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# 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 ( $\cdot\text{O}_2^-$ ) production contributes to endothelial dysfunction and hypertension in animal models of cardiovascular disease. Observations in experimental animals suggest that angiotensin II (Ang II) increases  $\cdot\text{O}_2^-$  production by activation of vascular NAD(P)H oxidase. We studied the sources of  $\cdot\text{O}_2^-$  production in human blood vessels and investigated whether, and by what mechanism, Ang II might alter vascular  $\cdot\text{O}_2^-$  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.  $\cdot\text{O}_2^-$  was measured by lucigenin chemiluminescence. Basal  $\cdot\text{O}_2^-$  concentrations were greater in IMAs than SVs. Inhibitors of NAD(P)H oxidase (10  $\mu\text{mol/L}$  to 200  $\mu\text{mol/L}$  diphenyleneiodonium) and xanthine oxidase (1 mmol/L allopurinol) caused reductions in  $\cdot\text{O}_2^-$  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  $\mu\text{mol/L}$ ). Ang II increased  $\cdot\text{O}_2^-$  production in IMAs at 4 hours of incubation (control,  $978 \pm 117$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$ ; 1  $\mu\text{mol/L}$  of Ang II,  $1690 \pm 213$  pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$ ; n=27,  $P=0.0001$ , 95% CI 336, 925) but not in SVs. This effect was completely inhibited by coincubation of IMAs with DPI (100  $\mu\text{mol/L}$ ), a nonspecific Ang II antagonist ([sar<sup>1</sup>, thre<sup>8</sup>]-Ang II, 1  $\mu\text{mol/L}$ ) and a specific Ang II type 1 (AT<sub>1</sub>) receptor antagonist (losartan, 1  $\mu\text{mol/L}$ ).

**Conclusions**— $\cdot\text{O}_2^-$  production is greater in human IMAs than in SVs. NAD(P)H oxidase and xanthine oxidase are sources of  $\cdot\text{O}_2^-$  production in these vessels. The vasoactive peptide Ang II increases  $\cdot\text{O}_2^-$  production in human arteries by an AT<sub>1</sub> receptor-dependent mechanism. (*Circulation*. 2000;101:2206-2212.)

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

Increased vascular superoxide anion ( $\cdot\text{O}_2^-$ ) production can lead to reduced bioavailable nitric oxide (NO)<sup>1</sup> and impaired endothelium-dependent relaxation,<sup>2,3</sup> a feature of disease states such as hypertension,<sup>4</sup> hypercholesterolemia,<sup>5</sup> diabetes,<sup>6</sup> and heart failure.<sup>7</sup>

In experimental animals, the cellular sources of vascular  $\cdot\text{O}_2^-$  production in animals are the endothelium,<sup>8</sup> vascular smooth muscle cells (VSMCs),<sup>9</sup> and adventitial fibroblasts.<sup>10</sup> The main enzymatic sources of  $\cdot\text{O}_2^-$  production within the vascular wall are NAD(P)H oxidase,<sup>11</sup> xanthine oxidase,<sup>12</sup> and endothelial NO synthase (eNOS) enzyme.<sup>13</sup> Superoxide can be removed by reaction with other free radicals, such as NO, to form peroxynitrite<sup>1</sup> or enzymatically by superoxide dismutase (SOD) to form H<sub>2</sub>O<sub>2</sub>.<sup>14</sup> 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  $\cdot\text{O}_2^-$  and enhancing the bioavailability of endothelial NO.<sup>15</sup>

Further evidence from animal studies suggests that the activity of one  $\cdot\text{O}_2^-$ -producing enzyme, NAD(P)H oxidase, may be modulated by angiotensin II (Ang II) and that  $\cdot\text{O}_2^-$ , in turn, may play a role in the physiological and pathophysiological actions of this peptide. Ang II increases VSMC  $\cdot\text{O}_2^-$  production by activation of a membrane-bound NAD(P)H oxidase.<sup>16</sup> In rats chronically infused with pressor and nonpressor doses of Ang II, increased vascular NAD(P)H oxidase  $\cdot\text{O}_2^-$  production contributed to the development of both endothelial dysfunction and hypertension.<sup>17</sup> In rabbits, activation of the renin-angiotensin system also contributes to increased  $\cdot\text{O}_2^-$  production and vascular dysfunction.<sup>18</sup>

