Investigation Into the Sources of Superoxide in Human Blood Vessels

Angiotensin II Increases Superoxide Production in Human Internal Mammary Arteries

Colin Berry, MD; Carlene A. Hamilton, PhD; M. Julia Brosnan, PhD; Fergus G. Magill, BSc; Geoffrey A. Berg, MD; John J.V. McMurray, MD; Anna F. Dominiczak, MD

Background—Increased vascular superoxide anion (·O$_2^-$) production contributes to endothelial dysfunction and hypertension in animal models of cardiovascular disease. Observations in experimental animals suggest that angiotensin II (Ang II) increases ·O$_2^-$ production by activation of vascular NAD(P)H oxidase. We studied the sources of ·O$_2^-$ production in human blood vessels and investigated whether, and by what mechanism, Ang II might alter vascular ·O$_2^-$ production.

Methods and Results—Internal mammary arteries (IMAs) and saphenous veins (SVs) were collected at the time of cardiac surgery. Vessels were incubated in Krebs buffer at 37°C. ·O$_2^-$ was measured by lucigenin chemiluminescence. Basal ·O$_2^-$ concentrations were greater in IMAs than SVs. Inhibitors of NAD(P)H oxidase (10 μmol/L to 200 μmol/L diphenyleneiodonium) and xanthine oxidase (1 mmol/L allopurinol) caused reductions in ·O$_2^-$ concentrations in both IMAs and SVs. Western blotting of superoxide dismutase proteins demonstrated similar expression in IMAs and SVs. Vessels were also incubated in the presence or absence of Ang II (1 pmol/L to 1 μmol/L). Ang II increased ·O$_2^-$ production in IMAs at 4 hours of incubation (control, 978±117 pmol · min$^{-1}$ · mg$^{-1}$; 1 μmol/L of Ang II, 1690±213 pmol · min$^{-1}$ · mg$^{-1}$; n=27, P=0.0001, 95% CI 336, 925) but not in SVs. This effect was completely inhibited by coincidence of IMAs with DPI (100 μmol/L), a nonspecific Ang II antagonist ([sar$^1$, thre$^8$]–Ang II–1 μmol/L) and a specific Ang II type 1 (AT$_1$) receptor antagonist (losartan, 1 μmol/L).

Conclusions—·O$_2^-$ production is greater in human IMAs than in SVs. NAD(P)H oxidase and xanthine oxidase are sources of ·O$_2^-$ production in these vessels. The vasoactive peptide Ang II increases ·O$_2^-$ production in human arteries by an AT$_1$ receptor–dependent mechanism. (Circulation. 2000;101:2206-2212.)

Key Words: free radicals ■ arteries ■ veins ■ angiotensin ■ receptors

Increased vascular superoxide anion (·O$_2^-$) production can lead to reduced bioavailable nitric oxide (NO) and impaired endothelium-dependent relaxation, a feature of disease states such as hypertension, hypercholesterolemia, diabetes, and heart failure.

In experimental animals, the cellular sources of vascular ·O$_2^-$ production in animals are the endothelium, vascular smooth muscle cells (VSMCs), and adventitial fibroblasts. The main enzymatic sources of ·O$_2^-$ production within the vascular wall are NAD(P)H oxidase, xanthine oxidase, and endothelial NO synthase (eNOS) enzyme. Superoxide can be removed by reaction with other free radicals, such as NO, to form peroxynitrite or enzymatically by superoxide dismutase (SOD) to form H$_2$O$_2$. Three mammalian SODs exist: copper/zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD), and extracellular SOD (EC-SOD). Mn-SOD and Cu/Zn-SOD are major vascular isoforms and are important in both scavenging vascular ·O$_2^-$ and enhancing the bioavailability of endothelial NO.

