Localization of Sites of Enhanced Expression of Endothelin-1 in the Kidney of DOCA-Salt Hypertensive Rats

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ABSTRACT

Although the role of endothelin-1, a potent vasoconstrictor peptide, in hypertension remains unclear, there is evidence of its involvement in deoxycorticosterone acetate (DOCA)-salt hypertensive rats, in which enhanced vascular production of endothelin-1 has been documented. The study presented here examined endothelin-1 gene expression in the kidney in DOCA-salt hypertensive rats by in situ hybridization histochemistry. A high specific activity 35S-labeled complementary RNA probe was used. Significant increases in abundance of endothelin-1 mRNA transcripts were found in the endothelium of renal vessels, and in capillary endothelial and mesangial cells of glomeruli of the remaining kidney of DOCA-salt hypertensive rats, in comparison with unilaterally nephrectomized control rats. Enhanced expression of the endothelin-1 gene in the kidney of DOCA-salt hypertensive rats may participate in abnormalities of renal function in this model of hypertension, and thus contribute to the development and maintenance of elevated blood pressure.

Key Words: Messenger RNA, in situ hybridization, glomeruli, endothelium, mesangial cells

Endothelin-1 (ET-1) is a member of a family of 21 amino acid peptides, which are potent vasoconstrictors originally isolated from the culture medium of porcine aortic endothelial cells (1,2). The biologically active form of ET-1 originates from the 212 amino acid precursor pre-proET-1. After signal peptide cleavage, pre-ET-1 is cleaved at a pair of basic amino acid residues to give rise to big ET-1, which is subsequently cleaved into mature ET-1 by a ET-converting enzyme (3,4). Although originally thought to be generated in the endothelium and to act primarily on vascular smooth muscle cells, endothelins have now been shown to be produced ubiquitously, and to exert numerous actions on different tissues. In the kidney, synthesis of endothelins has been demonstrated in glomeruli and inner medullary collecting ducts as well as in blood vessels (5-7). Locally or systemically produced endothelin exerts powerful effects on the kidney. It induces a constriction of large and small renal arteries, with reduction in RBF; it constricts afferent and, to a lesser degree, efferent arterioles, resulting in decreases in GFR (8-11), and affects tubular function, inhibiting AVP-stimulated osmotic water permeability in inner medullary collecting ducts and chloride and water reabsorption in cortical collecting ducts (12).

In deoxycorticosterone acetate (DOCA)-salt hypertensive rats, a model of mineralocorticoid-induced, volume-expanded experimental hypertension, immunoreactive ET-1 peptide (ir-ET-1) content (13), and ET-1 mRNA levels (14) were shown to be increased in the wall of aorta and mesenteric arteries. The cellular site of increased endothelin synthesis was shown to be the endothelium in these blood vessels (15). Endothelin-1 gene expression was also enhanced in the heart, and was localized to the endothelium of large and small coronary arteries (16). However, it is unknown whether enhanced endothelin-1 expression occurs in other tissues, or whether it is limited to vascular structures. The kidney plays an important role in hypertension, and is an heterogeneous tissue with blood vessels, glomeruli, and tubules, all of which may contribute to renal endothelin production (5). The potential contribution of endothelins to renal physiology in hypertension therefore prompted a study to answer the question of whether endothelin expression was elevated in the kidney of DOCA-salt hypertensive rats, and if so, which were the renal structures that contributed to ET-1 gene expression in this condition.

In the study presented here, in situ hybridization analysis of ET-1 mRNA was performed in the remaining kidney of DOCA-salt hypertensive rats and compared with unilaterally nephrectomized (Uni-Nx) control rats to establish whether there was an increment in ET-1 mRNA transcripts in the kidney and the cellular sites at which this occurred, and where a role could be played by ET-1 in renal functional alterations in this model 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. DOCA-salt hypertensive rats were induced by the method of Ormsbee and Ryan (17). Four male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada) weighing 200 g each were unilaterally nephrectomized under sodium pentobarbital anesthesia (40 mg/kg). Silicone rubber impregnated with DOCA (200 mg/rat) was implanted subcutaneously, and rats were given 1% saline to drink. Rats were killed 3 wk after becoming hypertensive (blood pressure >150 mm Hg). Four control animals were also unilaterally nephrectomized but received a silicone rubber implant without DOCA, and tap water to drink. The day before the rats were killed, blood pressure measurements were taken by the tail-cuff method on conscious restrained rats after warming, and were recorded on a Model 7 polygraph (Grass Instruments Co., Quincy, MA) fitted with a 7P8 preamplifier connected to a PCPB photoelectric pulse sensor. The average of three pressure readings was obtained. Rats were killed by decapitation. The kidneys were immediately removed from DOCA-salt and Uni-Nx rats. They were frozen in isopentane cooled to −35°C and then stored at −80°C, until processed for in situ hybridization histochemistry.

