Increased Expression of Endothelin-1 Gene in Blood Vessels of Deoxycorticosterone Acetate–Salt Hypertensive Rats

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We have recently shown that the content of immunoreactive endothelin-1 is increased in acid extracts from blood vessels of deoxycorticosterone acetate (DOCA)–salt hypertensive rats compared with uninephrectomized control rats. We have also found by immunohistochemistry a significant increase in immunoreactive endothelin-1 in endothelial cells of aorta and mesenteric arteries of DOCA-salt hypertensive rats. In the present study, we investigated preproendothelin-1 gene expression in blood vessels of DOCA-salt hypertensive rats and uninephrectomized control rats. Northern blot analysis using a specific \( ^{32} \text{P} \)-labeled complementary RNA probe for rat preproendothelin-1 of 319 base pairs revealed a fourfold to fivefold increase in abundance of preproendothelin-1 messenger RNA transcripts in both aorta and mesenteric arteries from DOCA-salt hypertensive rats. Thus, increased immunoreactive endothelin-1 content in blood vessels of DOCA-salt hypertensive rats is secondary to increased preproendothelin-1 gene expression. Exaggerated expression of the preproendothelin-1 gene in mineralocorticoid hypertension may contribute to the maintenance of elevated blood pressure.

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KEY WORDS • endothelins • endothelium • RNA, messenger • arteries • aorta • mesenteric arteries • hypertension, mineralocorticoid

Endothelin-1 (ET-1), a 21-amino acid peptide produced primarily in endothelial cells, is the most powerful endogenous vasoconstrictor agent known to date.\(^1\) Prepro-ET-1 messenger RNA (mRNA) was found to be expressed in vascular endothelial cells and also in vascular smooth muscle cells.\(^1^2\) Gene expression of prepro-ET-1 is increased in response to different agents, such as thrombin, angiotensin II, vasopressin, epinephrine, and transforming growth factor-\( \beta \).\(^1^2\) These agents augment ET-1 secretion from both endothelial and smooth muscle cells. ET-1 secretion is also stimulated by shear stress.\(^5\) Locally produced ET-1 exerts its effects in an autocrine and paracrine fashion to constrict blood vessels by acting on smooth muscle and also to stimulate production of other agents such as endothelium-derived relaxing factor.\(^6\) However, the role of ET-1 in hypertension remains unclear. Plasma ET-1 concentrations in experimental and human hypertension are similar or slightly higher than in normotensive controls.\(^5^10\) However, the role of circulating endothelin in the regulation of vascular tone is possibly minor. It is more likely that abluminal secretion of endothelin plays a more important role through its autocrine and paracrine effects. Moreover, it is likely that circulating ET-1 levels do not mirror the vascular production of endothelin. For this reason, we have recently investigated concentrations of ET-1 in the walls of arteries in hypertensive rats. We found that the content of immunoreactive ET-1 in blood vessels of deoxycorticosterone acetate (DOCA)–salt hypertensive rats was significantly increased relative to uninephrectomized control rats.\(^1^1\) In contrast, no such increase could be demonstrated in blood vessels of spontaneously hypertensive rats in the same study. The increase in immunoreactive ET-1 staining was shown to occur in endothelial cells of aorta and large and small mesenteric arteries of DOCA-salt hypertensive rats. We have speculated that increased immunoreactive ET-1 content may result from exaggerated expression of the gene of prepro-ET-1 in arteries of DOCA-salt hypertensive rats. We therefore have investigated in this study the abundance of mRNA transcripts for prepro-ET-1 in blood vessels of this model of experimental 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. DOCA-salt hypertension was induced by the method of Ormsbee and Ryan.\(^1^2\) Male Sprague-Dawley rats (Charles River Laboratories, St. Constant, Québec, Canada) weighing 200 g were uninephrectomized under sodium pentobarbital anesthesia (40 mg/kg) (Somnotol, MTC Pharmaceuticals, Cambridge, Ontario, 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. Rats were housed under conditions of constant temperature (22°C) and humidity (60%) and were exposed to a 12-hour light–dark cycle. Blood pressure was measured the day before experiments by the tail-cuff method on conscious semirestrained 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 ede-
tate and centrifuged. Plasma was stored at −20°C until plasma renin activity and plasma prepro-1 were assayed.

**Extraction of RNA From Tissues**

A 1.5-cm segment of thoracic aorta and the complete mesenteric vascular bed of DOCA-salt hypertensive rats and uninephrectomized controls were removed and dissected free of fat and connective tissue. Tissues were snap-frozen in liquid nitrogen and stored at −70°C until RNA extraction was performed. Total RNA was extracted by a guanidine isothiocyanate extraction, immediately followed by lithium chloride precipitation. The pellets were treated for 3 hours at 42°C with proteinase-K at a final concentration of 100 µg/mL in 0.5% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 7.5), and 5 mM ethylenediaminetetraacetic acid (EDTA), followed by two phenol-chloroform extractions and ethanol precipitation. The samples were resuspended in diethylpyrocarbonate-treated water, and the optical density was determined at 260 and 280 nm.