Further evidence from animal studies suggests that the activity of one ·O$_2^-$ -producing enzyme, NAD(P)H oxidase, may be modulated by angiotensin II (Ang II) and that ·O$_2^-$, in turn, may play a role in the physiological and pathophysiological actions of this peptide. Ang II increases VSMC ·O$_2^-$ production by activation of a membrane-bound NAD(P)H oxidase. In rats chronically infused with pressor and nonpressor doses of Ang II, increased vascular NAD(P)H oxidase ·O$_2^-$ production contributed to the development of both endothelial dysfunction and hypertension. In rabbits, activation of the renin-angiotensin system also contributes to increased ·O$_2^-$ production and vascular dysfunction.

Superoxide production has recently been measured in human arteries but not in veins. The enzymatic sources of...
Ang II Increases $\text{O}_2^-$ Release in Human Arteries

TABLE 1. Patient Characteristics, Including Risk Factors and Therapy

<table>
<thead>
<tr>
<th>Age, y</th>
<th>62±8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men, n (%)</td>
<td>179 (73)</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>66 (27)</td>
</tr>
<tr>
<td>Risk factors, n (%)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>43 (18)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>82 (34)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>26 (11)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>180 (74)</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dL (mean±SD)</td>
<td>212±58</td>
</tr>
<tr>
<td>Medication, n (%)</td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>203 (83)</td>
</tr>
<tr>
<td>$\beta$-Blockers</td>
<td>136 (56)</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>145 (60)</td>
</tr>
<tr>
<td>HMG-CoA reductase inhibitors</td>
<td>127 (52)</td>
</tr>
<tr>
<td>Nitroglycerin</td>
<td>48 (18)</td>
</tr>
<tr>
<td>Renin-angiotensin system inhibitors</td>
<td>54 (22)</td>
</tr>
</tbody>
</table>

$\text{O}_2^-$ generation in human blood vessels, however, have not been identified. Furthermore, it is not known whether Ang II alters $\text{O}_2^-$ production in human blood vessels. We hypothesized that angiotensin II, in addition to its known physiological and pathophysiological effects, is able to stimulate $\text{O}_2^-$ generation in human vessels and furthermore, that this mechanism can be inhibited with a specific Ang II type 1 (AT1) receptor antagonist.

Our aims in this study were first, to study the cellular and enzymatic sources of $\text{O}_2^-$ production in human blood vessels; second, to quantify the amount of SOD protein present in arteries and veins to assess whether SOD expression might account for any differences in $\text{O}_2^-$ measured; and finally, to investigate whether, and by what mechanism, Ang II might affect $\text{O}_2^-$ production in human blood vessels.

Methods

Patient Characteristics

Patients with coronary artery disease (CAD) who were undergoing elective CABG were included in this study. Patient characteristics were determined by review of case records. A history of current cigarette smoking, hypertension (blood pressure >140/90 mm Hg), diabetes mellitus, and hypercholesterolemia (plasma cholesterol >200 mg/dL) were considered risk factors for CAD. Approval from the West Glasgow Hospitals University Trust Ethics Committee was granted for this study.

Vessel Preparation

Distal segments of left internal mammary artery (IMA) and saphenous vein (SV), which were harvested at the time of routine coronary artery revascularization surgery, were immediately taken to the laboratory in Krebs-HEPES buffer on ice. At this point and in these conditions, the blood vessels were then carefully dissected free of loose connective tissue, divided into 4- to 5-mm segments, and weighed. The vessels were then incubated in Krebs buffer at pH 7.4±2 and maintained in atmospheric conditions (PO2 19±4 kPa; Pco2 3±4 kPa) at 37°C.

Measurement of Superoxide Anion Production

Vascular $\text{O}_2^-$ was measured by lucigenin-enhanced chemiluminescence in a liquid scintillation counter (Hewlett Packard model 2100TR) as previously described and recently revalidated by Li et al. Absolute counts were quantified with a xanthine/xanthine oxidase calibration curve for $\text{O}_2^-$ production in human IMAs and SVs, expressed as pmol·min⁻¹·mg tissue⁻¹.

