Vascular Superoxide Production and Vasomotor Function in Hypertension Induced by Deoxycorticosterone Acetate–Salt

Mark J. Somers, MD; Kreton Mavromatis, MD; Zorina S. Galis, PhD; David G. Harrison, MD

Background—Angiotensin II–induced hypertension is associated with increased vascular superoxide production, which contributes to hypertension caused by the octapeptide. In cell culture, stretch increases endothelial and vascular smooth muscle production of reactive oxygen species (ROS). In perfused isolated vessels, elevations of pressure can increase vessel angiotensin II production. The effects of low-renin hypertension on vascular ROS production remain unclear. Furthermore, the role of ROS in vascular function and hypertension in low-renin hypertension is undefined.

Methods and Results—Rats were treated with DOCA and saline drinking water for 3 weeks. Both systolic blood pressure (189 ± 4 versus 126 ± 2 mm Hg) and aortic superoxide production (3972 ± 257 versus 852 ± 287, P < 0.05) were increased compared with controls. Relaxes of vascular segments to acetylcholine (ACh, 100 ± 2% versus 75 ± 2%, P < 0.05) and the calcium ionophore A23187 (92 ± 2% versus 72 ± 3%, P < 0.05) were also impaired in DOCA-salt. Heparin-binding superoxide dismutase (1200 U/d IV for 3 days) had no effect on blood pressure but significantly improved relaxations to ACh and A23187. Losartan (25 mg · kg⁻¹ · day⁻¹ PO) for 7 days did not correct the hypertension or endothelium-dependent vessel relaxation in DOCA-salt rats, excluding a role of a local renin/angiotensin II system.

Conclusions—These findings indicate that increased vascular superoxide production occurs not only in angiotensin II–induced hypertension but also in hypertension known to be associated with low-renin states. Increased superoxide production alters large-vessel endothelium-dependent vascular relaxation but does not modulate blood pressure in low-renin hypertension. (Circulation. 2000;101:1722-1728.)

Key Words: hypertension • angiotensin • free radicals • endothelium

The production of reactive oxygen species by vascular cells seems to play a critical role in the genesis of vascular disease. Recently, it has become clear that angiotensin II can dramatically increase the activity of an NADH/NADPH-driven oxidase, a major source of reactive oxygen species in vascular cells. In rats, angiotensin II–induced hypertension is associated with an increase in vascular superoxide production, which in turn seems to reduce the biological activity of endothelium-derived nitric oxide (NO). This loss of NO bioactivity dramatically alters vascular reactivity and contributes to elevations of blood pressure caused by angiotensin II. Increased production of hydrogen peroxide in response to angiotensin II seems to be critical in the development of vascular smooth muscle hypertrophy caused by the peptide.

In previous studies, we have shown that short-term hypertension (5 days) caused by norepinephrine, unlike that caused by angiotensin II, was not associated with an increase in vascular production of superoxide and did not alter vascular reactivity. These findings suggested that angiotensin II may be unique as a cause of vascular oxidant stress, whereas other causes of hypertension may not have this effect on vascular function. This conclusion was supported by the observation that lower-dose administration of angiotensin II, not associated with an increase in blood pressure, also increased vascular superoxide production. An important caveat is that the lack of effect of norepinephrine on vascular superoxide production was observed during a relatively short term (5 days) of hypertension. Furthermore, hypertension caused by norepinephrine is unique in that it is associated with an increase in cardiac output and ultimately an increase in vascular shear stress, which may have independent effects on vascular function.

