased in the shoulder region of atherosclerotic plaques and in patients with unstable angina. We have extended these observations by showing that $O_2^{-}$ production is increased in segments of coronary arteries from CAD patients even when morphological features of atherosclerosis are absent. This indicates that CAD is associated with generalized vascular oxidative stress in the coronary circulation, not limited to the sites of overt atherosclerosis. In line with these observations are our previous findings that superoxide production and endothelial dysfunction are also present in the peripheral systemic circulation in atherosclerotic individuals and actually precedes the development of overt lesion formation in experimental animals with either hypertension or hypercholesterolemia.
Despite the systemic nature of oxidative stress and endothelial dysfunction, certain areas of coronary tree are more prone for atherosclerosis. In particular, increased endothelial dysfunction and plaque burden have been found at bifurcations. We show that superoxide production is almost doubled at bifurcations. This likely is caused by disturbed flow at these sites leading to increased NAD(P)H oxidase expression and activity. Moreover, oscillatory shear stress stimulates both NADPH oxidase and XO activity in cultured endothelial cells.

The cellular sources of superoxide in coronary arteries appear to be complex. Inflammatory cell infiltrates are likely to be a quantitatively important source. However, dihydroethidium staining, presented here, shows that superoxide production is evenly increased in cells within endothelium, media, and adventitia in CAD, showing that inflammatory cells are not the sole source of \( \text{O}_2^\cdot^- \) in CAD.

Our studies show that NAD(P)H oxidases are major sources of \( \text{O}_2^\cdot^- \) production in human coronary arteries, and their inhibitors (DPI and apocynin) abolish the difference between CAD and non-CAD. Based on our studies with oxypurinol, xanthine oxidase appears to be a significant source of superoxide in CAD patients. We also made the novel observations that levels of xanthine oxidase protein are increased in CAD vessels, whereas the expression of dehydrogenase form is not different. This is important because XO is responsible for superoxide and hydrogen peroxide production, whereas XDH used NAD⁺ as a substrate to form NADH.

Arachidonic acid metabolism may also contribute to vascular \( \text{O}_2^\cdot^- \) production, as indicated by inhibition by indomethacin, although this effect may reflect the importance of arachidonic acid in NAD(P)H oxidase activation. These results show that the enzymatic sources of \( \text{O}_2^\cdot^- \) in coronary arteries are similar to those of peripheral arteries. Our findings are also in agreement with Spiekermann et al, who showed increased activity of both NAD(P)H oxidase and xanthine oxidase in CAD.

The mechanisms regulating superoxide production in coronary arteries appear to be similar to peripheral vasculature. The protein kinase C inhibitor, chelerythrine, caused significant inhibition of both basal and NADPH stimulated superoxide production in coronary arteries. Angiotensin II increased both basal and NADPH stimulated superoxide production in coronary arteries and this was inhibited by losartan. This finding suggests an important role for the AT1 receptor in stimulating NAD(P)H oxidase activity and supports the therapeutic potential of AT1 receptor blockade in CAD. This finding may be particularly important in coronary arteries, as the effects of angiotensin II may vary in different human vessels.

Increased NAD(P)H oxidase activity in CAD was associated with increased p22phox mRNA and protein levels and increased nox2 mRNA levels, suggesting that these subunits are major determinants of vascular superoxide production in human CAD. The protein levels of classical NAD(P)H oxidase subunits were increased including p22phox, p67phox, and p47phox. In part, this difference is likely to be related to increased inflammatory cell infiltration, indicated by McCSFR expression.

The nox proteins, which are recently described gp91phox homologues, represent the catalytic subunits of the NAD(P)H oxidases, but their relative importance in human coronary artery disease has remained less clear. Our results indicate that expression of nox2 is most strongly increased in CAD. We confirmed that expression of nox2 mRNA in coronary arteries correlates strongly with McCSFR mRNA, whereas nox4 correlates with \( \alpha \)-smooth muscle actin. The elevation of nox2 likely reflects the presence of inflammatory cells in CAD, whereas nox4 expression is likely to be determined by smooth muscle cells or myofibroblasts. Both endothelial and adventitial oxidases are also nox2-based and may play important roles in overall vascular \( \text{O}_2^\cdot^- \) production. The relationship between nox4 expression and NAD(P)H oxidase activity in human coronary arteries cannot be simply explained by the content of \( \alpha \)-smooth muscle actin-expressing cells. When corrected for \( \alpha \)-smooth muscle actin expression in individual samples, nox4 expression was even more strongly associated with NAD(P)H oxidase activity. This observation suggests that the cellular regulation of nox4 expression is either a direct determinant, or at least a marker of increased coronary artery NAD(P)H oxidase activity. The lack of significant difference in nox4 mRNA between CAD and non-CAD samples may suggest that nox4 is upregulated in both groups, for example in relation to heart failure that is known to be associated with endothelial dysfunction. In the present study, our non-CAD group also had heart failure, which could affect vascular \( \text{O}_2^\cdot^- \) production. To examine the importance of this, we also measured \( \text{O}_2^\cdot^- \) production in coronary arteries obtained from accident victims without heart failure or CAD. In this group, coronary \( \text{O}_2^\cdot^- \) production was only slightly and nonsignificantly lower that that observed in the heart failure, non-CAD group. Thus, the presence or absence of CAD plays a major role in determining the level of coronary \( \text{O}_2^\cdot^- \) production whether heart failure is present or absent. However, although our comparisons reflect oxidative status in the presence and absence of CAD in heart failure patients, it is important to note these results cannot be extrapolated to subjects that do not have heart failure.

