Endothelin Vascular Receptors and Responses in Deoxycorticosterone Acetate–Salt Hypertensive Rats

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The vasoconstrictor effect, the binding, and the response of inositol phosphates to endothelin-1 (ET-1) were investigated in blood vessels of deoxycorticosterone acetate (DOCA)–salt hypertensive rats within 2 weeks of development of hypertension and in uninephrectomized control rats. In DOCA-salt and uninephrectomized rats, plasma levels of endothelin were similar (1.2±0.1 fmol/ml). Thoracic aorta and mesenteric artery rings devoid of endothelium presented significantly decreased responses to increasing concentrations of ET-1. Binding of ET-1 to mesenteric artery membranes was significantly lower in DOCA-salt rats (106±22 fmol/mg protein) than in uninephrectomized rats (172±19 fmol/mg protein, p<0.05), whereas affinity was similar. Phosphoinositide metabolism was examined in aorta and mesenteric arteries after incubation with \[^{3}H\]myoinositol. Inositol phosphates were separated by high-performance liquid chromatography. In response to 100 nmol/l ET-1, accumulation of inositol 1,4,5-trisphosphate after 20 seconds and of inositol monophosphate, inositol bisphosphate, and inositol 1,3,4-trisphosphate after 30 minutes (in the presence of 25 mmol/l LiCl) were significantly lower in DOCA-salt hypertensive than in uninephrectomized control rats, in both aorta and mesenteric arteries. In conclusion, decreased density of ET-1 receptors in DOCA-salt hypertensive rats results in decreased activation of phospholipase C and, consequently, reduced vasoconstriction induced by ET-1. Because the decrease in vasoconstrictor effects of ET-1 is found in the absence of endothelium, it is likely that receptor downregulation rather than prior receptor occupancy underlies these findings. (Hypertension 1992;19[Suppl II]:II-98–II-104)

A possible factor contributing to the elevated vascular resistance seen in hypertension is altered reactivity of blood vessels to vasoactive substances. Increased responsiveness to vasoconstrictor agents has been demonstrated in different models of experimental hypertension, such as the renovascular hypertensive rat and the deoxycorticosterone acetate (DOCA)–salt hypertensive rat. The endothelium synthesizes and releases paracrine hormones that regulate the contraction and physiology of vascular smooth muscle cells. In addition to the well-described vasorelaxant factors (i.e., endothelium-derived relaxing factor and prostacyclin), it has been shown that the endothelium also releases vasoconstrictor compounds, and one of these is the peptide endothelin. Endothelin-1 (ET-1), originally isolated from the supernatant of cultured porcine aortic endothelial cells, is a 21-amino acid peptide and one of the most potent vasoconstrictor agents thus far described. It probably acts more as a local rather than a circulating hormone, and it might be involved in the control of blood flow and pressure because of its potent microvascular constrictor effect demonstrated in vivo. Greater reactivity to ET-1 has been observed in the isolated arteries of spontaneously hypertensive rats. Thus, enhanced reactivity to this agent might be expected in other models of experimental hypertension, such as the DOCA-salt hypertensive rat.

Initial studies suggested that endothelins act on vascular smooth muscle cells by increasing calcium influx from the extracellular fluid through voltage-dependent calcium channels. It later became evident that activation of phospholipase C and breakdown of membrane phosphoinositides, resulting in
release of calcium from the endoplasmic reticulum to produce a rise in cytosolic calcium and activation of protein kinase C, were as or more important in the intracellular signal transduction of endothelin receptors. Secondary activation of a nonselective cationic channel permeable to calcium may result in membrane potential changes that activate L-type Ca\textsuperscript{2+} channels. The role of phosphoinositide turnover and activation of protein kinase C appears to be critical in rabbit aorta, as shown by the inhibition of protein kinase C.

In this study, we therefore have investigated the reactivity to ET-1 of blood vessels (aorta and mesenteric arteries) in DOCA-salt hypertensive rats, concurrently with the binding of ET-1 to vascular membranes and postreceptor mechanisms such as the production of inositol phosphates.

**Methods**

**Materials**

ET-1 (endothelin 1-21), (human, porcine) was purchased from Peninsula Laboratories, Inc., Belmont, Calif. d-Myo-2-[\textsuperscript{3}H]inositol, d-myo-l-[\textsuperscript{3}H(N)]inositol 1,3,4-trisphosphate, and d-myo-2-[\textsuperscript{3}H]inositol 1,3,4,5-tetrakisphosphate were obtained from Amer sham, Oakville, Canada, and Dupont New England Nuclear, Boston, Mass. All reagents used were of the highest reagent grade available.

