Increased Endothelin-1 Content in Blood Vessels of Deoxycorticosterone Acetate–Salt Hypertensive but Not in Spontaneously Hypertensive Rats

Richard Larivière, Gaétan Thibault, and Ernesto L. Schiffrin

Endothelin-1 (ET-1) is a powerful vasoconstrictor peptide produced in the endothelium of blood vessels that may play an important role in the control of local blood flow and could be involved in the pathogenesis of hypertension. We investigated immunoreactive ET-1 (ir-ET-1) levels in acid extracts from blood vessels of deoxycorticosterone acetate (DOCA)–salt and spontaneously hypertensive rats. We found that segments of thoracic aorta and the mesenteric vascular bed contain significantly more ir-ET-1 (11.84±0.84 and 17.30±1.89 fmol, respectively) than uninephrectomized control rats (1.78±0.20 and 9.19±0.63 fmol, respectively; p<0.001). High performance liquid chromatography showed that ir-ET-1 of blood vessels of DOCA-salt hypertensive rats eluted in the same position as synthetic ET-1. Significantly increased ir-ET-1 was localized by immunohistochemistry in endothelial cells of aorta and large and small mesenteric arteries of DOCA-salt hypertensive rats. In contrast to the latter, in spontaneously hypertensive rats, vascular content of ir-ET-1 was similar to that of blood vessels of Wistar-Kyoto control rats, at both 6 and 16 weeks of age. High levels of vascular ET-1 may explain the downregulation of vascular endothelin receptors previously described in DOCA-salt hypertensive rats. Furthermore, this suggests that ET-1 may be involved in the maintenance of high blood pressure in mineralocorticoid hypertension. (Hypertension 1993;21:294–300)

Key Words • endothelins • aorta • mesenteric arteries • endothelium

Endothelin-1 (ET-1) is the most powerful endogenous vasoconstrictor agent known to date. ET-1 is a 21–amino acid peptide produced primarily in endothelial cells but also in vascular smooth muscle cells. Locally produced ET-1 exerts its effects in an autocrine and paracrine fashion to constrict blood vessels by acting on smooth muscle. Through its effect on endothelial cells, it induces the production of endothelium-derived relaxing factor and prostacyclin, which relax underlying smooth muscle cells. The balance between these responses may contribute to the control of blood pressure. The role of ET-1 in hypertension, however, remains unclear. Plasma ET-1 levels in different models of hypertension in the rat and in essential hypertension in humans have been found to be similar or slightly higher in comparison to normotensive controls. Recently, we have shown that arteries of deoxycorticosterone acetate (DOCA)–salt hypertensive rats respond less to ET-1 than those of uninephrectomized control rats. This was in part explained by downregulation of vascular endothelin receptors, accompanied by a reduction of phospholipase C activity. We have speculated that downregulation of endothelin receptors could be secondary to exaggerated production of ET-1 in blood vessels of DOCA-salt hypertensive rats. Here, we show that aorta and mesenteric arteries of DOCA-salt hypertensive rats contain greater amounts of immunoreactive ET-1 (ir-ET-1), suggesting that vascular ET-1 may be involved in some forms of hypertension.

Methods

Animal Experiments

Animal experiments were performed following the recommendations of the Canadian Council of Animal Care and were approved by the Animal Care Committee of the Clinical Research Institute of Montreal. Spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were bought from Taconic Farms ( Germantown, N.Y.) and were studied at 6 and 16 weeks of age. Rats were housed under conditions of constant temperature (22°C) and humidity (60%), exposed to a 12-hour light/dark cycle, and offered tap water to drink. DOCA-salt hypertension was induced by the method of Ormsbee and Ryan. Male Sprague-Dawley rats, purchased from Charles River Laboratories (St. Constant, Canada) and weighing 200 g, were uninephrectomized under sodium
pentobarbital anesthesia (40 mg/kg) (Somnotol, MTC Pharmaceuticals, Cambridge, Canada). Silicone rubber impregnated with DOCA (130 mg per rat) was implanted subcutaneously, and rats were offered 1% saline to drink. Rats were studied 2–3 weeks after becoming hypertensive (blood pressure >150 mm Hg). Another group of uninephrectomized rats having silicone rubber implanted without DOCA and receiving tap water to drink served as controls. Blood pressure was measured the day before experiments by the tail-cuff method on conscious semi-restrained rats after warming and was recorded on a model 7 polygraph (Grass Instrument Co., Quincy, Mass.) fitted with a 7P8 preamplifier and Grass model 1010 crystal microphone as a pulse detector. The average of three pressure readings was obtained. Rats were killed by decapitation. Blood was collected from the trunk in Vacutainer tubes (Becton Dickinson, Rutherford, N.J.) containing potassium edetate and was centrifuged. Plasma was stored at −20°C until plasma renin activity and plasma ir-ET-1 were assayed.