Figure 1. $\text{O}_2^-$ production in human IMAs and SVs, expressed as pmol·min⁻¹·mg tissue⁻¹. Results are shown as mean±SEM. Shaded bar indicates IMA (n=55); open bar, SV (n=58).
Western Blotting

Five-millimeter segments of vessels were homogenized in 200 μL of a boiling vanadate buffer. After centrifugation at 14 000g for 60 seconds, the supernatant was withdrawn and the protein concentration measured. Ten micrograms of protein and prestained molecular weight standards were separated by SDS-PAGE with a 12% gel. The proteins were transferred overnight onto a polyvinylidene difluoride membrane and sequentially probed with sheep monoclonal anti–CuZn-SOD or rabbit polyclonal anti–Mn-SOD or anti-actin antibodies. Protein bands were visualized by enhanced chemiluminescence (GS-525 Biorad-Laboratories Ltd).

Organ Bath Studies

Studies of the isometric tension development of isolated IMA and SV rings were performed by methods previously described. Ang II dose-response curves (10 pmol/L to 100 nmol/L) were performed in the presence and absence of 10 μmol/L losartan and 10 μmol/L [sar¹, thre⁶]–Ang II.

Materials

Xanthine, xanthine oxidase, lucigenin, DETCA, Tiron, DPI, L-NAME, ALP, [sar¹, thre⁶]–Ang II, and norepinephrine were purchased from Sigma-Aldrich. Losartan was obtained as a gift from Professor Taniguchi (University of Osaka, Japan) and anti–CuZn-SOD was purchased from Calbiochem.

Statistical Analyses

All data are presented as mean±SEM or as percent change from controls to facilitate comparison between groups. Statistical analyses of vascular -O₂⁻ concentrations were undertaken by use of the Wilcoxon signed-rank test. The relationships between risk factors and basal vascular -O₂⁻ concentrations were determined by use of the Pearson correlation coefficient (r). A value of P<0.05 was considered statistically significant.

Results

Patient Characteristics

The study population consisted of 244 consecutive patients with CAD who underwent CABG in our hospital over a 14-month period. Data on age, sex, risk factors, and drug therapy are given in Table 1. Patient age ranged from 33 to 80 years. Seventy-five percent of patients had ≥1 risk factors for CHD, and 92% of patients were on ≥1 types of antianginal therapy.

Basal -O₂⁻ Production in Human Arteries and Veins

Superoxide production was greater in human arteries than in veins: IMA, 1922±235 pmol · min⁻¹ · mg⁻¹ (n=55); SV, 662±179 pmol · min⁻¹ · mg⁻¹ (n=58); P<0.0001; 95% CI, 570, 1421 (Figure 1). Basal -O₂⁻ production in human IMA was weakly associated with patient age (r=0.19, P<0.1). No other relationships existed between basal -O₂⁻ production in either IMA or SV and any other patient characteristic.

Characterization of -O₂⁻ Production

The free radical scavenger Tiron (10 mmol/L) reduced basal -O₂⁻ concentrations in both IMA (control, 1937±412; Tiron, 10 mmol/L 901±94; n=9, P=0.018; 95% CI, −1919, −238) and SV (control, 350±85; Tiron 10 mmol/L, 149±59; n=13, P=0.002; 95% CI, −311, −95). DETCA (100 μmol/L), an inhibitor of SOD, increased -O₂⁻ steady-state concentrations in IMA (control, 853±208; DETCA 100 μmol/L, 1492±347; n=8, P=0.021; 95% CI, 186, 1149) and SV (control, 551±145; DETCA 100 μmol/L, 945±245; n=14, P=0.002; 95% CI, 67, 694). The NAD(P)H oxidase inhibitor DPI (concentration range, 10 to 200 μmol/L) attenuated -O₂⁻ generation in both arteries (Table 2) and veins (Table 3). The xanthine oxidase inhibitor ALP (1 mmol/L) attenuated -O₂⁻ production in both IMA and SV (Tables 2 and 3).