**Animal Experiments**

The study was approved by the Animal Care Committee of the Clinical Research Institute. DOCA-salt hypertension was induced by the method of Ormsbee and Ryan. In brief, male Sprague-Dawley rats (Charles River Laboratories, St. Constant, Canada) weighing 200 g were uninephrectomized under ether anesthesia. Silicone rubber impregnated with DOCA (150 mg/rat) was implanted subcutaneously, and rats were offered 1% saline to drink. Rats were studied 1-2 weeks after becoming hypertensive. Another group of uninephrectomized rats receiving silicone rubber without DOCA impregnation served as control. This group received tap water to drink. Blood pressure measurements were by the tail-cuff method. With rats under light anesthesia, blood pressure was measured the day before experiments and recorded on a Model 7 polygraph (Grass Instrument Co., Quincy, Mass.) fitted with a 7P8 preamplifier and a Grass Model 1010 crystal microphone as a pulse detector. The average of three pressure readings was obtained.

**Constrictor Response of Blood Vessels to Endothelin-1**

Segments of rat thoracic aorta and mesenteric arteries from both experimental groups were removed and dissected free of connective tissue. The first 0.5 cm of the proximal end of the aortic strips was discarded, and rings (4 mm in length) were prepared from the next 2.5 cm for incubation in a glass-jacketed, 15-ml tissue bath at 37°C. Rings were subjected to a passive force of 2.0 g for aorta and 1.0 g for mesenteric arteries. During preparation, the luminal surface was gently rubbed to eliminate the endothelial layer. The bathing medium was Kreb’s bicarbonate with the following composition (mmol/l): NaCl 118, MgSO\textsubscript{4} 1.18, KH\textsubscript{2}PO\textsubscript{4} 1.18, dextrose 5.5, NaHCO\textsubscript{3} 25.0, CaCl\textsubscript{2} 2.5, and KCl 4.7. The solution was bubbled with a mixture of 95% O\textsubscript{2}-5% CO\textsubscript{2}. Tissues were equilibrated for 90 minutes. They were challenged with 1 \textmu mol/l norepinephrine, and relaxation was attempted with 0.1 mmol/l acetylcholine to verify absence of endothelium. A cumulative concentration-response curve (at 15-minute intervals between each dose) to ET-1 (10 pmol/l to 100 nmol/l) was obtained. Isometric contractions were recorded as changes in grams of force on a Grass Model 7 polygraph. All drugs were prepared immediately before use in concentrations such that volumes no greater than 100 \mu l were added to tissue baths.

**Endothelin-1 Binding Assay**

Mesenteric arteries were used to prepare vascular membranes for binding experiments as described previously for other binding studies. In brief, mesenteric arteries were homogenized with a polytron (setting 8 for 10 seconds, twice) in a 0.25 mol/l sucrose solution. The homogenate was centrifuged at 1,000g for 10 minutes, the supernatant then centrifuged at 104,000g for 30 minutes, and the pellet resuspended in 0.05 mol/l Tris-HCl (pH 7.2) containing 5 mmol/l MgCl\textsubscript{2}, 1 \mu mol/l aprotinin, 0.1% bacitracin, and 0.5 mmol/l phenylmethylsulfonyl fluoride. After proteins were measured by the Coomassie blue method, bovine serum albumin was added to a concentration of 0.5%. Fifty micrograms of membrane protein was used per tube, with 40 pmol/l \textsuperscript{125}I-ET-1 and increasing concentrations of unlabeled ET-1 (1 pmol/l to 1 \mu mol/l) in a final volume of 0.25 ml. In preliminary experiments, it was shown that binding was linear between 20 and 100 \mu g protein per tube. \textsuperscript{125}I-ET-1 was prepared by the lactoperoxidase method as previously described and had a specific activity of approximately 1,000 Ci/mmol. All assays were performed in duplicate at 22°C for 120 minutes. At this time interval, binding was at steady state and there was no degradation of labeled ET-1 as judged by high-performance liquid chromatography. Separation of bound and free radioactivities was achieved by rapid filtration through GF/C filters (Whatman Inc., Clifton, N.J.). The filters were washed twice with 3 ml Tris-HCl (pH 7.4), allowed to dry, and counted in a Rackgamma LKB counter (Turku, Finland) with 80% efficiency. In preliminary experiments, we found that binding was specific for ET-1, with an inhibition constant \(K_i\) of 0.4 nmol/l, whereas endothelin-3 had a \(K_i\) of 2.5 nmol/l. Neither angiotensin II, vasopressin, atrial natriuretic peptide, nor other unrelated peptides displaced ET-1 binding.
Inositol Phosphate Measurements