**Measurement of Immunoreactive Endothelin-1 in Blood Vessels**

A 1.5-cm segment of thoracic aorta and the complete mesenteric vascular bed from all experimental groups of rats were removed, dissected free of fat and connective tissue, and quickly frozen in liquid nitrogen. Frozen tissues were homogenized with a Polytron (Kinematica, Lucerne, Switzerland) for 15 seconds in 2 mL ice-cold extraction solution containing 1N HCl, 1% formic acid, 1% trifluoroacetic acid, and 1% NaCl. The homogenate was centrifuged at 1,500g for 30 minutes at 4°C. The supernatant was passed through a C18 Sep-Pak cartridge preactivated with 1 M acetic acid. After washing with 10 mL of 1 mol/L acetic acid, ir-ET-1 was eluted with 3 mL 80% acetonitrile. The samples were dried overnight in a Speed-Vac (Savant Instruments Inc., Farmingdale, N.Y.) and reconstituted in 300 μL radioimmunoassay (RIA) buffer containing 50 mM sodium phosphate, pH 7.4, 0.1% bovine serum albumin, 150 mM NaCl, and 0.1% Triton X-100. ET-1 (Peninsula Laboratories, Belmont, Calif.) was reconstituted in 1% trifluoroacetic acid and loaded onto a Vydac protein C4 column (Separation Group, Hesperia, Calif.) for 30 minutes. The sections were then incubated for 60 minutes in the ABP solution prepared by adding 25 μL avidin DH and 25 μL biotinylated peroxidase to 4 mL dilution buffer (0.05 mol/L phosphate plus 2.5 mg/mL bovine serum albumin). Peroxidase activity was visualized by incubation in a medium containing 3 mg of 3,3′-diaminobenzidine and 10 mL of 0.05 mol/L Tris-HCl (pH 7.6) and 0.1% H2O2. The sections were dehydrated in graded alcohols and xylol and coverslipped with Permount. Control sections were treated with antisera previously adsorbed with 10 μg ET-1 to establish the specificity of the immunochemical reaction.

**High Performance Liquid Chromatography of Endothelin-1 in Blood Vessels**

Acid extracts obtained from five aortas or entire mesenteric vascular beds were prepared and passed through a C18 Sep-Pak cartridge as described above. After evaporation in a Speed-Vac, the samples were reconstituted in 1% trifluoroacetic acid and loaded onto a Vydac protein C4 column (Separation Group, Hesperia, Calif.). The samples were separated over a linear gradient of 15–50% acetonitrile (1%/min) and 1% trifluoroacetic acid. One-minute fractions were collected, dried, and resuspended in 100 μL RIA buffer for ir-ET-1 measurement.

**Immunohistochemical Demonstration of Endothelin in Blood Vessels**

The rats were first perfused in situ with 20 mL Ringer-Locke fluid. The aorta and mesenteric vascular bed were then fixed for 12–24 hours in Bouin’s fluid and, after dehydration, were embedded in paraffin. The vascular tissues were serially cut in sections 5 μm thick. The sections were mounted on glass slides and, after deparaffinization and hydration, were immunostained according to the avidin-biotin-peroxidase (ABP) technique. The sections were sequentially exposed to normal goat serum (diluted in phosphate buffered saline) for 1 hour then to ET-1 antiserum (Peninsula Laboratories) at a final dilution of 1:100 for 48 hours. They were treated with a 1:400 dilution of biotinylated goat anti-rabbit IgG (ABP kit, Vector Laboratories, Burlingame, Calif.) for 30 minutes. The sections were then incubated for 60 minutes in the ABP solution prepared by adding 25 μL avidin DH and 25 μL biotinylated peroxidase to 4 mL dilution buffer (0.05 mol/L phosphate plus 2.5 mg/mL bovine serum albumin). Peroxidase activity was visualized by incubation in a medium containing 3 mg of 3,3′-diaminobenzidine and 10 mL of 0.05 mol/L Tris-HCl (pH 7.6) and 0.1% H2O2. The sections were dehydrated in graded alcohols and xylol and coverslipped with Permount. Control sections were treated with antisera previously adsorbed with 10 μg ET-1 to establish the specificity of the immunochemical reaction.