The effects on -O₂⁻ steady-state concentrations in IMA and SV of endothelial denudation by rubbing and inhibition of eNOS by incubation with L-NAME were more variable (Tables 2 and 3). Incubation of IMA with L-NAME reduced -O₂⁻ concentrations in 7 of 10 and 6 of 10 patients, respectively. In SV, these treatments corresponded to a reduction in -O₂⁻ concentrations in 12 of 16 and 10 of 14 patients. Sufficient tissue was available in veins to study the effects of L-NAME treatment and endothelial removal by rubbing in vessel segments from the same patients. In these studies, both manipulations had similar effects on -O₂⁻ concentrations in individual patients (r=0.85, n=12, P<0.001). Furthermore, the difference in -O₂⁻ concentrations between IMA and SV was maintained after both endothelial denudation and eNOS inhibition.

Quantification of SOD Proteins

Immunoblotting showed that single bands were detected for both Mn-SOD and CuZn-SOD (Figure 2). The intensity of

| TABLE 2. Effect of Inhibition of NAD(P)H Oxidase, Xanthine Oxidase, and NOS and of Endothelial Denudation on -O₂⁻ Production in IMA |
|---------------------------------|-----------------|-----------------|-----------------|
| Treatment | n  | Control | Treated | % Change | P*  | 95% CI  |
| DPI 10 μmol/L | 8  | 965±150 | 616±212 | −36±17 | 0.14 | −808, 239 |
| DPI 100 μmol/L | 8  | 2723±696 | 1568±430 | −39±13 | 0.03 | −2515, −137 |
| ALP 1 mmol/L | 9  | 2120±629 | 1024±290 | −42±10 | 0.013 | −2020, −330 |
| L-NAME 100 μmol/L | 10 | 1990±508 | 1420±304 | −29±16 | 0.1 | −1421, 67 |
| Endothelial denudation | 10 | 643±134 | 647±145 | −3±10 | 0.55 | −183, 153 |

*DPI, ALP, and L-NAME are inhibitors of NAD(P)H oxidase, xanthine oxidase, and eNOS enzymes, respectively. Data are shown as mean±SEM.
CuZn-SOD bands relative to an actin control was 4.04±0.31 in arteries versus 3.59±0.23 in veins (P=0.055). Mn-SOD expression was found to have relative intensities of 3.14±0.25 versus 3.87±0.42 (P=0.056) in arteries and veins, respectively.

Effect of Ang II on ∙O₂⁻ Production

Ang II at concentrations of 1 µmol/L, 1 nmol/L, and 1 pmol/L increased ∙O₂⁻ production in IMA but not SV (Figure 3, Table 4). In IMA, Ang II had no effect on ∙O₂⁻ production after 15 minutes of incubation (control, 1173±239 pmol ⋅ min⁻¹ ⋅ mg tissue⁻¹; 1 µmol/L of Ang II, 918±170 pmol ⋅ min⁻¹ ⋅ mg⁻¹; n=11, P=0.12; 95% CI, −680, 155) but increased ∙O₂⁻ production after 1 and 4 hours, respectively. Norepinephrine (1 µmol/L), which was used as a positive control, had no effect on ∙O₂⁻ generation in either arteries (control, 1581±899 pmol ⋅ min⁻¹ ⋅ mg⁻¹; norepinephrine, 738±241 pmol ⋅ min⁻¹ ⋅ mg⁻¹; n=11, P=0.45; 95% CI, −3590, 164) or veins (control, 360±218 pmol ⋅ min⁻¹ ⋅ mg⁻¹; norepinephrine, 249±118 pmol ⋅ min⁻¹ ⋅ mg⁻¹; n=7, P=0.18; 95% CI, −391, 45).