Superoxide production has recently been measured in human arteries but not in veins.<sup>19</sup> The enzymatic sources of

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**TABLE 1. Patient Characteristics, Including Risk Factors and Therapy**

Age, y	62±8
Men, n (%)	179 (73)
Women, n (%)	66 (27)
Risk factors, n (%)	
Smoking	43 (18)
Hypertension	82 (34)
Diabetes mellitus	26 (11)
Hypercholesterolemia	180 (74)
Plasma cholesterol, mg/dL (mean±SD)	212±58
Medication, n (%)	
Aspirin	203 (83)
$\beta$ -Blockers	136 (56)
Calcium channel blockers	145 (60)
HMG-CoA reductase inhibitors	127 (52)
Nitroglycerin	148 (61)
Renin-angiotensin system inhibitors	54 (22)

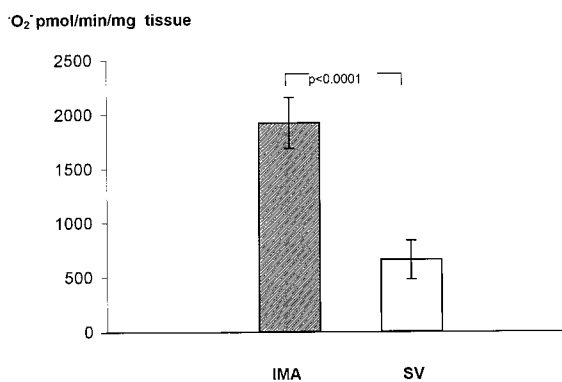
$\cdot\text{O}_2^-$  generation in human blood vessels, however, have not been identified. Furthermore, it is not known whether Ang II alters  $\cdot\text{O}_2^-$  production in human blood vessels. We hypothesized that angiotensin II, in addition to its known physiological and pathophysiological effects, is able to stimulate  $\cdot\text{O}_2^-$  generation in human vessels and furthermore, that this mechanism can be inhibited with a specific Ang II type 1 ( $\text{AT}_1$ ) receptor antagonist.

Our aims in this study were first, to study the cellular and enzymatic sources of  $\cdot\text{O}_2^-$  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  $\cdot\text{O}_2^-$  measured; and finally, to investigate whether, and by what mechanism, Ang II might affect  $\cdot\text{O}_2^-$  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



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

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 ( $\text{PO}_2$  19±4 kPa;  $\text{PCO}_2$  3±4 kPa) at 37°C.

### Measurement of Superoxide Anion Production

Vascular  $\cdot\text{O}_2^-$  was measured by lucigenin-enhanced chemiluminescence in a liquid scintillation counter (Hewlett Packard model Tricarb 2100TR) as previously described<sup>8,12</sup> and recently revalidated by Li et al.<sup>20</sup> Absolute counts were quantified with a xanthine/xanthine oxidase calibration curve for  $\cdot\text{O}_2^-$  generation and reported as pmol  $\cdot$  min $^{-1}$   $\cdot$  mg tissue $^{-1}$ . In all experiments,  $\cdot\text{O}_2^-$  production was measured in paired samples with a matched control from the same vessel in every case.