**Preparation of Rat Preproendothelin-1 Probe**

The rat prepro-ET-1 probe was prepared by polymerase-
chain reaction (PCR) amplification using first strand
complementary DNA (cDNA) from rat lung. First strand cDNA was synthesized using 5 µg total RNA in 50 µL reaction mixture containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl2, 10 mM dithiothreitol, 1 mM deoxynucleosides 5'-triphosphate (dNTPs), 0.5 mM spermidine, 1 µg oligo (dT)12-18 (Pharmacia, Baie d'Urfe, Quebec, Canada), and 10 units/µg RNA avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, Wis.) for 1 hour at 42°C. The tubes were then boiled 5 minutes, and bovine pancreas ribonu-
clease (Pharmacia) was added to a final concentra-
tion of 1 µg/mL for 30 minutes at 37°C. Five microliters of first strand cDNA was then applied to PCR amplifi-
cation in 50 µL reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 200 µM dNTPs, 100 pmol of each primer, and 2 units of Thermus aquaticus (Tag) DNA polymer-
ase (Promega). PCR amplification was performed in an Easy Cycler (Eicropic, Inc., San Diego, Calif.) programed as follows: 5 minutes initial denaturation at 95°C, 30 cycles each including 1 minute of denaturation at 95°C, 1 minute annealing at 50°C, and 1 minute elongation at 72°C followed by a final elongation for 10 minutes at 72°C. A 319-bp rat prepro-ET-1 PCR prod-
uct was obtained using a 5' forward primer, 5'-CTAG-
GCTAAGCGATCCTTTG-3', and a 3' reverse primer, 5'-TTCCTGTCCTGAGTGTAGTCC-3', located at nucleotides 266–285 and 565–584 of the coding sequence of the rat prepro-ET-1 cDNA, respectively. This PCR product was then cloned into pGEM-7zf (+) plasmid (Promega). The sequence was determined by the Sanger method of dieoxy-mediated chain termination and was identical to the sequence of the cloned rat prepro-ET-1.

Radiolabeled riboprobes were prepared using [32P]uridine 5'-triphosphate (UTP) (800 Ci/mmol; Am-
bersham, Arlington Heights, Ill.). Transcription reaction mixtures contained 160 µCi (200 pmol) [32P]UTP, 150 mM nucleoside 5'-triphosphate–UTP, 12.5 mM dithio-
thereitol, 20 units RNase inhibitor, the linearized plas-
mid preparation in 1 µL (1 µg/µL concentration), and 6 units of T; RNA polymerase in a total volume of 10 µL. Reactions were carried out for 60–90 minutes at 37°C.

**Northern Analysis**

Total RNA samples (20 µg per lane) were run on a horizontal gel apparatus on a 1.2% agarose gel containing 20 mM HEPES (pH 7.8), 1 mM EDTA, and 6% formaldehyde. The gel was submerged in 20 mM HEPES (pH 7.8), 1 mM EDTA, and 6% formaldehyde. With each set of samples, a 2 µg RNA ladder (9.5, 7.5, 4.4, 2.4, 1.4, and 0.24 kb; Gibco/BRL) was included in an adjacent lane. The samples were heated for 10 minutes at 65°C before loading. The electrophoresis was monitored by the migration of bromophenol blue and xylene cyanol added to each of the samples. Typical run times were between 4 and 5 hours. The samples were transferred from the gel to a nylon membrane (Nytran, Schleicher & Schuell, Inc., Keene, N.H.) by capillary action. After blotting, the filters were completely dried, and the RNA was fixed to the membranes by long-wave UV irradiation. To verify evenness of loading in each lane and to identify the location of the 18S and 28S ribosomal RNA species as well as the position of each marker in the RNA ladder, we stained the filters with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.5) for 3–5 minutes, followed by rapid destaining in diethylpyrocarbonate-treated water. After each of the marker positions was recorded, the membranes were destained in 0.1× sodium chloride, sodium citrate (SSC) and 1% sodium dodecyl sulfate for 15 minutes. The membranes were prehybridized at 62°C for 2 hours in 400 mM sodium phosphate buffer (pH 7.2) containing 5% sodium dodecyl sulfate, 1 mM EDTA, 1 mg/mL bovine serum albumin, and 50% formamide. Hybridization began with addition of the [32P]UTP-labeled complementatory RNA (cRNA) probe, which was carried out for 12–16 hours at 62°C. The membranes were washed in 0.1× SSC, 0.1% sodium dodecyl sulfate, and 1 mM EDTA at 75°C for 2–3 hours. They were then patted dry with paper towels, wrapped in plastic wrap, and exposed to x-ray film with intensifying screens at −80°C for 7 days. Each of the autoradiograms was analyzed with Macintosh-based image-analysis software (Image 1.41, W. Rasband, National Institutes of Health, Bethesda, Md.).
Biochemical Measurements

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

Analysis of Data

Statistical significance was determined by two-tailed Student's t test, and differences were considered significant at a value of p<0.05.