Losartan (1 µmol/L), a competitive AT₁-specific receptor antagonist, had no effect on basal ∙O₂⁻ production but blocked the Ang II–mediated increase of ∙O₂⁻ production (Figure 4). [Sar¹, thre³]–Ang II (1 µmol/L), a nonspecific Ang II receptor antagonist, also blocked Ang II–mediated increase of ∙O₂⁻ production ([Sar¹, thre³]–Ang II, 1 µmol/L, 1252±276 pmol ⋅ min⁻¹ ⋅ mg⁻¹; [Sar¹, thre³]–Ang II, 1 µmol/L + Ang II, 1 µmol/L, 1281±204 pmol ⋅ min⁻¹ ⋅ mg⁻¹; n=15, P=0.63; 95% CI, −200, 305). Isometric tension studies in IMA rings confirmed that both antagonists were effective in blocking Ang II–induced vasoconstriction. Incubation of IMA for 4 hours with DPI 100 µmol/L, but not DPI 10 µmol/L, blocked Ang II–mediated increase of ∙O₂⁻ production (Figure 5).

Discussion

We have shown that basal ∙O₂⁻ concentrations are greater in human IMA than SV and have demonstrated that both NAD(P)H oxidase and xanthine oxidase enzymes contribute to basal ∙O₂⁻ production in these vessels. Furthermore, we report that SOD proteins are quantitatively similar in human IMA and SV. This is the first demonstration that Ang II can increase ∙O₂⁻ production in human arteries. This effect is AT₁ receptor–dependent, as it was completely blocked by an AT₁ receptor antagonist. We have also demonstrated that this Ang II–mediated increase of ∙O₂⁻ is mediated by NAD(P)H oxidase, as it was inhibited by DPI. This is a clinically important observation, as physiological concentrations (pmol) of Ang II induced an increase in ∙O₂⁻ production in human arteries, and this effect was blocked by losartan.

In the present study, the higher basal ∙O₂⁻ concentrations in arteries, compared with those in veins, were maintained after endothelial denudation by rubbing. This suggests that VSMCs may be an important source of ∙O₂⁻ generation, as arteries have a proportionately greater content of VSMCs than veins. The balance between ∙O₂⁻ generation and degra-

Table 3. Effect of Inhibition of NAD(P)H Oxidase, Xanthine Oxidase, and NOS and of Endothelial Denudation on ∙O₂⁻ Production in SV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number</th>
<th>Control</th>
<th>Treated</th>
<th>% Change</th>
<th>P, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPI 10 µmol/L</td>
<td>10</td>
<td>184±25</td>
<td>116±28</td>
<td>−34±16</td>
<td>0.037; −124, −7</td>
</tr>
<tr>
<td>DPI 100 µmol/L</td>
<td>14</td>
<td>759±140</td>
<td>469±94</td>
<td>−37±6</td>
<td>0.001; −519, −103</td>
</tr>
<tr>
<td>DPI 200 µmol/L</td>
<td>8</td>
<td>452±126</td>
<td>228±73</td>
<td>−47±7</td>
<td>0.014; −394, −59</td>
</tr>
<tr>
<td>ALP 1 mmol/L</td>
<td>13</td>
<td>759±173</td>
<td>426±107</td>
<td>−32±8</td>
<td>0.003; −673, −57</td>
</tr>
<tr>
<td>L-NAME 100 µmol/L</td>
<td>16</td>
<td>284±44</td>
<td>214±36</td>
<td>−20±13</td>
<td>0.06; −148, 5</td>
</tr>
<tr>
<td>Endothelial denudation</td>
<td>14</td>
<td>324±48</td>
<td>250±40</td>
<td>−15±9</td>
<td>0.04; −144, −8</td>
</tr>
<tr>
<td>Endothelial denudation+DPI</td>
<td>9</td>
<td>787±259</td>
<td>477±136</td>
<td>−29±9</td>
<td>0.018; −671, −39</td>
</tr>
</tbody>
</table>

DPI, ALP, and L-NAME are inhibitors of NAD(P)H oxidase, xanthine oxidase, and eNOS enzymes, respectively. Data are shown as mean±SEM.
TABLE 4. Effect of Ang II Concentration (1 pmol/L, 1 nmol/L, and 1 μmol/L) on
\( \text{O}_2^- \) Production in IMA After Incubation Periods of 1 and 4 Hours