### Effect of Varying Lucigenin Concentrations on $\cdot\text{O}_2^-$ Production

Lucigenin itself has been reported to generate  $\cdot\text{O}_2^-$  at higher concentrations.<sup>21</sup> Therefore, a range of lucigenin concentrations was used to assay  $\cdot\text{O}_2^-$  in a series of samples from single vessels by use of xanthine/xanthine oxidase calibration curves with the appropriate concentration of lucigenin in each sample. Superoxide production in IMA was similar, with a lucigenin concentration of either 5  $\mu\text{mol/L}$  (1715±343 pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$ ) or 250  $\mu\text{mol/L}$  (1410±93 pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$ ; n=6, P=0.59). In SV, sufficient tissue was available to assess the effect of lucigenin on  $\cdot\text{O}_2^-$  production at lucigenin concentrations of 5 to 250  $\mu\text{mol/L}$ . Superoxide production was 311±67 pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$  with 5  $\mu\text{mol/L}$  lucigenin, 208±27 pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$  with 15  $\mu\text{mol/L}$  lucigenin, 300±33 pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$  with 50  $\mu\text{mol/L}$  lucigenin, and 241±50 pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$  with 250  $\mu\text{mol/L}$  lucigenin (n=10 for each lucigenin concentration; P=NS between all groups). These data show that  $\cdot\text{O}_2^-$  production in IMA and SV was not affected by the concentration of lucigenin under these conditions.

### Experimental Design

IMA and SV tissues were incubated at 37°C in the absence (control) or presence of an inhibitor of SOD (diethylenethiocarbamate [DETCA], 100  $\mu\text{mol/L}$ ), a free radical scavenger (4,5-dihydroxy-1,3-benzene disulfonic acid salt [Tiron], 10 mmol/L), an inhibitor of NAD(P)H oxidase (diphenyleneiodonium [DPI], 10, 100, and 200  $\mu\text{mol/L}$ ), an inhibitor of eNOS (*N*<sup>ω</sup>-nitro-L-arginine methyl ester [L-NAME], 100  $\mu\text{mol/L}$ ), or an inhibitor of xanthine oxidase (allopurinol [ALP], 1 mmol/L) for 1 hour before quantification of  $\cdot\text{O}_2^-$ . In addition, some vessels were denuded of endothelium by rubbing. The absence of the endothelium and the integrity of the vessel wall were confirmed by histological analysis in a subset of 20 vessels. Vessels were also incubated in the absence (control) or presence of 1 pmol/L, 1 nmol/L, and 1  $\mu\text{mol/L}$  of Ang II for 1 and 4 hours. Functional integrity of the vessels was not compromised by this incubation. IMA tissues were also incubated with 1  $\mu\text{mol/L}$  of Ang II for 15 minutes. To assess the effects of a positive control, IMAs were incubated in the absence or presence of norepinephrine 1  $\mu\text{mol/L}$  for 4 hours. In addition, IMAs were coincubated with 1  $\mu\text{mol/L}$  of Ang II and either an  $\text{AT}_1$ -specific and competitive receptor antagonist (losartan, 1  $\mu\text{mol/L}$ ) or a nonspecific receptor antagonist (sar<sup>1</sup>, three<sup>8</sup> Ang II, 1  $\mu\text{mol/L}$ ).

**TABLE 2. Effect of Inhibition of NAD(P)H Oxidase, Xanthine Oxidase, and NOS and of Endothelial Denudation on  $\cdot\text{O}_2^-$  Production in IMA**

Treatment	n	$\cdot\text{O}_2^-$ Generation, pmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$			
		Control	Treated	% Change	P; 95% CI
DPI 10 $\mu\text{mol/L}$	8	965 $\pm$ 150	616 $\pm$ 212	-36 $\pm$ 17	0.14; -808, 239
DPI 100 $\mu\text{mol/L}$	8	2723 $\pm$ 696	1568 $\pm$ 430	-39 $\pm$ 13	0.03; -2515, -137
ALP 1 mmol/L	9	2120 $\pm$ 629	1024 $\pm$ 290	-42 $\pm$ 10	0.013; -2020, -330
L-NAME 100 $\mu\text{mol/L}$	10	1990 $\pm$ 508	1420 $\pm$ 304	-29 $\pm$ 16	0.1; -1421, 67
Endothelial denudation	10	643 $\pm$ 134	647 $\pm$ 145	-3 $\pm$ 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 $\pm$ SEM.

### Western Blotting

Five-millimeter segments of vessels were homogenized in 200  $\mu\text{L}$  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.<sup>3</sup> Ang II dose-response curves (10 pmol/L to 100 nmol/L) were performed in the presence and absence of 10  $\mu\text{mol/L}$  losartan and 10  $\mu\text{mol/L}$  [sar<sup>1</sup>, thre<sup>8</sup>]-Ang II.