In contrast to the above-described findings, recent studies have shown that stretch of vascular cells can enhance production of both superoxide and H₂O₂. These findings raise the possibility that the direct effects of hypertension, which directly increases stretch of vascular smooth muscle cells in vivo, might also increase vascular production of reactive oxygen species. To examine this, we studied rats with deoxycorticosterone acetate (DOCA)-salt hypertension. This model of hypertension is associated with markedly depressed plasma renin activity and thus provides an opportunity to study the effect of hypertension in the absence of angiotensin II on vascular superoxide production and vascular reactivity.
Methods

Materials
Deoxycorticosterone acetate (DOCA) pellets and control pellets were purchased from Innovative Research of America. Heparin was purchased from Upjohn Corp. Pentobarbital and ketamine were purchased from Abbot Laboratories. Xyline was purchased from Bayer. Nitroglycerin was purchased from American Regent Laboratories. Losartan was provided as a gift from Merck. Heparin-binding superoxide dismutase (HB-SOD) was provided by Dr Margaret Tarpey, Birmingham, Ala. Dihydroethidium was purchased from Molecular Probes. All other chemicals were purchased from Sigma-Aldrich.

DOCA-Salt Rats
Male Sprague-Dawley Rats (250 to 300 g, Harlan Sprague Dawley Inc, Indianapolis, Ind) were anesthetized with intraperitoneal ketamine (80 mg/kg) and xyline (10 mg/kg). A midscapular incision was made, and a 100-mg slow-release DOCA pellet was inserted subcutaneously. A right flank incision was made and a right nephrectomy performed. Drinking water was replaced by 1% saline. Control rats underwent nephrectomy and implantation with a sham pellet and were given water ad libitum. A separate group of rats underwent DOCA pellet implantation alone without saline to determine the direct effects of DOCA on endothelial function. On day 16, the vessels were acquired 1 hour in the plethysmography unit. On day 21 after operation, blood pressures were measured by tail-cuff plethysmography, and the rats were euthanized with intraperitoneal sodium pentobarbital.

In some experiments, rats were treated with HB-SOD. HB-SOD is a recombinant form of SOD that accumulates in the vascular extracellular matrix and potently scavenges O$_2^-$ produced by vascular cells. The rats were anesthetized on day 15 after DOCA pellet implantation, and a Tygon catheter was implanted in the left carotid artery with the tip advanced to the ascending aorta. A second catheter was implanted into the left jugular vein. The catheters were externalized through a second incision and plugged with a nylon pin. On days 18 to 21, the rats underwent intravenous injection with 600 U/kg of HB-SOD every 12 hours. On the day of death, the blood pressure was monitored with the aortic catheter connected to a Gould pressure transducer and an oscillographic recorder (Gould RS3600).

AT$_1$ Receptor Antagonist Studies
In some rats, on day 10 to 21 after pellet implantation, the rats were given 25 mg/kg of the specific angiotensin II receptor type 1 antagonist losartan in their drinking fluid.

Vessel Preparation
The aorta was placed into chilled modified Krebs/HEPES buffer (composition in mmol/L: NaCl 99.01, KCl 4.69, CaCl$_2$ 1.87, MgSO$_4$ 1.20, K$_2$HPO$_4$ 1.03, NaHCO$_3$ 25.0, Na-HEPES 20.0, and glucose 11.1; pH 7.4). The aorta was placed into chilled modified Krebs/HEPES buffer and allowed to equilibrate for 30 minutes at 37°C. Scintillation vials containing 2 mL Krebs/HEPES buffer with 5 mmol/L lucigenin were placed into a scintillation counter switched to out-of-coincidence mode. After dark adaptation, background counts were recorded, and a vascular segment was added to the vial. Scintillation counts were then recorded every minute, and the counts from 15 to 19 minutes were averaged (steady-state production). The vessels were then dried in an oven at 90°C for 24 hours, and the counts were expressed as counts above background per milligram dry tissue.

Additional studies were performed with in situ dihydroethidium fluorescence as described previously. Three frozen 30-μm tissue sections from each of 3 matched pairs of DOCA-salt and control rat aortas were placed on glass slides. The sections were submerged in 2 μmol/L dihydroethidium in Krebs/HEPES buffer and incubated at 37°C for 30 minutes in a dark, humidified container. Tissue sections were then visualized with a Bio-Rad MRC 1024 argon confocal microscope with fluorescence detected with a 585-nm long-pass filter, and images were collected and stored digitally. Paired aortas from DOCA-salt and control rats were processed in parallel, and images were acquired with identical acquisition parameters.