<table>
<thead>
<tr>
<th></th>
<th>1 Hour</th>
<th>4 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 pmol/L (n=10)</td>
<td>1 nmol/L (n=9)</td>
</tr>
<tr>
<td></td>
<td>1 pmol/L (n=15)</td>
<td>1 nmol/L (n=11)</td>
</tr>
<tr>
<td>Control</td>
<td>1516±253</td>
<td>1604±322</td>
</tr>
<tr>
<td>Ang II</td>
<td>1380±138</td>
<td>1752±323</td>
</tr>
<tr>
<td>P</td>
<td>0.8</td>
<td>0.52</td>
</tr>
<tr>
<td>95% CI</td>
<td>−690, 396</td>
<td>−430, 540</td>
</tr>
<tr>
<td>% Change</td>
<td>12±22</td>
<td>15±16</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM.

Figure 3. Effect of Ang II on \( \text{O}_2^- \) production in human IMAs and SVs. Blood vessels were incubated in presence or absence of 1 μmol/L Ang II for 4 hours. Results are expressed as pmol · min⁻¹ · mg tissue⁻¹ and are shown as mean±SEM. Shaded bars indicate IMA (n=27); open bars, SV (n=8).

Figure 4. Effect of losartan, a specific AT₁ receptor antagonist, on Ang II–mediated increase in \( \text{O}_2^- \) production. IMAs were incubated in presence or absence of 1 μmol/L Ang II (n=13) or 1 μmol/L losartan or were coincubated with 1 μmol/L Ang II and 1 μmol/L losartan (n=15) for 4 hours. Results are expressed as pmol · min⁻¹ · mg tissue⁻¹ and are shown as mean±SEM. Open bars indicate control or losartan; shaded bars, IMA incubated with Ang II and/or losartan.
nation for our observations may be that the intracellular mechanisms by which Ang II activates NAD(P)H oxidase may differ in human arteries and veins. In vitro studies in human VSMCs have suggested that Ang II may increase \( \cdot \text{O}_2^- \) production in these cells by activation of NAD(P)H oxidase.²⁸ Our observations may also be substantiated by a report that the pressor effect of intrabrahial artery infusion of Ang II in humans is attenuated by the coinfusion of vitamin C.²⁹ This study, however, failed to identify a specific Ang II receptor or to evaluate a positive control (ie, another vasoconstrictor, such as norepinephrine).

Experimental studies have demonstrated that the Ang II–mediated increase in \( \cdot \text{O}_2^- \) production is of functional importance. The Ang II–stimulated increase in \( \cdot \text{O}_2^- \) production contributes to its trophic effect of on rat VSMCs³⁰ and mesangial cells³⁰ and also contributes to the pressor effect of this hormone in a hypertensive rat model.¹⁷

We have shown that Ang II increases \( \cdot \text{O}_2^- \) production in human arteries by an AT₁-dependent mechanism. The mechanism of Ang II–mediated increase of \( \cdot \text{O}_2^- \) production differs in other species. An AT₁-dependent Ang II–mediated increase of \( \cdot \text{O}_2^- \) production has been reported in the aorta¹⁷ and mesangium³⁰ of Sprague-Dawley rats and aorta of hypercholesterolemic rabbits.¹⁸ However, the Ang II–induced increase in \( \cdot \text{O}_2^- \) production in normocholesterolemic rabbit aortic adventitial fibroblasts is mediated by a non-AT₁–non-AT₂-receptor mechanism.¹⁸,³¹ The species differences in the mechanism of Ang II–mediated increase in \( \cdot \text{O}_2^- \) production emphasize the importance of human investigations, as is the case in the present study.

In conclusion, we have characterized cellular and enzyme sources of \( \cdot \text{O}_2^- \) production in human arteries and veins. We have demonstrated that Ang II increases \( \cdot \text{O}_2^- \) production at physiological and pharmacological concentrations in human arteries. This effect, which is mediated by NAD(P)H oxidase, is completely inhibited by the AT₁ receptor antagonist losartan. These observations suggest a putative therapeutic role for AT₁ receptor antagonists in reducing oxidative stress in cardiovascular disease.

**Acknowledgments**

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