Sorescu et al showed that nox4 expression increased with early progression of atherosclerotic plaque, but decreased in more advanced lesions. The role of nox1 in human vasculature remains unclear. Both our findings and those of Sorescu et al indicate only very low levels of nox1 mRNA expression in human coronary arteries.

The association of nox2 and p22phox expression with NAD(P)H oxidase activity was not evident in the small number of vessels with very high monocyte/macrophage content (judged by McCSFR mRNA). Inflammatory cells contain >100-fold more p22phox and nox2 mRNA than vascular cells, but changes in mRNA encoding NAD(P)H oxidase membrane subunits appear to have only minor or no effect on \( \text{O}_2^\cdot^- \) production from human leukocytes, thus obscuring a relationship between the p22phox or nox2 mRNA and oxidase activity. In contrast, increases of p22phox and nox mRNA levels in vascular cells are associated with increased NAD(P)H oxidase activity. While analyzing these relationships, it is important to remember that mRNA levels,
although readily quantified by fluorescent real-time reverse-transcription PCR, may not be directly related to protein levels, because post-translational regulatory steps. Further studies relating nox homologue protein levels with oxidative activity would be warranted.

One of the inevitable limitations of any study using human blood vessels is the fact that patients receive treatment with statins and other medications which might affect vascular oxidative stress.\(^{22}\) However, such treatments might be expected to reduce rather than increase the magnitude of observed differences between CAD and non-CAD patients; therefore, it is reasonable to assume that the differences without treatments could have been even more pronounced.

In conclusion, we show that \(O_2^-\) production in human coronary arteries is almost doubled in CAD. Nox2-based and Nox4-based NAD(P)H oxidases appear to be the predominant contributors to oxidative stress in human coronary arteries, whereas xanthine oxidase also contributes in CAD patients. Our studies identify some of pathways that regulate the expression and activity of NAD(P)H oxidases in the pathogenesis of oxidative stress in human CAD.

Acknowledgments
This work was supported by a Wellcome Trust International Research Development Award (to T.G. and K.M.C.) and by the British Heart Foundation and Polish Committee for Scientific Research grant (to T.G.).

References
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SUPPLEMENTARY MATERIALS AND METHODS

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Oxidative Fluorescent Microtopography
In situ superoxide generation was visualized in vascular cryosections (30µm) with the SOD-inhibitable dihydroethidium (DHE; 2 µmol/L) fluorescence, as described previously\cite{12, 13}. Images were obtained with a BioRad MRC 1024 scanning confocal microscope using the excitation/emission wavelengths 488nm/610nm. In each case,
paired segments of CAD and non-CAD vessels that had been collected and stored under identical conditions were analyzed in parallel with identical imaging parameters.

**Western Immunoblotting**

Portions of vascular homogenates (20 ug protein) were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes for 12h at 40 mA at 4°C or for 2h at 100V in RT. NAD(P)H oxidase components were detected using mouse monoclonal antibodies against p67phox or p47phox, (BD Pharmedingen) or by rabbit polyclonal antibodies against p22phox (kindly provided by Dr. Imajoh-Ohmi, Japan).

Xanthine dehydrogenase (150kDa)/Xanthine oxidase (130kDa) was detected as described before. Briefly 100ug of protein homogenate was used and was separated on 10% SDS-PAGE gel. and transferred to nitrocellulose membranes for 2h at 100V in RT. XDH/XO was detected using using rabbit polyclonal antibody (kindly provided by Dr. JR Hoidal; University of Utah).

Bands were detected by horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies (Promega, UK), visualized using a chemiluminescence detection system (Supersignal®, Pierce, USA) and analyzed using NIH Image software.

**RT-PCR and quantitative real-time RT-PCR**

RNA was isolated from snap-frozen coronary artery segments using Tri-reagent and re-purified using RNA easy kit with DNase digestion. RNA quantity was determined using the RiboGreen assay. cDNA was synthesized using ImProm® RT System (Promega) with random primers. The cDNA synthesized from 20ng total RNA was subjected to quantitative PCR using SYBR®Green PCR Quantitec kit (Quiagen) and Rotorgene 3000 flourescent real-time PCR system (Corbett Reseach, Australia). Mg²⁺ concentrations were 1.5 mmol/l for all primers except 4mmol/l was employed when nox4 mRNA was quantified. Annealing temperatures were 58°C for all primers except nox4 (68°C). All primers were used as described by Sorescu et al (for nox1, nox2, nox-4, MfCSFR, α-sma), except for p22phox. The housekeeping gene MLN-51 was used to further normalize for reverse transcription and PCR efficiencies, although the variability was minimal (not shown). Relative amounts of RNA were determined using Rotorgene v. 4.6 software using serial dilutions of total RNA isolated from THP1 cells as standards for nox2 and p22phox, or from hMEC for nox1 and nox4.
**Statistical analysis**

All data are expressed as means ± SEM with n equal to the number of patients. Comparisons between groups of patients or treatments were made using student t-test or one-way ANOVA, followed by the Student-Newman-Keuls post-hoc test. Correlation between oxidase activity and nox expression was assessed by simple linear regression. Values of $p < 0.05$ were considered statistically significant.