Isolated Vascular Ring Experiments
Four 5-mm ring segments of the thoracic aorta were suspended in individual organ chambers filled with Krebs buffer of the following composition (mmol/L): NaCl 99.01, KCl 4.69, CaCl$_2$ 1.87, MgSO$_4$ 1.20, K$_2$HPO$_4$ 1.03, NaHCO$_3$ 25.0, Na-HEPES 20.0, and glucose 11.1; pH 7.4. The solution was aerated continuously with a 95% O$_2$/5% CO$_2$ mixture and maintained at 37°C. Care was taken not to injure the endothelium during preparation of the rings. Tension was recorded with a linear force transducer. Over a period of 1 hour, the resting tension was gradually increased until the optimal tension for generating force during isometric contraction was achieved; this proved to be 4.0 g in all subsets of animals. The vessels were left at this resting tension throughout the remainder of the study. To prevent synthesis of prostaglandins, we performed all experiments in the presence of 10 μmol/L indomethacin. The vessels were then precontracted with l-phenylephrine (10$^{-7}$ mol/L). After a stable contraction plateau was reached, the rings were exposed to either acetylcholine (1 nmol/L to 3 μmol/L), the calcium ionophore (1 nmol to 3 μmol/L), or nitroglycerin (1 nmol to 3 μmol/L).

Data Analysis
Data are expressed as mean±SEM. Comparisons between groups of animals or treatments were made by 1-way ANOVA. When significance was indicated, a Student-Newman-Keuls post hoc analysis was used.

Results
Effects of DOCA-Salt on Blood Pressure and Vascular Superoxide Production
Treatment with DOCA-salt caused a significant increase in systolic blood pressure after 21 days, from 126±2 mm Hg in sham-operated rats to 189±4 mm Hg in DOCA-salt–treated
rats (Figure 1A). The amount of superoxide measured by lucigenin chemiluminescence was significantly higher in aortas from DOCA-salt rats (3972 ± 2257 counts mg⁻¹ min⁻¹) than that in aortas from sham-operated animals (852 ± 287 counts mg⁻¹ min⁻¹) (Figure 1B).

To examine vascular O₂⁻ production by an independent approach and to gain insight into the vascular localization of O₂⁻ production, we also used dihydroethidium fluorescent staining. Aortas of DOCA-salt rats consistently showed increased red fluorescence, indicating increased superoxide levels, compared with controls (n=3 pairs of DOCA-salt and sham-operated rats, Figure 2). Of note, dihydroethidium fluorescence was increased in all cell layers of vessels from DOCA-salt rats compared with controls.

**Effects of DOCA-Salt on Vascular Relaxation**

As shown in Figure 3 and the Table, DOCA-salt produced significant impairment in endothelium-dependent and -independent vascular relaxations. The maximum relaxation induced by acetylcholine, calcium ionophore A23187, and nitroglycerin was all significantly reduced. Sensitivity to acetylcholine, as reflected by the EC₅₀, was also reduced.

**Role of Superoxide in Hypertension and Vascular Relaxations in DOCA-Salt Hypertension**

The above-cited data show that DOCA-salt hypertension is associated with an increase in vascular O₂⁻ production and altered endothelium-dependent vascular relaxations. In angiotensin II–induced hypertension, both hypertension and altered endothelium-dependent vascular relaxation can be improved by treatment with membrane-targeted forms of superoxide dismutase (SOD). We therefore examined the role of increased vascular O₂⁻ in hypertension and altered endothelium-dependent vascular relaxation in DOCA-salt hyperten-