In Situ Hybridization Histochemistry

Frozen sections of kidney (10-μm thick) were obtained on a Bright-Hacker cryostat (Bright Instrument Co., Huntingdon, UK), thaw-mounted on polylysine-coated glass slides, and stored at −80°C until processed for in situ hybridization as described previously (15,16,18). Frozen sections were fixed in 0.1 M phosphate-buffered 4% formaldehyde solution, pH 7.2, for 60 min and then washed three times in 0.05 M phosphate-buffered saline, pH 7.4, for 10 min each. Denaturation was carried out with proteinase K (0.1 μg/ml) for 5 min at 37°C. Slides were transferred to 0.1 M triethanolamine, pH 8.0, and incubated in the same solution containing acetic anhydride (0.25% vol/vol) for 10 min at room temperature. Sections were then rinsed in 2× saline-sodium citrate (SSC) and dehydrated in ethanol (50% to 100%). Sense and antisense strand radioactive ET-1 RNA probes were diluted in hybridization buffer (75% formamide, 10% dextran sulfate, 3× SSC, 50 mM NaPO₄, pH 7.4, 1× Denhardt’s reagent (0.02% each of Ficoll 400, polyvinylpyrrolidone, BSA), 0.1 mg/ml yeast RNA) to a final concentration of 40×10⁶ dpm/μL. Dithiothreitol was added to a final concentration of 10 mM. Hybridization mix (25 μL per slide) was applied and the sections coveredslipped. The tissues were incubated in the hybridization oven at 55°C for 16 h. The next day, coverslips were removed in 2× SSC. Sections were treated with RNase A (40 μg/ml) at 37°C for 45 min to remove single-stranded RNA molecules. Successive washes at room temperature followed. In 2×, 1×, and 0.5× SSC for 10 min each and in 0.1× SSC at 60°C for 1 h. The tissue was then dehydrated and sections were dipped in Kodak NTB2 nuclear emulsion (diluted 1:1 with H₂O₂) and stored at 4°C. After exposure times of 6 wk, autoradiograms were developed in Kodak D19 (Eastman Kodak, Rochester, NY) at full strength for 2 min and fixed in 30% sodium thiosulfate for 4 min. To identify cellular structures, the sections were counterstained with cresyl violet. Specificity of labeling was established by incubating adjacent sections with the sense strand ET-1 RNA probe of the same size and specific activity as the antisense strand probe. Specific labeling was never encountered, using the sense strand ET-1 RNA probe. Tissues from eight different animals were sectioned (4 DOCA-salt and 4 Uni-Nx) and multiple sections for each group were examined (minimum of 12 tissue sections).

Preparation of the Rat ET-1 Probe

The rat ET-1 sense and antisense RNA probes were prepared by RNA transcription reaction, using T7 or SP6 RNA polymerase, respectively, and a CDNA plasmid construction previously described (14). In brief, a polymerase chain reaction product, obtained by amplification of cDNA from rat lung using specific oligonucleotide primers (14) derived from the cloned rat prepro-ET-1 sequence (19), was subcloned into pGEM7zf(+). From this construction, single-strand sense or antisense ET-1 RNA probes of 319 base pairs were generated. Radiolabeled riboprobes were prepared using 35S-UTP and 35S-CTP (1.250 Ci/mmol; Amersham, Arlington Hills, IL) in the same labeling reaction. Transcription reaction mixtures contained 250 μCi (200 pmol) each of 35S-UTP and 35S-CTP, 150 μM ATP and 150 μM GTP, 12.5 mM dithiothreitol, 20 U RNAse inhibitor, the appropriate linearized plasmid preparation in 1 μL (1 μg/μL concentration), and 6 U of SP6 or T7 RNA polymerase in a total volume of 10 μL. The reactions were carried out for 60 to 90 min at 37°C. The specific activity of each of these probes is estimated to be approximately 2×10⁶ Ci/μmol.