### Materials

Xanthine, xanthine oxidase, lucigenin, DETCA, Tiron, DPI, L-NAME, ALP, [sar<sup>1</sup>, thre<sup>8</sup>]-Ang II, and norepinephrine were purchased from Sigma-Aldrich. Losartan was obtained as a gift from Merck, Sharp & Dohme (UK). DPI and ALP were dissolved in DMSO, and all other drugs were dissolved in buffer. For studies with DPI and ALP, the appropriate concentration of DMSO was added to control samples. Anti-Mn-SOD was 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 $\pm$ SEM or as percent change from controls to facilitate comparison between groups. Statistical analyses of vascular  $\cdot\text{O}_2^-$  concentrations were undertaken by use of the Wilcoxon signed-rank test. The relationships between risk factors and basal vascular  $\cdot\text{O}_2^-$  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  $\geq 1$  risk factors for CHD, and 92% of patients were on  $\geq 1$  types of antianginal therapy.

### Basal $\cdot\text{O}_2^-$ Production in Human Arteries and Veins

Superoxide production was greater in human arteries than in veins: IMA, 1922 $\pm$ 235 pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$  (n=55); SV, 662 $\pm$ 179 pmol  $\cdot$  min $^{-1}$   $\cdot$  mg $^{-1}$  (n=58); *P*<0.0001; 95% CI, 570, 1421 (Figure 1). Basal  $\cdot\text{O}_2^-$  production in human IMA was weakly associated with patient age (*r*=0.19, *P*<0.1). No other relationships existed between basal  $\cdot\text{O}_2^-$  production in either IMA or SV and any other patient characteristic.

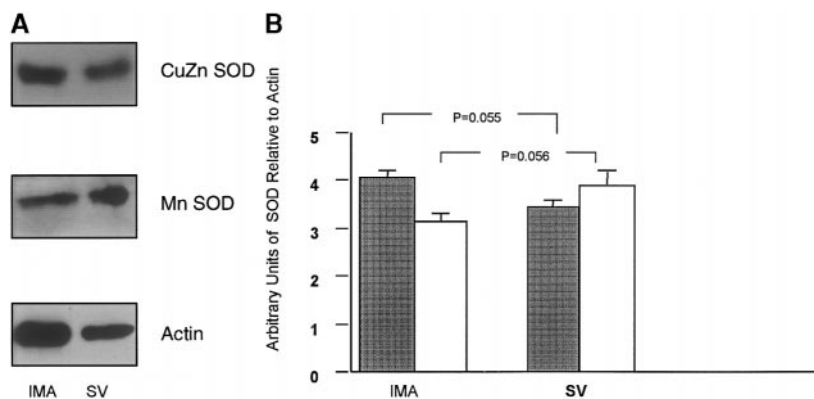
### Characterization of $\cdot\text{O}_2^-$ Production

The free radical scavenger Tiron (10 mmol/L) reduced basal  $\cdot\text{O}_2^-$  concentrations in both IMA (control, 1937 $\pm$ 412; Tiron, 10 mmol/L 901 $\pm$ 94; n=9, *P*=0.018; 95% CI, -1919, -238) and SV (control, 350 $\pm$ 85; Tiron 10 mmol/L, 149 $\pm$ 39; n=13, *P*=0.002; 95% CI, -311, -95). DETCA (100  $\mu\text{mol/L}$ ), an inhibitor of SOD, increased  $\cdot\text{O}_2^-$  steady-state concentrations in IMA (control, 853 $\pm$ 208; DETCA 100  $\mu\text{mol/L}$ , 1492 $\pm$ 347; n=8, *P*=0.021; 95% CI, 186, 1149) and SV (control, 551 $\pm$ 145; DETCA 100  $\mu\text{mol/L}$ , 945 $\pm$ 245; n=14, *P*=0.002; 95% CI, 67, 694). The NAD(P)H oxidase inhibitor DPI (concentration range, 10 to 200  $\mu\text{mol/L}$ ) attenuated  $\cdot\text{O}_2^-$  generation in both arteries (Table 2) and veins (Table 3). The xanthine oxidase inhibitor ALP (1 mmol/L) attenuated  $\cdot\text{O}_2^-$  production in both IMA and SV (Tables 2 and 3).