Preparation of blood vessels. After careful removal of fat, aorta and mesenteric arteries were placed in a modified Krebs-Ringer solution with the following composition (mmol/l): NaCl 118, KCl 4.7, MgSO4 1.18, KH2PO4 1.18, CaCl2 2.50, dextrose 10.0, NaHCO3 25.0, and 2% bovine serum albumin. They were then incubated in the presence of 50 µCi/ml [3H]myoinositol for a total of 5 hours at 37°C in an atmosphere containing 95% O2-5% CO2 under constant stirring. The excess radioactivity was then washed by incubation in a solution of Krebs' bicarbonate containing 10 mmol/l unlabeled myoinositol. Tissues were then weighed and, for long-term stimulations, incubated in the same solution for 30 minutes in the presence of LiCl (25 mmol/l) to inhibit inositol monophosphatase and optimize the accumulation of inositol monophosphate during long-term stimulation.

Inositol phosphate stimulation and extraction. Stimulation of phosphatidylinositol hydrolysis was initiated by dipping the tissue in a solution containing ET-1 (100 nmol/l) after an incubation of 2 hours at pH 6.5, as previously described. Plasma renin activity was measured by radioimmunoassay of angiotensin I after an incubation of 2 hours at pH 6.5, as previously described. Briefly, plasma acidified with 0.1 mol/l acetic acid was extracted by passage through activated Sep-Pak cartridges (Waters Associates, Milford, Mass.) and eluted with 80% acetonitrile. Antibody from Peninsula Laboratories was used at a dilution of 1:120,000 and had 10% cross-reactivity with big endothelin and 7% with endothelin-3.

Data Analysis

Binding data were analyzed by computer-assisted nonlinear regression analysis using the LIGAND program for determination of density and affinity of binding sites. The ALLFIT program based on the four-parameter logistic equation was used to fit the data from dose-response curves for the determination of the maximum response and effective concentration producing 50% of the maximum response (EC50). Results are reported as mean±SEM. Statistical differences between means were analyzed by Student’s t test. Differences were considered significant at a value of p<0.05.

Results

Blood Pressure, Plasma Renin Activity, and Plasma Endothelin-1 Concentration

Systolic blood pressure was significantly higher (p<0.01) in the DOCA-salt hypertensive rats than in the uninephrectomized control rats (Table 1). DOCA-salt rats had suppressed plasma renin activity as expected. Plasma ET-1 levels were similar in DOCA-salt hypertensive rats and uninephrectomized controls.

Constrictor Response of Aortic and Mesenteric Artery Rings to Endothelin-1

Aortic and mesenteric artery rings were contracted with 1 µmol/l norepinephrine after the equilibration period of 90 minutes. Responses of aortic rings were similar in uninephrectomized rats and in DOCA-salt hypertensive rats (0.77±0.17 versus 0.70±0.11 g, respectively). Mesenteric artery rings of DOCA-salt hypertensive rats, in contrast, exhibited diminished maximum responses to norepinephrine (0.21±0.05 versus 0.39±0.04 g in controls, p<0.03). The rings...
were challenged with 0.1 nmol/l acetylcholine, and none relaxed, indicating the effectiveness of the removal of endothelium. ET-1-induced contractions developed slowly, reaching a maximum within 10-15 minutes, and were difficult to wash out. The maximum response to ET-1 in vascular rings of DOCA-salt hypertensive rats was significantly depressed to 66% of controls in aorta and 33% of controls in mesenteric artery rings (Figure 1). In mesenteric artery rings, reactivity to norepinephrine was reduced, so the decrease in response to ET-1 to 33% of controls was in actual fact to 66% in DOCA-salt hypertensive rats, after normalization according to norepinephrine responsiveness, and thus was similar to the reduction in response found in aortic rings. There were no significant differences in EC50 between DOCA-salt and uninephrectomized rats.