Observation and Photography

Labeled tissue sections were observed using a Zeiss Axioplan microscope equipped with a Darklite illuminator (Micro Video Instruments, Inc., Avon, MA). All photographs were taken using double-exposure settings. The autoradiographic grains were first exposed to Kodak Tungsten 64 ASA slide film (Eastman Kodak) under darkfield illumination, thus resulting in the white grain appearance. The second exposure was then taken under brightfield exposure with the lamp temperature set at 3200° and using two neutral gray filters. This second exposure shows the underlying tissue structure counterstained with cresyl violet. The camera was set on automatic exposure at 800 ASA for the darkfield exposure and 200 ASA for the brightfield exposure. Use of a double exposure also permits the proper focusing adjustment on the autoradiographic grains of the underlying tissue structure. Color prints were produced using the color slides obtained as negatives.

Biochemical Methods

ET-1 concentration was measured in plasma obtained from trunk blood, by RIA after extraction on C₁₈ Sep-Pak (Millipore, Milford, MA) as previously described (13).

Analysis of Data

Results are presented as means ± SE. Statistical differences were evaluated using two-tailed t test and results were considered different if P < 0.05.

RESULTS

Systolic blood pressure were significantly elevated in DOCA-salt hypertensive rats (189 ± 3 compared with 108 ± 3 mm Hg in Uni-Nx control rats, P < 0.01). Body weights were also significantly different (P <
0.01) in DOCA-salt hypertensive rats (275 ± 8 g) as compared with Uni-Nx rats (405 ± 10 g). Plasma ir-ET-1 levels were similar in this group of DOCA-salt hypertensive (2.9 ± 0.2 pM) and in normotensive rats (2.8 ± 0.1 pM).

Representative microphotographs of the in situ hybridization study of ET-1 mRNA expression in the kidney of DOCA-salt hypertensive rats and control Uni-Nx rats are shown in Figure 1. Similar results were observed in kidneys of four hypertensive and four control rats. ET-1 mRNA was not detectable in small cortical vessels or in glomeruli of Uni-Nx control rats using an ET-1 antisense probe (Figure 1, A and D). The grains present were similar in density to those obtained with the ET-1 sense probe, which suggested little ET-1 mRNA expression in these kidney structures of control rats. In DOCA-salt hypertensive rats, in contrast, a strong density of autoradiographic grains was observed in the endothelium of large and small arteries (Figure 1, B and C, arrows) and on mesangial cells and some capillary endothelial cells in glomeruli (Figure 1E, arrows, and F, arrows omitted for clarity). The ET-1 sense probe showed the level of background labeling, and no increase in signal was observed with the sense probe on the endothelial cells of blood vessels (not shown) or in glomeruli (Figure 1G) in the kidneys of the DOCA-salt hypertensive rats, demonstrating the specificity of the enhanced labeling found with the ET-1 antisense probe. In the medulla and papilla of Uni-Nx control rats (Figure 1H), some labeling with the antisense probe was found on vasa rectae. In DOCA-salt hypertensive rats, a moderate increase in the density of grains could be observed with the antisense probe in the vasa rectae in the medulla and papilla (Figure 1I) in comparison with controls (arrows).

DISCUSSION

This study suggests that excess local production of ET-1 in DOCA-salt hypertensive rats, present despite the absence of increased circulating levels (14, this study), occurs in defined kidney structures that may result in renal physiopathological effects of ET-1 in this model of experimental hypertension. These results also indicate that in the DOCA-salt hypertensive rat and, presumably, in other hypertensive models in which increased expression of the endothelin-1 gene may be demonstrated, the enhanced abundance of ET-1 mRNA transcripts is a generalized phenomenon affecting many endothelin-producing tissues, which may potentially locally affect function via endothelin-dependent effects. Although endothelin-like immunoreactivity has been demonstrated in the brush border of the most proximal portion of the proximal convoluted tubules, and in papillary collecting duct epithelium as well as in endothelium of blood vessels (5), no enhancement in expression could be noted in tubules either in the renal cortex, the medulla, or the papilla in the study presented here. Thus, increased expression in the kidney in DOCA-salt hypertensive rats occurs predominantly in the endothelium of blood vessels (large arteries, small cortical arteries, vasa rectae) and in glomeruli. In the latter, the distribution of grains has a mesangial cell pattern (Figure 1, E and F), although increased density of grains is also found over capillary endothelial cells. Thus, mesangial cells, which resemble smooth muscle cells, exhibit enhanced endothelin-1 gene expression, in agreement with the reported ability of smooth muscle cells to produce endothelins (20).