**Effects of DOCA-Salt, SOD Treatment, and Losartan on Vascular Reactivity**

<table>
<thead>
<tr>
<th>Intervention</th>
<th>ACh ED₅₀</th>
<th>ACh Relaxation</th>
<th>A23187 ED₅₀</th>
<th>A23187 Relaxation</th>
<th>NTG ED₅₀</th>
<th>NTG Relaxation</th>
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<tbody>
<tr>
<td>Sham</td>
<td>-7.45±0.05</td>
<td>100±2</td>
<td>-7.25±0.09</td>
<td>91±2</td>
<td>-7.58±0.06</td>
<td>95±2</td>
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<tr>
<td>DOCA</td>
<td>-7.00±0.09*</td>
<td>75±2</td>
<td>-7.22±0.08</td>
<td>68±3*</td>
<td>-7.18±0.05*</td>
<td>85±2*</td>
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<tr>
<td>Sham+SOD</td>
<td>-7.56±0.13</td>
<td>102±3</td>
<td>-7.47±0.07</td>
<td>91±7</td>
<td>-7.86±0.09*</td>
<td>100±2</td>
</tr>
<tr>
<td>DOCA+SOD</td>
<td>-6.92±0.06*</td>
<td>88±2*</td>
<td>-7.12±0.06</td>
<td>82±3</td>
<td>-7.15±0.04*</td>
<td>90±1</td>
</tr>
<tr>
<td>Sham+losartan</td>
<td>-7.43±0.10</td>
<td>99±3</td>
<td>-7.32±0.13</td>
<td>97±2</td>
<td>-7.66±0.10</td>
<td>97±2</td>
</tr>
<tr>
<td>DOCA+losartan</td>
<td>-6.86±0.11*</td>
<td>71±7*</td>
<td>-6.92±0.16</td>
<td>76±8*</td>
<td>-7.27±0.16*</td>
<td>83±6*</td>
</tr>
</tbody>
</table>

ACh indicates acetylcholine; NTG, nitroglycerine.
*Significantly different from Sham.
ED₅₀ are log molar concentrations. Relaxations are percent preconstriction.
sion. Treatment with heparin-binding SOD did not significantly decrease the blood pressure in rats with DOCA-salt hypertension (189±4 mm Hg without SOD versus 195±3 mm Hg with SOD) (Figure 4). Treatment with heparin-binding SOD significantly improved endothelium-dependent vascular relaxations to A23187 and acetylcholine in DOCA-salt–treated rats compared with similar responses in DOCA-salt rats not treated with SOD (Figure 5 and Table).

Role of a Local Renin-Angiotensin System in DOCA-Salt Hypertension
It has been shown in an ex vivo animal model that perfusion of vessels at hypertensive pressures activates production of angiotensin II by a local renin-angiotensin system.17 Furthermore, angiotensin II increases production of reactive oxygen species by vascular smooth muscle cells in both cultured cells and intact vessels. Thus, it is possible that increased local levels of angiotensin II produced in the vessel wall might mediate the increase in vascular O$_2^\cdot$ observed in DOCA-salt hypertension. As shown in Figure 6, however, losartan did not alter blood pressure or endothelium-dependent vascular relaxation in rats with DOCA-salt hypertension.

Effect of DOCA Alone on Superoxide, Pressure, and Vascular Relaxation
DOCA-salt may increase vascular superoxide production by a direct mineralocorticoid effect rather than stretch. To investigate this possibility, a separate group of rats were implanted with a DOCA pellet and given water ad libitum. No significant changes were seen in blood pressure, vascular O$_2^\cdot$ production, or vascular relaxation compared with placebo.

Discussion
The new findings in this study are that in DOCA-salt hypertension, there is an increase in vascular O$_2^\cdot$ production that at least in part contributes to alterations of endothelium-dependent vascular relaxation. This increase in O$_2^\cdot$ production occurs in a model of hypertension known to be associated with suppression of the systemic renin-angiotensin system, suggesting that it was independent of locally pro-

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**Figure 3.** Effect of DOCA-salt on vascular ring relaxation. Aortic rings from DOCA-salt and placebo control rats were preconstricted with phenylephrine, and relaxations to acetylcholine, calcium ionophore A23187, and nitroglycerin (NTG) were measured. Significant impairment was seen in relaxation to all 3 agents.