The effects on  $\cdot\text{O}_2^-$  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 (100  $\mu\text{mol/L}$ ) and endothelial denudation reduced  $\cdot\text{O}_2^-$  concentrations in 7 of 10 and 6 of 10 patients, respectively. In SV, these treatments corresponded to a reduction in  $\cdot\text{O}_2^-$  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 endothelium removal by rubbing in vessel segments from the same patients. In these studies, both manipulations had similar effects on  $\cdot\text{O}_2^-$  concentrations in individual patients (*r*=0.85, n=12, *P*<0.001). Furthermore, the difference in  $\cdot\text{O}_2^-$  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



**Figure 2.** Western blot analysis of CuZn-SOD and Mn-SOD in human IMAs and SVs. A, Representative autoradiograms showing single bands for Mn-SOD at 25 kD and CuZn-SOD at 19 kD. Actin loading control is also shown. B, Summary data for protein expression. Shaded bars represent Mn-SOD; open bars, CuZn-SOD (n=6 for each). Mn-SOD and CuZn-SOD are quantitatively similar in human IMA and SV.

CuZn-SOD bands relative to an actin control was  $4.04 \pm 0.31$  in arteries versus  $3.59 \pm 0.23$  in veins ( $P=0.055$ ). Mn-SOD expression was found to have relative intensities of  $3.14 \pm 0.25$  versus  $3.87 \pm 0.42$  ( $P=0.056$ ) in arteries and veins, respectively.

### Effect of Ang II on $\cdot\text{O}_2^-$ Production

Ang II at concentrations of  $1 \mu\text{mol/L}$ ,  $1 \text{ nmol/L}$ , and  $1 \text{ pmol/L}$  increased  $\cdot\text{O}_2^-$  production in IMA but not SV (Figure 3, Table 4). In IMA, Ang II had no effect on  $\cdot\text{O}_2^-$  production after 15 minutes of incubation (control,  $1173 \pm 239 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg tissue}^{-1}$ ;  $1 \mu\text{mol/L}$  of Ang II,  $918 \pm 170 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; n=11,  $P=0.12$ ; 95% CI, -680, 155) but increased  $\cdot\text{O}_2^-$  production after 1 and 4 hours, respectively. Norepinephrine ( $1 \mu\text{mol/L}$ ), which was used as a positive control, had no effect on  $\cdot\text{O}_2^-$  generation in either arteries (control,  $1581 \pm 899 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; norepinephrine,  $738 \pm 241 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; n=11,  $P=0.45$ ; 95% CI, -3590, 164) or veins (control,  $360 \pm 218 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; norepinephrine,  $249 \pm 118 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; n=7,  $P=0.18$ ; 95% CI, -391, 45).

Losartan ( $1 \mu\text{mol/L}$ ), a competitive  $\text{AT}_1$ -specific receptor antagonist, had no effect on basal  $\cdot\text{O}_2^-$  production but blocked the Ang II-mediated increase of  $\cdot\text{O}_2^-$  production (Figure 4). [Sar<sup>1</sup>, thre<sup>8</sup>]-Ang II ( $1 \mu\text{mol/L}$ ), a nonspecific Ang II receptor antagonist, also blocked Ang II-mediated increase of  $\cdot\text{O}_2^-$  production ([sar<sup>1</sup>, thre<sup>8</sup>]-Ang II,  $1 \mu\text{mol/L}$ ,  $1252 \pm 276 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; [sar<sup>1</sup>, thre<sup>8</sup>]-Ang II,  $1 \mu\text{mol/L}$  + Ang II,

$1 \mu\text{mol/L}$ ,  $1281 \pm 204 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; 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  $100 \mu\text{mol/L}$ , but not DPI  $10 \mu\text{mol/L}$ , blocked Ang II-mediated increase of  $\cdot\text{O}_2^-$  production (Figure 5).