**Measurement of Plasma Immunoreactive Endothelin-1 and Plasma Renin Activity**

Plasma ir-ET-1 was measured by RIA after plasma extraction by passage through C18 Sep-Pak cartridges as described previously. Plasma renin activity was measured by RIA of angiotensin I produced after a 2-hour incubation of plasma at 37°C and pH 6.5, as previously described.

**Analysis of Data**

Results are represented as mean±SEM. Statistical differences between groups were determined by two-tailed Student's t test. Differences were considered significant at a value of p<0.05.

**Results**

Body weight was lower and systolic blood pressure was significantly higher in DOCA-salt hypertensive rats and in SHR than in uninephrectomized and WKY control rats, respectively (Table 1). Plasma renin activ-
In this article we show for the first time that blood vessels from DOCA-salt hypertensive rats contain greater amounts of ir-ET-1 than vessels of uninephrectomized control rats. Content of ir-ET-1 was increased more than sixfold in aorta and almost twofold in mesenteric arteries of DOCA-salt rats in comparison to uninephrectomized rats. Amounts of ET-1 present in the blood vessel wall were determined from acid extracts prepared with aorta segments of similar length or with entire mesenteric vascular beds and were assayed individually. In consequence, results were expressed per total vascular tissue assayed and not normalized per unit weight, because the objective was to ascertain total amounts of ET-1 contained in the blood vessel wall independent of whether the wall was thickened. This is because ir-ET-1 is contained in endothelial cells that, even if hypertrophied in the hypertensive animals, are not hypertrophied to the degree that the intima or media is (Figures 2 and 3). The latter contribute most of the increase in weight and thus dilute the elevated immunostaining found in the endothelial cell layer of the DOCA-salt vessels at high magnification (Figures 2C and 3C). The immunostaining was specific for ET-1, because it was almost completely abolished by addition of excess synthetic ET-1 (Figures 2D and 3D).

In contrast to DOCA-salt rats, no significant difference in endothelial cell layer ET-1 immunostaining could be shown in either aorta or mesenteric arteries between SHR and WKY rats (not shown).

**Discussion**

**TABLE 1. Body Weight, Blood Pressure, Plasma Renin Activity, and Plasma Endothelin-1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Systolic blood pressure (mm Hg)</th>
<th>PRA (ng Ang I/mL/hr)</th>
<th>Plasma ET-1 (fmol/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uni Nx</td>
<td>411±5</td>
<td>107±2</td>
<td>2.42±0.25</td>
<td>0.94±0.05</td>
</tr>
<tr>
<td>DOCA-salt hypertensive</td>
<td>274±5*</td>
<td>196±4*</td>
<td>0.43±0.10*</td>
<td>1.03±0.07</td>
</tr>
<tr>
<td>WKY (6-week-old)</td>
<td>154±2</td>
<td>101±2</td>
<td>ND</td>
<td>1.11±0.24</td>
</tr>
<tr>
<td>SHR (6-week-old)</td>
<td>111±1*</td>
<td>133±2*</td>
<td>ND</td>
<td>1.39±0.23</td>
</tr>
<tr>
<td>WKY (16-week-old)</td>
<td>441±5</td>
<td>106±3</td>
<td>ND</td>
<td>0.62±0.08</td>
</tr>
<tr>
<td>SHR (16-week-old)</td>
<td>288±2*</td>
<td>179±3*</td>
<td>ND</td>
<td>1.33±0.22†</td>
</tr>
</tbody>
</table>

PRA, plasma renin activity; Ang I, angiotensin I; ET-1, endothelin-1; Uni Nx, uninephrectomized rats; DOCA, deoxycorticosterone acetate; WKY, Wistar-Kyoto rat; ND, not determined; SHR, spontaneously hypertensive rat. Values are mean±SEM. *p<0.001, †p<0.05.