**Figure 4.** Blood pressure response in DOCA-salt rats treated with HB-SOD. Hypertension seen in DOCA-salt hypertension was unchanged after 3 days of twice-daily treatment with 1200 U · kg$^{-1} ·$d$^{-1}$ of HB-SOD.

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Systolic Blood Pressure (mm Hg)
duced angiotensin II. Furthermore, unlike the effect on endothelium-dependent vascular relaxation, the reducing vascular $O_2$ production did not ameliorate the hypertension caused by DOCA-salt.

In previous studies, we found that hypertension caused by angiotensin II markedly increased vascular $O_2$ production, which in turn diminished endothelium-dependent vascular relaxation and contributed to the elevation of blood pressure caused by the octapeptide.3,4 These properties were not shared by norepinephrine-induced hypertension, suggesting that angiotensin II–induced hypertension may have unique untoward effects on vascular function. These previous studies compared hypertension caused by rather short-term (5-day) infusions of angiotensin II or norepinephrine but did not address the possibility that longer-term hypertension of any cause might also increase vascular production of reactive oxygen species. The present studies show that long-term hypertension, in the presence of a suppressed renin-angiotensin system, can also dramatically increase vascular production of $O_2$.

The increase in vascular $O_2$ production detected by lucigenin-enhanced chemiluminescence was confirmed by in situ dihydroethidium staining. Oxidation of dihydroethidium to ethidium, as detected by this fluorescent technique, has been shown to be specific for $O_2$.18 Interestingly, fluorescence was particularly increased in endothelial and adventitial cells but was also increased in vascular smooth muscle cells of DOCA-salt rats compared with paired images of sham-treated rats. The mechanisms and enzyme systems responsible for increased $O_2$ production in these various cell layers remain unidentified and may vary. A major source of vascular $O_2$ production has been shown to be an NADH/NADPH oxidase.19,20 In cultured cells, this oxidase is activated by cyclic stretch,8 and it is conceivable that the direct mechanical effect of hypertension on the vessel wall has a similar effect in vivo. It has recently been shown that all of the components of the neutrophil NADPH oxidase exist in endothelial and adventitial cells.21,22 In contrast, only p22phox and p47phox have been definitively identified in vascular smooth muscle cells to date.23,24 Other enzyme systems may also be involved, including NO synthase, which can produce $O_2$ in the absence of either l-arginine or tetrahydrobiopterin.25,26

Figure 5. Effect of HB-SOD on DOCA-salt vascular ring relaxation. Aortic rings from DOCA-salt and placebo control rats were preconstricted with phenylephrine, and relaxations to acetylcholine, calcium ionophore A23187, and nitroglycerin (NTG) were measured. HB-SOD caused a significant improvement in peak relaxation to calcium ionophore A23187 and acetylcholine.

Figure 6. Top, Effect of losartan on DOCA-salt hypertension. Losartan failed to significantly correct hypertension seen in DOCA-salt. Bottom, Effect of losartan on DOCA-salt vascular ring relaxation. Aortic rings from DOCA-salt and placebo control rats were preconstricted with phenylephrine, and relaxations to acetylcholine (ACH), calcium ionophore A23187, and nitroglycerin were measured. Losartan caused no significant improvement in vascular relaxations.
Our findings are compatible with previous studies showing that endothelium-dependent vascular relaxations are altered in both conductance and resistance vessels in DOCA-salt hypertension.\textsuperscript{27} The present findings add to these previous studies by showing that this abnormality of vascular function is in part caused by an increase in vascular $O_2^{\cdot -}$, because these responses were improved by treatment with heparin-binding SOD. In rat aorta, the major endothelium-derived relaxing factor is NO, which rapidly reacts with $O_2^{\cdot -}$ to form the peroxynitrite anion.\textsuperscript{28} The fact that SOD improved these responses is consistent with a role of $O_2^{\cdot -}$ in alteration of NO bioavailability. Interestingly, responses to nitroglycerin were improved by treatment with heparin-binding (HB)-SOD in sham-treated but not in DOCA-salt rats. This may be related to the sites of nitroglycerin bioconversion, the location at which the HB-SOD accumulates, and the site of $O_2^{\cdot -}$ production in vessels of sham-treated and DOCA-salt rats.