Previous studies using the immunoperoxidase technique have localized endothelin-like immunoreactivity in the endothelium of blood vessels in the cortex, medulla, and papilla of the rat kidney (5). In glomeruli, endothelin immunostaining was found predominantly in endothelial capillary cells with a smaller amount of reaction over mesangial cells. Some immunostaining was also found over proximal tubules and papillary collecting ducts. In our study, no immunohistochemical detection of endothelin was attempted because evaluation of immunoreactive ET-1 levels by immunohistochemistry assumes that enough peptide was produced and stored in the cell for differences to be detected. Dramatic changes in peptide synthesis may be occurring, but will not be efficiently detected if the release rate is high. The importance of storage both quantitatively and temporally in endothelium and other endothelin-producing cells has not been assessed. However, storage does not appear to be prominent in endothelial cells because typical secretory granules do not exist. Translation may also be altered, and increased mRNA could result in substantially less peptide synthesis than judged from measurement of mRNA abundance. In the blood vessels of DOCA-salt hypertensive rats (14), a disproportionately large abundance of ET-1 mRNA was found in relation to the

Figure 1. In situ hybridization histochemistry of the kidney using ET-1 cRNA probes. (A) Blood vessel from unilaterally nephrectomized (Uni-Nx) control rat, using ET-1 antisense probe (original magnification, × 100). (B) Blood vessel from DOCA-salt hypertensive rat, using ET-1 antisense probe, shows endothelial location (arrows) of specific grains (original magnification, ×100). (C) Darkfield photograph of (B). (D) Glomerulus from Uni-Nx control rat, using ET-1 antisense probe (original magnification, ×80). (E) Glomerulus from DOCA-salt hypertensive rat, using ET-1 antisense probe, shows specific grains localized in mesangial cells (arrows) and, to a lesser degree, in capillary endothelial cells (original magnification, ×80). (F) Darkfield photograph of (E) (arrows omitted for clarity). (G) Absence of labeling in glomerulus of DOCA-salt hypertensive rats when using the ET-1 sense probe (original magnification, ×80). (H) Moderate labeling of vasa recta in the inner medulla in Uni-Nx, using the ET-1 antisense probe (original magnification, ×40). (I) Increased density of grains in vasa recta in the inner medulla in DOCA-salt hypertensive rats, using the ET-1 antisense probe (original magnification, ×40).
amounts of immunoreactive ET-1 detected (13). This could be the result of either rapid secretion of synthesized peptide or of absence of a tight coupling between regulation of transcription or of mRNA stability, both of which could affect mRNA concentration, and translation or processing of the peptide. With this caveat in mind, detection of ET-1 mRNA levels may be a good index of cellular activity in the production of ET-1, as performed in this study and in previous studies (15,16). Furthermore, in situ hybridization histochemistry identifies unambiguously the presence of ET-1 expression, whereas antibodies exhibit crossreactivity between the different endothelins, and, thus, immunohistochemistry does not allow a conclusive identification of which endothelin is overproduced. The study presented here demonstrates that, in the kidney, ET-1 overexpression in DOCA-salt hypertensive rats is primarily confined to endothelial cells of both glomeruli and blood vessels, although in glomeruli, enhancement of expression occurs predominantly in mesangial cells, which agrees with the prior localization of endothelin-like immunostaining to these (5).