### Discussion

We have shown that basal  $\cdot\text{O}_2^-$  concentrations are greater in human IMA than SV and have demonstrated that both NAD(P)H oxidase and xanthine oxidase enzymes contribute to basal  $\cdot\text{O}_2^-$  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  $\cdot\text{O}_2^-$  production in human arteries. This effect is  $\text{AT}_1$  receptor-dependent, as it was completely blocked by an  $\text{AT}_1$  receptor antagonist. We have also demonstrated that this Ang II-mediated increase of  $\cdot\text{O}_2^-$  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  $\cdot\text{O}_2^-$  production in human arteries, and this effect was blocked by losartan.

In the present study, the higher basal  $\cdot\text{O}_2^-$  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  $\cdot\text{O}_2^-$  generation, as arteries have a proportionately greater content of VSMCs than veins. The balance between  $\cdot\text{O}_2^-$  generation and degra-

**TABLE 3. Effect of Inhibition of NAD(P)H Oxidase, Xanthine Oxidase, and NOS and of Endothelial Denudation on  $\cdot\text{O}_2^-$  Production in SV**

Treatment	Number	$\cdot\text{O}_2^-$ Generation, $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$			
		Control	Treated	% Change	P; 95% CI
DPI $10 \mu\text{mol/L}$	10	$184 \pm 25$	$116 \pm 28$	$-34 \pm 16$	0.037; -124, -7
DPI $100 \mu\text{mol/L}$	14	$759 \pm 140$	$469 \pm 94$	$-37 \pm 6$	0.001; -519, -103
DPI $200 \mu\text{mol/L}$	8	$452 \pm 126$	$228 \pm 73$	$-47 \pm 7$	0.014; -394, -59
ALP $1 \text{ mmol/L}$	13	$759 \pm 173$	$426 \pm 107$	$-32 \pm 8$	0.003; -673, -57
L-NAME $100 \mu\text{mol/L}$	16	$284 \pm 44$	$214 \pm 36$	$-20 \pm 13$	0.06; -148, 5
Endothelial denudation	14	$324 \pm 48$	$250 \pm 40$	$-15 \pm 9$	0.04; -144, -8
Endothelial denudation+DPI $100 \mu\text{mol/L}$	9	$787 \pm 259$	$477 \pm 136$	$-29 \pm 9$	0.018; -671, -39

DPI, ALP, and L-NAME are inhibitors of NAD(P)H oxidase, xanthine oxidase, and eNOS enzymes, respectively. Data are shown as mean  $\pm$  SEM.

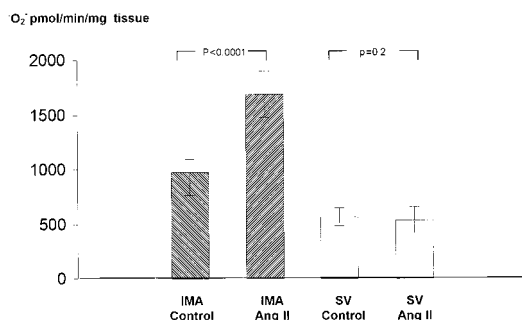
**TABLE 4. Effect of Ang II Concentration (1 pmol/L, 1 nmol/L, and 1  $\mu$ mol/L) on  $\cdot\text{O}_2^-$  Production in IMA After Incubation Periods of 1 and 4 Hours**

	$\cdot\text{O}_2^-$ Generation, pmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$					
	1 Hour			4 Hours		
	1 pmol/L (n=10)	1 nmol/L (n=9)	1 $\mu$ mol/L (n=11)	1 pmol/L (n=15)	1 nmol/L (n=11)	1 $\mu$ mol/L (n=27)
Control	1516 $\pm$ 253	1604 $\pm$ 322	1331 $\pm$ 232	1511 $\pm$ 324	1360 $\pm$ 293	978 $\pm$ 117
Ang II	1380 $\pm$ 138	1752 $\pm$ 323	2572 $\pm$ 708	2090 $\pm$ 358	2285 $\pm$ 502	1690 $\pm$ 213
<i>P</i>	0.8	0.52	0.012	0.057	0.004	0.0001
95% CI	-690, 396	-430, 540	170, 2672	-25, 1098	315, 1545	336, 925
% Change	12 $\pm$ 22	15 $\pm$ 16	76 $\pm$ 22	83 $\pm$ 30	93 $\pm$ 27	99 $\pm$ 24