Results

Systolic blood pressure was significantly elevated in DOCA-salt hypertensive rats (189±6 compared with 108±3 mm Hg in uninephrectomized control rats, p<0.01, n=4 in each group). DOCA-salt hypertensive rats weighed significantly less (275±8 g) than their age-matched controls (405±10 g, p<0.01). Plasma renin activity was significantly depressed in DOCA-salt rats as expected (0.32±0.11 versus 2.05±0.35 ng angiotensin I·mL⁻¹·hr⁻¹ in controls, p<0.01). Plasma immunoreactive ET-1 was similar in DOCA-salt hypertensive rats and in normotensive control rats (0.99±0.08 compared with 0.95±0.05 pm in controls).

Northern blot analysis of total RNA extracted from segments of aorta and the complete mesenteric vascular bed, using a specific PCR probe for prepro-ET-1 mRNA, revealed a single band of 2.3 kb, in agreement with the size of prepro-ET-1 transcripts previously described. The intensity of the prepro-ET-1 band was much greater in lanes corresponding to total RNA from DOCA-salt hypertensive rats (top panels of Figures 1 and 2), although the amount of RNA loaded was the same. Densitometric scanning of the autoradiograms showed that abundance of prepro-ET-1 mRNA was fivefold greater in aorta and fourfold greater in mesenteric blood vessels of DOCA-salt hypertensive rats than in controls (bottom panels of Figures 1 and 2). These increases are most likely underestimated in the hypertensive rats compared with controls, because in order for the bands in the vessels from normotensive rats to be visualized, exposure had to be prolonged, resulting in saturation of the bands observed in the case of the DOCA-salt rats.

Discussion

In this study, we have investigated the abundance of mRNA for prepro-ET-1 in blood vessels of DOCA-salt hypertensive rats and demonstrated significant increased expression of the prepro-ET-1 gene. The study of expression of the prepro-ET-1 gene was motivated by previous results from our laboratory showing an increased content of immunoreactive ET-1 in acid extracts of aortic and mesenteric arteries of DOCA-salt hypertensive rats. Also, we had found in the same study significant exaggeration in the immunohistochemical reaction for ET-1 in endothelium of aorta and large and small mesenteric arteries of these hypertensive rats. No specific immunohistochemical reaction was observed in smooth muscle, indicating that the location of the exaggerated production of ET-1 was the endothelium. Because increased content of the peptide may be due to increased production or decreased release from endothelial cells, our present data showing greater abundance of prepro-ET-1 mRNA in total RNA from vessels of the DOCA-salt rats suggest that increased production of endothelin occurs in this model of experimental hypertension. Whether increased transcription or greater stability of prepro-ET-1 mRNA results in its greater abundance needs to be established. However, the very large difference found suggests that increased transcription is the main factor in the greater concentration of mRNA found, although differences in stability may also contribute in part. The present study demonstrates greater abundance of transcripts for prepro-ET-1, but in our previous study and other investigations of the vascular changes in DOCA-salt hypertension as well as in other models of hypertension, it has been clearly demonstrated that a hypertrophic response occurs in the blood vessel wall. This hypertrophy affects to a certain degree the endothelium, as well as smooth muscle cells and other components of the blood vessel wall. It remains to be determined whether the increase in ET-1 production is thus part of a general hypertrophic phenomenon occurring in the walls of
The present study did not examine the consequences of exaggerated expression of the prepro-ET-1 gene in blood vessels of this model of experimental hypertension. However, previous work suggests that ET-1 may downregulate endothelin receptors in smooth muscle cells. We have previously shown that in DOCA-salt hypertensive rats, responses of aorta and large and small mesenteric arteries to exogenous ET-1 are blunted. Furthermore, binding of ET-1 to mesenteric arteries is decreased, and signal transduction is also blunted in both mesenteric arteries and aorta. This suggests that, indeed as a consequence of increased production of ET-1 in blood vessels of DOCA-salt hypertensive rats, endothelin receptors are downregulated, resulting in depressed responses to exogenous and probably endogenous endothelin. Because the walls of blood vessels in DOCA-salt rats are significantly hypertrophied and their lumen reduced, vasodilator responses are magnified in resistance vessels and thus increased production of ET-1 may play a role in the maintenance of hypertension in this model despite depressed responsiveness to ET-1. Because ET-1 is also a potent mitogen, increased vascular production of ET-1 in DOCA-salt hypertensive rats could be in part responsible for the very important vascular hypertrophy present in this hypertensive model.

In conclusion, this study demonstrates that there is increased abundance of mRNA transcripts for prepro-ET-1 in aorta and mesenteric arteries of DOCA-salt hypertensive rats, providing further evidence for increased production of ET-1 in the walls of blood vessels in this model of experimental hypertension. Together with previous data showing abnormal function of the endothelium in DOCA-salt hypertensive rats, the present findings provide further evidence of the involvement of the endothelium in the mechanisms underlying elevated blood pressure in some forms of hypertension.

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References


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