Endothelin-1 Binding Sites in Mesenteric Vasculature

Density of ET-1 binding sites in membranes of mesenteric arteries of DOCA-salt hypertensive rats was significantly decreased (p<0.05) as compared with that of controls (Table 1). Figure 2 shows representative binding curves. The apparent dissociation constant (KD) was not different in the two groups. The pseudo-Hill coefficient derived from the computer analysis using the LIGAND program was close to unity, reflecting the presence of only one population of binding sites, of high affinity.

Production of Inositol Phosphates

The mass of the aorta and mesenteric arteries from DOCA-salt hypertensive rats was significantly higher than in uninephrectomized control rats (Table 2). Results were therefore normalized according to weights. At 20 seconds of stimulation with 100 nmol/l ET-1, the amount of [3H]inositol 1,4,5-trisphosphate detected in aorta and mesenteric arteries was significantly lower (p<0.05) in DOCA-salt rats than in control uninephrectomized rats (Figure 3). After 30 minutes of stimulation with ET-1, in the presence of
Table 2. Stimulation of Inositol Phosphate Accumulation by Endothelin-1 (100 nmol/l) in Aorta and Mesenteric Arteries of Deoxycorticosterone Acetate–Salt Hypertensive Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Total IP (Δcpm/mg tissue [wet wt])</th>
<th>IP₁ (Δcpm/mg tissue [wet wt])</th>
<th>IP₂ (Δcpm/mg tissue [wet wt])</th>
<th>1,3,4-IP₃ (Δcpm/mg tissue [wet wt])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thoracic aorta (Δcpm/mg tissue [wet wt])</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninephrectomized (n=5)</td>
<td>2,542±364</td>
<td>2,029±288</td>
<td>482±108</td>
<td>39±5</td>
</tr>
<tr>
<td>DOCA-salt (n=5)</td>
<td>1,239±253*</td>
<td>1,133±184*</td>
<td>99±35*</td>
<td>12±8*</td>
</tr>
<tr>
<td>% of control</td>
<td>49%</td>
<td>56%</td>
<td>21%</td>
<td>31%</td>
</tr>
<tr>
<td>Mesenteric arteries (Δcpm/mg tissue [wet wt])</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninephrectomized (n=2)</td>
<td>4,854±817</td>
<td>2,383±240</td>
<td>2,430±560</td>
<td>11±5</td>
</tr>
<tr>
<td>DOCA-salt (n=2)</td>
<td>2,134±335</td>
<td>1,656±309</td>
<td>463±86</td>
<td>3±3</td>
</tr>
<tr>
<td>% of control</td>
<td>44%</td>
<td>69%</td>
<td>19%</td>
<td>27%</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Wet weight of aortas was 78±3 mg in controls and 97±6 mg in deoxycorticosterone acetate (DOCA)-salt hypertensive rats (p<0.01); that of mesenteric arteries was 61±4 mg in controls and 87±8 mg in DOCA-salt rats (p<0.01). IP, inositol phosphate; IP₁, inositol monophosphate; IP₂, inositol bisphosphate; 1,3,4-IP₃, inositol 1,3,4-trisphosphate.

25 mmol/l LiCl both in thoracic aorta and in mesenteric arteries, total inositol phosphates as well as each of the inositol phosphates (inositol monophosphate, inositol bisphosphate, and inositol 1,3,4-trisphosphate) showed a marked decrease in DOCA-salt rats compared with controls (Table 2).

Discussion

In this study, we observed a decreased efficacy (maximal response) of ET-1 without change in sensitivity in endothelium-denuded aortic and mesenteric rings of DOCA-salt hypertensive rats, a model of renin-suppressed, volume-expanded hypertension, as compared with uninephrectomized control rats. This was accompanied by a reduction in the density of detectable ET-1 binding sites in mesenteric artery membrane preparations as well as in production of inositol phosphates in response to a maximal dose of ET-1.

The decreased reactivity to ET-1 found in this study is in contrast to the enhanced response to vasoconstrictor agents frequently demonstrated in different models of experimental hypertension. In DOCA-salt hypertensive rats, blood vessels have been shown to have greater sensitivity (or reactivity) to norepinephrine, vasopressin, and angiotensin II. Different mechanisms, such as upregulation of receptors or exaggerated postreceptor mechanisms, appear to underlie these enhanced vasoconstrictor responses. In this study, reactivity to norepinephrine of aortic rings of DOCA-salt hypertensive rats was similar to that of controls, whereas that of mesenteric artery rings was reduced. The response to ET-1 in mesenteric artery rings was decreased to 33%, but after correction according to norepinephrine responsiveness, it was diminished in DOCA-salt rats to 66%, similar to the decrease in response found in aortic rings, in ET-1 receptor density, and in generation of inositol phosphates. This also agrees with the percent decrement in response to ET-1 of small mesenteric arteries (i.d. 220–260 μm) from DOCA-salt hypertensive rats that we have found in a parallel study.