Data are shown as mean $\pm$ SEM.

dation determines superoxide steady-state concentrations. In this study, the levels of Mn-SOD and CuZn-SOD proteins were quantitatively similar in arteries and veins. The activity and amount of EC-SOD protein are significantly higher in human arteries than veins.<sup>22</sup> Taken together with our results, this would suggest that the elevated concentrations of  $\cdot\text{O}_2^-$  observed in arteries compared with veins were not a consequence of a reduced capacity for enzymatic removal but rather occurred through increased  $\cdot\text{O}_2^-$  production.

We also sought to characterize the mechanisms of  $\cdot\text{O}_2^-$  production in human IMA and SV. Endothelial NOS activity was inhibited by removal of the endothelium by rubbing and by incubating vessels with L-NAME. This failed to reduce  $\cdot\text{O}_2^-$  steady-state concentrations in all patients. The lack of effect of these treatments on  $\cdot\text{O}_2^-$  production in the blood vessels of some patients suggests that VSMCs and adventitial fibroblasts were alternative sources of  $\cdot\text{O}_2^-$  generation. Inhibition of NAD(P)H oxidase and xanthine oxidase, by incubation of arteries and veins with DPI and ALP, respectively, resulted in similar reductions in basal  $\cdot\text{O}_2^-$  steady-state concentrations in both of these tissues. This suggests that both NAD(P)H and xanthine oxidases are sources of  $\cdot\text{O}_2^-$  generation in human arteries and veins. Observations from in vitro studies suggest that the activity of xanthine oxidase may be increased in endothelial cells subject to ischemia-reperfusion injury.<sup>23</sup> Findings from in vivo human studies suggest that patients with risk factors for CAD also have increased vascular xanthine oxidase-mediated  $\cdot\text{O}_2^-$  pro-

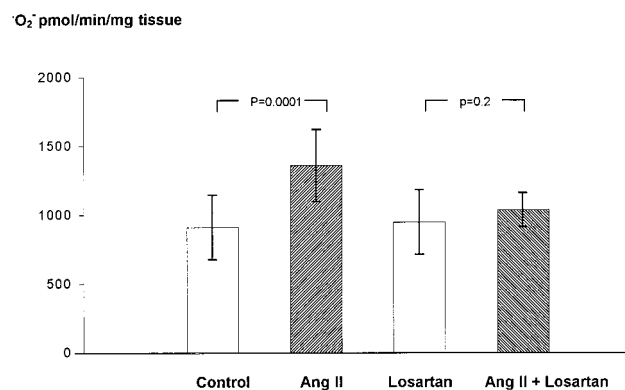


**Figure 3.** Effect of Ang II on  $\cdot\text{O}_2^-$  production in human IMAs and SVs. Blood vessels were incubated in presence or absence of 1  $\mu$ mol/L Ang II for 4 hours. Results are expressed as pmol  $\cdot$  min $^{-1}$   $\cdot$  mg tissue $^{-1}$  and are shown as mean $\pm$ SEM. Shaded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

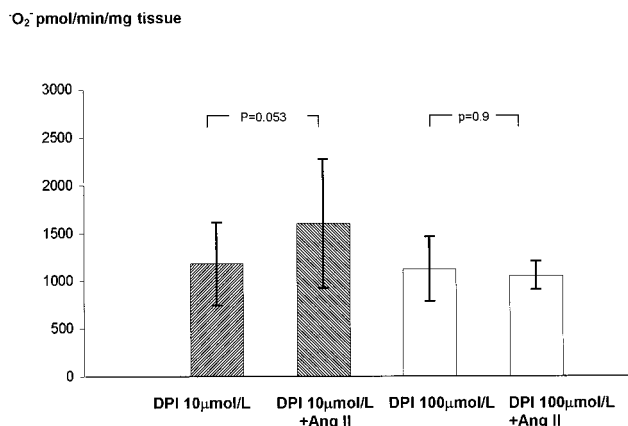
duction, which may contribute to impaired endothelium-dependent vasodilation in these patients.<sup>24</sup> Considered together with our findings, this suggests that xanthine oxidase may be an important source of vascular  $\cdot\text{O}_2^-$  production in patients who have CAD.