**TABLE 2. Wet Weight and Endothelin-1 Content of Blood Vessels in DOCA-Salt Hypertensive Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Wet weight (mg)</th>
<th>ET-1 content (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aorta</td>
<td>Mesenteric vascular bed</td>
</tr>
<tr>
<td>Uni Nx</td>
<td>44.7±2.3</td>
<td>82.0±2.8</td>
</tr>
<tr>
<td>DOCA-salt hypertensive</td>
<td>65.7±2.5*</td>
<td>244.9±7.8*</td>
</tr>
</tbody>
</table>

ET-1, endothelin-1; Uni Nx, uninephrectomized rats; DOCA, deoxycorticosterone acetate. Values are mean±SEM. Number of rats studied was 10 in each group. ET-1 content is expressed as femtomoles per segment of aorta or whole mesenteric vascular bed. *p<0.001.
TABLE 3. Wet Weight and Endothelin-1 Content of Blood Vessels in Spontaneously Hypertensive Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Wet weight (mg)</th>
<th>ET-1 content (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aorta</td>
<td>Mesenteric vascular bed</td>
</tr>
<tr>
<td>WKY (6-week-old)</td>
<td>20.1 ± 0.9</td>
<td>55.6 ± 4.5</td>
</tr>
<tr>
<td>SHR (6-week-old)</td>
<td>15.4 ± 0.7*</td>
<td>50.1 ± 3.2</td>
</tr>
<tr>
<td>WKY (16-week-old)</td>
<td>37.3 ± 3.1</td>
<td>70.3 ± 5.7</td>
</tr>
<tr>
<td>SHR (16-week-old)</td>
<td>30.9 ± 1.3</td>
<td>71.0 ± 4.7</td>
</tr>
</tbody>
</table>

ET-1, endothelin-1; WKY, Wistar-Kyoto rat; SHR, spontaneously hypertensive rat. Values are mean ± SEM. Number of rats studied was eight in each group. ET-1 content is expressed as femtomoles per segment of aorta or whole mesenteric vascular bed.

*p < 0.05, †p < 0.001.

in hypertensive rats), this distorts the conclusion to be drawn from these results as explained above. Thus, the possible physiological consequence of elevated absolute amounts of ET-1 in these arteries would be missed.

High levels of ET-1 may be involved in the arterial structural changes found in DOCA-salt hypertensive rats. ET-1 has been shown to possess growth factor activity on vascular smooth muscle cells, because it is a potent mitogen and stimulates DNA synthesis in vascular smooth muscle cells, which may thus contribute to vascular hypertrophy.

Although endothelium was initially recognized as a source of ET-1, it is now appreciated that vascular smooth muscle cells may also produce ET-1. Thus, it is conceivable that a certain proportion of the increased vascular content of ET-1 originates in smooth muscle cells. However, no significant ir-ET-1 staining was found in the media of blood vessels from DOCA-salt hypertensive or uninephrectomized rats. A very large increase in ET-1 immunostaining was localized only in the endothelial cell monolayer, which is therefore responsible for significantly elevating ir-ET-1 content measured in the blood vessel acid extracts.

Ir-ET-1 measured in acid extracts of blood vessels and the immunostaining reaction shown probably represent authentic ET-1, because the immunoreactive peak eluted at the same position as synthetic ET-1 on HPLC. Furthermore, the ET-1 antisera used has very low cross-reactivity with other endothelins (such as endothelin-2 and endothelin-3). Therefore, it is highly likely that the increased ir-ET-1 content and immunostaining found in DOCA-salt vascular tissues represent ET-1. Big endothelin coeluted with ET-1 in the HPLC system used. Thus, it is impossible to establish from this method the proportion of big endothelin present in these blood vessels.

Increased production of ir-ET-1 in endothelial cells of DOCA-salt hypertensive rats may be related to shear stress, which has been found to be an important stimulator of ET-1 secretion. Endogenous factors such as thrombin, angiotensin II, vasopressin and norepinephrine, and transforming growth factor-β may also stimulate ET-1 production and release from endothelial cells. However, it is not clear what induces increased vascular ir-ET-1 production in DOCA-salt hypertensive rats. Vasopressin effects have been shown to be exaggerated in the mesenteric circulation of these hypertensive rats. Thus, vasopressin is a possible candidate for this role. The sympathetic nervous system is also activated in DOCA-salt hypertension and may also be involved in activating expression of ET-1, but this remains to be determined.