Little data is available on the regulation of ET-1 receptors in blood vessels in this model of hypertension. In this study, although there is a clear decrease in ET-1 binding capacity of mesenteric artery membrane preparations, there is no evidence of change in receptor affinity for [125I]-ET-1, suggesting that the receptor itself is unchanged. Similar to our findings in mesenteric arteries of DOCA-salt rats, a decreased density of endothelin binding sites was shown in spontaneously hypertensive rats. The decrease in binding capacity is not associated with a significant increase in circulating plasma ET-1 in DOCA-salt rats, in contrast to other circulating hormonal agonists, such as vasopressin or atrial natriuretic peptide, whose receptors are found to be downregu-
lated in this model of hypertension. However, circulating levels of endothelin may not be representative of abluminal release from endothelial cells, and it is possible that secretion of endothelin is indeed increased; however, this remains to be proven. The finding of essentially similar plasma endothelin levels in this study corroborates previous findings.26

ET-1 binding sites are of high affinity, with equilibrium dissociation constants in the high picomolar range, in both the DOCA-salt and the control group. Because cellular responses to ET-1 can be elicited at similar concentrations,27 it is likely that these binding sites represent true receptors. The high affinity of the peptide for its receptor is reflected in the observation by other investigators that $^{125}$I-ET-1 binding is difficult to reverse by extensive washing. Tachyphylaxis of isolated arteries to ET-1 is also long-lasting.27 Because of the unusually tight binding of ET-1 to its receptors, prior occupancy could lead in vitro to both decreased detectable binding capacity, reduced inositol phosphate responses, and vascular reactivity to ET-1. However, the decreased response of blood vessels to ET-1 in these DOCA-salt hypertensive rats is found in vessels from which endothelium has been removed, thus eliminating an important source of endogenous endothelin from the rings for at least 3 or more hours before they were challenged with exogenous ET-1. During this time of manipulation and repeated washing, it would be expected that reactivity to ET-1 should have recovered if prior receptor occupancy did play a role.27 The persistence of decreased response after 3 hours of removal of endothelium, a major source of endogenous endothelin, suggests that downregulation of endothelin receptors may instead be involved. Evidence for downregulation has already been provided in cultured vascular smooth muscle cells. Downregulation is present after 30 minutes of incubation and persists for 18 hours.28 Downregulation of ET-1 binding sites thus could modulate the vascular activity of ET-1.

In other studies, it has been shown that large vessels from DOCA-salt hypertensive rats do not exhibit differences in sensitivity to ET-1, whereas very small arterioles of less than 15 $\mu$m in internal diameter exhibit increased sensitivity.7 Furthermore, phosphoinositide breakdown has been shown to be enhanced in this model in response to ET-1 in atria and in mesenteric arteries.29 In these studies, however, blood vessels were examined after a longer period of hypertension, whereas in our study, rats were investigated within 2 weeks of developing hypertension. This may suggest that in DOCA-salt hypertensive rats, an initial period of decreased maximal response could be followed by normalization of responsiveness as phospholipase C-dependent responses, which were decreased because of downregulated ET-1 receptors, become exaggerated. This later exaggeration could be the result of changes undergone by the blood vessel wall as a consequence of hypertension, resulting in enhanced responses even in the presence of downregulated receptors, as found with other peptides such as vasopressin,3 or other mechanisms that remain to be established. With respect to the behavior of microvessels with an inner diameter of 15 $\mu$m in the study by De Carvalho et al,7 it should be pointed out that in a study parallel to the present one, we have found that small arterioles of 220–260 $\mu$m in internal diameter exhibit decreased responses to ET-1 in DOCA-salt hypertensive rats, whether or not they have intact endothelium.24 This underlines the need to consider separately different segments of vascular beds, which may behave differently under the same pathophysiological conditions.

In conclusion, early in the course of DOCA-salt hypertension, reduced response of endothelium-de-nuded aortic and mesenteric artery rings was found, together with decreased density of detectable ET-1 binding sites and responses of phosphoinositide turnover to ET-1, suggesting downregulation of ET-1 receptors. The mechanism of decrease in the number of receptors remains to be established.

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