In the present study, basal  $\cdot\text{O}_2^-$  concentrations in IMA were weakly related to patient age. The variation in basal vascular  $\cdot\text{O}_2^-$  concentrations observed in this and other human studies<sup>19</sup> and the lack of correlation of  $\cdot\text{O}_2^-$  production with some atherosclerotic risk factors, treatment with nitrates, and other therapies may be due to the heterogeneous clinical characteristics of patients with CAD. Such patients have differences in their genetic backgrounds, their atherosclerotic risk factors, their disease duration and severity, and their drug therapy.

We also investigated whether a vasoactive hormone, such as Ang II, could affect  $\cdot\text{O}_2^-$  production in human blood vessels. Ang II induced isometric contractions in both arteries and veins but increased  $\cdot\text{O}_2^-$  production only in arteries. These effects are unlikely to be due to a reduction in AT<sub>1</sub> receptor expression in veins, because the constrictor effect of Ang II in human saphenous veins, which is known to be AT<sub>1</sub>-dependent,<sup>25</sup> may be greater in SV than IMA.<sup>26</sup> Ang II exerts different effects in distinct vascular beds.<sup>27</sup> One expla-



**Figure 4.** Effect of losartan, a specific AT<sub>1</sub> receptor antagonist, on Ang II-mediated increase in  $\cdot\text{O}_2^-$  production. IMAs were incubated in presence or absence of 1  $\mu$ mol/L Ang II (n=13) or 1  $\mu$ mol/L losartan or were coincubated with 1  $\mu$ mol/L Ang II and 1  $\mu$ mol/L losartan (n=15) for 4 hours. Results are expressed as pmol  $\cdot$  min $^{-1}$   $\cdot$  mg tissue $^{-1}$  and are shown as mean $\pm$ SEM. Open bars indicate control or losartan; shaded bars, IMA incubated with Ang II and/or losartan.



**Figure 5.** Effect of NAD(P)H oxidase inhibition on Ang II-induced increase in  $\cdot\text{O}_2^-$  production in human IMA. IMAs were incubated with 10  $\mu\text{mol/L}$  ( $n=8$ ) and 100  $\mu\text{mol/L}$  ( $n=10$ ) DPI in presence or absence of 1  $\mu\text{mol/L}$  Ang II for 4 hours. Results are shown as mean  $\pm$  SEM. Shaded bars indicate IMA incubated with 10  $\mu\text{mol/L}$  DPI; open bars, IMA incubated with 100  $\mu\text{mol/L}$  DPI.

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.<sup>28</sup> Our observations may also be substantiated by a report that the pressor effect of intrabrachial artery infusion of Ang II in humans is attenuated by the coinfusion of vitamin C.<sup>29</sup> 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 VSMCs<sup>16</sup> and mesangial cells<sup>30</sup> and also contributes to the pressor effect of this hormone in a hypertensive rat model.<sup>17</sup>

We have shown that Ang II increases  $\cdot\text{O}_2^-$  production in human arteries by an  $\text{AT}_1$ -dependent mechanism. The mechanism of Ang II-mediated increase of  $\cdot\text{O}_2^-$  production differs in other species. An  $\text{AT}_1$ -dependent Ang II-mediated increase of  $\cdot\text{O}_2^-$  production has been reported in the aorta<sup>17</sup> and mesangium<sup>30</sup> of Sprague-Dawley rats and aorta of hypercholesterolemic rabbits.<sup>18</sup> However, the Ang II-induced increase in  $\cdot\text{O}_2^-$  production in normocholesterolemic rabbit aortic adventitial fibroblasts is mediated by a non- $\text{AT}_1$ -non- $\text{AT}_2$ -receptor mechanism.<sup>18,31</sup> 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  $\text{AT}_1$  receptor antagonist losartan. These observations suggest a putative therapeutic role for

$\text{AT}_1$  receptor antagonists in reducing oxidative stress in cardiovascular disease.

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