ET-1 may downregulate endothelin receptors on vascular smooth muscle cells. Thus, elevated production of vascular ET-1 in DOCA-salt rats is consistent with previous observations showing downregulation of endothelin receptors in blood vessels of this hypertensive model. Small mesenteric arteries from DOCA-salt hypertensive rats mounted on a wire myograph as well as large conduit arteries such as aortic or mesenteric artery rings respond with reduced wall stress to ET-1 in comparison to vessels from uninephrectomized control rats. Increased vascular ET-1 content in DOCA-salt hypertensive rats may increase receptor occupancy, leading to rapid endothelin-receptor internalization in vascular smooth muscle cells, downregulation of vas-
FIGURE 2. Photomicrographs show immunohistochemical localization of immunoreactive endothelin-1 in the wall of thoracic aorta of uninephrectomized (panel A) and doxycorticosterone acetate–salt hypertensive rats (panel B). A significant increase in immunostaining was found in the aortic endothelial cell layer from hypertensive rats (panels B and C). Immunostaining of endothelial cells was almost completely abolished by addition of excess synthetic endothelin-1 (panel D). Magnification of panels A, B, and D, ×250; panel C, ×1,000. L, lumen; m, media.

Our observations suggest that plasma ET-1 concentrations are not representative of vascular ET-1 production and therefore of its possible contribution to elevation of blood pressure. Plasma ET-1 levels were similar in DOCA-salt hypertensive rats and in uninephrectomized control rats. This was also observed in different models of hypertension in the rat and in human essential hypertension. ET-1 detected in plasma may represent only spillover from endothelial cells, which may not, however, correlate with levels detected in vascular tissues possibly because of its rapid elimination from the bloodstream. Therefore, locally produced ET-1 rather than circulating ET-1 may be a better indicator of its involvement in the regulation of vascular resistance.

In contrast to our findings in DOCA-salt hypertensive rats, no increase in vascular content of ir-ET-1 could be detected in young or adult SHR by either RIA of acid extracts or immunohistochemistry. The reasons for this difference may relate to stimulation mechanisms of ET-1 production in both hypertensive models (such as vasopressin, sympathetic activity, etc.), which may be more active in the DOCA-salt hypertensive rat than in SHR, although in the latter they may also play a role. Endothelial dysfunction could play a role in DOCA-salt hypertensive rats, resulting in exaggerated production of ET-1, but endothelial dysfunction is also present in SHR, and thus it is unlikely that the difference lies at this level. The reasons for the lack of increase of ir-ET-1 in blood vessels of SHR in contrast to increased content in DOCA-salt hypertensive rats thus remain to be explained.

ET-1 produces multiple effects on vascular tissues. Infusion of ET-1 into intact animals produces a transient hypotension related to increased endothelium-derived relaxing factor and prostacyclin production and release from vascular endothelial cells stimulated by ET-1. This phase is followed by a long-lasting hypertension due to the constrictor effect of ET-1 on vascular smooth muscle cells. The vasopressor effect of ET-1 is increased after destruction of endothelium. In hypertension, the vasorelaxing response mediated by endothelium-derived relaxing factor is impaired. The increased vascular ET-1 content in DOCA-salt hypertensive rats may then act unopposed on hypertrophied smooth muscle cells to contribute to increased peripheral resistance. These results indicate that ET-1 may play a role in the maintenance of high blood pressure in...
cular endothelin receptors,\(^6\) and decreased phospholipase C response to ET-1 activity,\(^6,10\) which in turn results in reduced ET-1 vascular responsiveness.\(^6,10\)

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ET-1 detected in plasma may represent only spillover from endothelial cells, which may not, however, correlate with levels detected in vascular tissues possibly because of its rapid elimination from the bloodstream.\(^26\) Therefore, locally produced ET-1 rather than circulating ET-1 may be a better indicator of its involvement in the regulation of vascular resistance.

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ET-1 produces multiple effects on vascular tissues. Infusion of ET-1 into intact animals produces a transient hypotension related to increased endothelium-derived relaxing factor and prostacyclin production and release from vascular endothelial cells stimulated by ET-1.\(^4\) This phase is followed by a long-lasting hypertension due to the constrictor effect of ET-1 on vascular smooth muscle cells. The vasopressor effect of ET-1 is increased after destruction of endothelium.\(^10\) In hypertension, the vasorelaxing response mediated by endothelium-derived relaxing factor is impaired.\(^29\) The increased vascular ET-1 content in DOCA-salt hypertensive rats may then act unopposed on hypertrophied smooth muscle cells to contribute to increased peripheral resistance. These results indicate that ET-1 may play a role in the maintenance of high blood pressure in
DOCA-salt hypertensive rats and perhaps in other forms of hypertension. Our results also show that increased production of ir-ET-1 is not found in either young or adult SHR, which, in contrast to DOCA-salt rats, does not provide evidence of a role of ET-1 in the pathogenesis of hypertension in SHR.

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