Treatment with heparin-binding SOD failed to lower blood pressure in rats with DOCA-salt hypertension. This is in contrast to the effect of membrane-targeted forms of SOD and other antioxidants on blood pressure in angiotensin II--induced hypertension and in spontaneously hypertensive rats.\textsuperscript{3,29,30} The reasons for these differences remain unclear. DOCA-salt hypertension is largely mediated by increased intravascular volume and circulating catecholamines, factors that are most likely independent of vascular superoxide production. Indeed, Huang et al.\textsuperscript{31,32} showed that the severity of hypertension in the DOCA-salt model is relatively independent of systemic vascular resistance. In these previous studies, the potent vasodilator minoxidil lowered systemic vascular resistance by 20% but did not alter blood pressure in rats with DOCA-salt hypertension. Thus, even if the bioactivity of NO in the resistance circulation were to be improved by heparin-binding SOD, this might not affect blood pressure in DOCA-salt hypertension.

It has recently been shown that ex vivo perfusion of vessels at hypertensive pressures activates production of angiotensin II by a local renin-angiotensin system.\textsuperscript{17} Consequently, it is possible that locally produced angiotensin II, even in the absence of increased plasma angiotensin II, might have contributed to the increase in vascular $O_2^{\cdot -}$ production in DOCA-salt hypertension. To exclude a role of local angiotensin II, some animals were treated with the AT$_1$ receptor antagonist losartan. Blockade of this angiotensin II receptor failed to block the effects of DOCA-salt on blood pressure or vascular relaxation. These data exclude the role of a local renin–angiotensin II system in the effects of DOCA-salt on vascular reactivity.

Although these studies show that the vascular production of reactive oxygen species is increased by chronic hypertension in the absence of an elevation of angiotensin II, they do not discount an important role of angiotensin II in conditions in which it is elevated. Angiotensin II activates the NADH/NADPH oxidase in vascular smooth muscle cells both in culture\textsuperscript{1} and when infused in vivo in low concentrations that only minimally affect blood pressure.\textsuperscript{3} Recent studies suggest that locally produced angiotensin II may increase vascular $O_2^{\cdot -}$ production in atherosclerotic vessels in the absence of an increase in blood pressure.\textsuperscript{13,33} Taken together with our present findings, these data strongly suggest that hypertension per se can increase vascular production of reactive oxygen species but that angiotensin II can produce this effect via 2 mechanisms. These include a direct action of the octapeptide on vascular smooth muscle cells and an increase in blood pressure, which in turn stimulates vascular production of reactive oxygen species.

In summary, the present studies show that hypertension associated with low levels of angiotensin II can increase in vascular $O_2^{\cdot -}$ production, which in turn reduces the bioactivity of endothelium-derived NO. In light of previous studies of renovascular hypertension,\textsuperscript{34} genetic hypertension,\textsuperscript{29,30,35} and hypertension caused by exogenous angiotensin II,\textsuperscript{2–4} these studies suggest that hypertension of almost any cause can increase vascular oxidant stress. An important aspect of this phenomenon is a reduction of NO bioactivity. NO has several effects on vascular homeostasis that inhibit the atherogenic process. These include inhibition of expression of the vascular cell adhesion molecule-1,\textsuperscript{1,36} and the monocyte chemoattractant protein-1,\textsuperscript{37} suppression of nuclear factor-$\kappa$B activation,\textsuperscript{38} and inhibition of platelet adhesion and vascular smooth muscle cell proliferation. Loss of these effects via an increase in vascular $O_2^{\cdot -}$ production could contribute to the proatherogenic effect of hypertension and emphasizes the importance of blood pressure lowering to minimize the development of vascular disease.

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**References**


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