The mechanisms that account for the selective increase in expression of the ET-1 gene in the kidney of DOCA-salt hypertensive rats are unclear. Vasoactive peptides and growth factors (21,22) and pressure (23,24) could play a role in the enhanced production ET-1. In renal pathological processes, the expression of transforming growth factor beta (25) and other growth factors, such as platelet-derived growth factor, is increased (26), and platelet-derived growth factor has also been shown to stimulate ET-1 expression (27). It is likely that, as in blood vessels (28), renal expression of these growth factors is enhanced in DOCA-salt hypertensive rats and could contribute to the activation of ET-1 gene expression in the kidney. Because DOCA-salt hypertensive rats are unilaterally nephrectomized, compensatory hypertrophy of the remaining kidney could also contribute to increased renal expression of ET-1. However, in this study, unilaterally nephrectomized control rats did not exhibit increased renal expression of ET-1 mRNA, negating this potential explanation. Blood pressure may play a role in enhancing ET-1 expression (23,24), and it is interesting that the structures that exhibit increased abundance of ET-1 mRNA in this study—large and small arteries, and glomeruli—are the ones exposed to elevated blood pressure. Vasa rectae present small increases and tubular structures do not appear to exhibit any increase. This suggests that DOCA and salt alone do not appear to be responsible for the increases in ET-1 expression, and that blood pressure could contribute to the stimulation of ET-1 production. This is in agreement with previous studies in which we demonstrated that DOCA and salt did not stimulate ET-1 expression if blood pressure was not significantly elevated, whereas some hypertensive models did not exhibit increased ET-1 expression in the absence of DOCA and salt (29). We have recently found that in one-kidney one-clip Goldblatt hypertensive rats, there is enhanced expression of the endothelin-1 gene in blood vessels (30), suggesting that DOCA does not play a direct role. Because this experimental model is like DOCA-salt hypertension (a model of volume-expanded, low-renin hypertension) and two-kidney one-clip Goldblatt hypertensive rats (which are renin-dependent and are not volume-expanded), endothelin-1 overexpression in blood vessels does not occur (30), this may suggest a role for salt retention and volume expansion in the mechanisms leading to enhanced endothelin-1 gene expression in hypertension.

Endothelin secretion from vascular endothelial cells is abluminal (31), and even if many tissues exhibit significant increases in endothelin production, circulating levels of immunoreactive endothelin in plasma may remain within normal limits, as found in this study. Enhanced ET-1 production may downregulate ET receptors (32). The final effect of enhanced renal endothelin synthesis will depend on the balance between the increased production and the reduced receptor density. Renal ET receptors have a differential distribution within the kidney. ETA receptors are present in glomeruli, and in vasa recta and arcuate arteries, whereas the ETB receptor is found predominantly in the initial and terminal inner medullary collecting duct and the glomerulus (33). ET-1 is a powerful vasoconstrictor of renal blood vessels and increases renal vascular resistance (10,11), reduces GFR by preferential afferent arteriolar constriction and by reduction in the capillary ultrafiltration coefficient (8,9), and also affects tubular function, albeit with the opposite effect on renal function. ET-1 inhibits AVP-stimulated osmotic water permeability in inner medullary collecting ducts and chloride and water reabsorption in cortical collecting ducts (12), and could thus induce natriuresis and diuresis. DOCA-salt hypertensive rats exhibit an important polyuria, to which enhanced renal ET-1 synthesis may contribute. Increased ET-1 production in blood vessels and glomeruli may participate in blood pressure elevation in DOCA-salt hypertensive rats. Chronic treatment with the orally active ETA/ETB endothelin receptor antagonist bosentan blunts the development of elevated blood pressure in DOCA-salt hypertensive rats (34), which suggests a role of ET-1 in the development and maintenance of this experimental model of hypertension. The extent to which the renal effects of enhanced ET-1 production in the kidney in DOCA-salt hypertensive rats participate in the pathophysiology of high blood pressure must await results of studies of renal function with endothelin antagonists in these rats. It is already known that in a variety of experimental renal diseases, endothelin receptor antagonism improves renal function (35,36).

In summary, the study presented here demonstrates enhanced ET-1 gene expression in the kidney of DOCA-salt hypertensive rats in the endothelium of
large and small cortical renal arteries and in medul-
mary and papillary vasa rectae, as well as in mesangial cells and some capillary endothelial cells of glomeruli. This suggests that ET-1 actions in the kidney may contribute to the functional abnormalities leading to the development or the maintenance of elevated blood pressure in this and, potentially, in other models of hypertension. The results of this study localize the sites in the kidney at which endothelins or their antagonists should be applied in physiological studies to understand the contribution of endothelins to altered renal function in hypertension.

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