Gene expression of natriuretic peptide receptors in rats with DOCA-salt hypertension

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1Department of Medicine, Hôpital St.-Luc, Université de Montréal, Montreal, Quebec H2X 3J4; 2Institut de Recherche Clinique de Montréal, Université de Montréal, Montreal, Quebec H2W 1R7; and 3Department of Medicine, Royal Victoria Hospital, McGill University, Montreal, Quebec, Canada H3A 1A1

Nuglozeh, Edem, Majambu Mbikay, Duncan J. Stewart, and Louis Legault. Gene expression of natriuretic peptide receptors in rats with DOCA-salt hypertension. Am. J. Physiol. 273 (Cell Physiol. 42): C1427–C1434, 1997.—In our previous studies, we found that the atrial natriuretic peptide (ANP) binding and guanylyl cyclase activity of A-type natriuretic peptide receptors (NPR-A) were upregulated in renal papillae but downregulated in vascular tissues and glomeruli of rats with deoxycorticosterone acetate (DOCA)-salt hypertension [E. Nuglozeh, G. Gauquelin, R. García, J. Tremblay, and E. L. Schiffrin. Am. J. Physiol. 259 (Renal Fluid Electrolyte Physiol. 28): F130–F137, 1990]. To further understand the molecular significance of these regulations, we measured the relative abundance of the transcripts of NPR-A and NPR-B by Northern blot in the aorta, mesenteric arteries, adrenal cortex, renal papillae, and lungs in DOCA-salt hypertensive and control rats. In renal papillae we also examined the translation and transcription of NPR-A by ribosome loading and run-on assay. Compared with controls, the steady-state levels of mRNA for NPR-A were increased in the aorta and mesenteric arteries but were decreased in the adrenal cortex and renal papillae in DOCA-salt-treated rats. NPR-B mRNA was decreased in the aorta, mesenteric arteries, and adrenal cortex in hypertensive rats. In lungs the mRNA for both receptors was unchanged. Translation of NPR-A mRNA, as assessed by ribosome loading, was reduced in renal papillae. Transcriptional activity of its gene was not detectable in these tissues. Guanosine 3′,5′-cyclic monophosphate (cGMP) accumulation in a large number of tissues, including glomeruli (2) and medullary and papillary collecting duct cells (17), an effect produced through the activation of particulate guanylyl cyclase (16). Binding studies, Northern blot studies, and enzymatic studies have demonstrated the presence of ANP receptor mRNA and cGMP generation in vascular tissues and vascular smooth muscle cells (30, 33). Subsequently, it has been reported that vascular smooth muscle cells produce CNP, which may have autocrine functions on these cells (13). Membrane preparations from other organs, e.g., adrenals and lungs, avidly bind ANP with dissociation constants of 30–1,800 pM (16) and 6.5–660 pM, respectively (12). Molecular cloning studies have identified three different natriuretic peptide receptors (NPRs) (8, 12, 28). Two of these receptors, NPR-A and NPR-B, also called GC-A and GC-B, constitute a newly described family of receptor guanylyl cyclase. These receptors are single transmembrane proteins with extracellular domains that share 44% identity. The intracellular regions of these proteins can be divided into two domains. The first domain of NPR-A and NPR-B, which is 280 amino acids long, is 30% homologous to protein kinase. The COOH-terminal portion, which is 250 amino acids long, is the guanylyl cyclase catalytic domain, which is activated on binding of the appropriate natriuretic peptide to the extracellular domain. This catalytic region exhibits the highest amount of sequence identity (88%) between NPR-A and NPR-B.

The third member of the NPR family is NPR-C. Molecular cloning (12) showed that this receptor contains a very short 37-amino acid cytoplasmic tail that bears no homology to the intracellular domain of any other known receptor. Its intracellular domain, however, is ~30% identical to that of NPR-A and NPR-B and is negatively coupled to adenylate cyclase (1).

The three NPR subtypes recognize the three known natriuretic peptides differentially. ANP and brain natriuretic peptide can effectively stimulate NPR-A, whereas...
NPR-B is efficiently stimulated by CNP (18, 19). NPR-C binds all three known natriuretic peptides with high affinity in assuming clearance and buffering functions (20).

Studying deoxycorticosterone acetate (DOCA)-salt hypertensive rats, we previously showed an increase in the number of receptors and cGMP responses to ANP in renal papillae of DOCA-salt hypertensive rats but a downregulation of ANP receptors in glomeruli and mesenteric arteries (22). From the former studies, it seems that NPR regulation is tissue specific; therefore, we undertook this study to determine the molecular aspect of this regulation in different tissues of this animal model.

MATERIALS AND METHODS

Materials. All materials were of the highest reagent grade available. Tris(hydroxymethyl)aminomethane (Tris)-HCl, Triton X-100, glycerol, diethyl pyrocarbonate, diethiothreitol, proteinase K, ammonium acetate, bovine serum albumin, and DOCA were from Sigma Chemical (St. Louis, MO); guanidine available. Tris(hydroxymethyl)aminomethane (Tris)·HCl, Tris(ethylenediamine)polyaldehyde, sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethyl-enediamine, acrylamide, bisacrylamide, acrylamide, bisacrylamide, EDTA, ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetate acid, NaCl, MgCl₂, KCl, KOH, NaOH, β-mercaptoethanol, agarose, formaldehyde, sodium lauryl sarcosine, sodium dithioate, and deoxycholate from Fisher (Nepean, ON, Canada); formamide, rNTP, dNTP, ribonuclease A, and yeast tRNA from Boehringer Mannheim (Laval, PQ, Canada); and α-UTP and γ-ATP from Amersham (Oakville, ON, Canada). X-Omat XRPF6 film was from Eastman Kodak (Rochester, NY). Taq DNA polymerase was from Cetus. Synthetic rat ANP (99–126) was purchased from Institut Armand-Frappier (Laval, PQ, Canada).

Animal experiments. DOCA-salt hypertonation was induced by the method of Ormsbee and Ryan (23). Male Sprague-Dawley rats (Charles River Laboratories, St.-Constant, PQ, Canada) weighing 200 g were uninephrectomized under isoflurane anesthesia. Silicone rubber impregnated with 13% NaCl was inserted into the renal cavity of anesthetized rats. The rats were given 3% saline to drink. A group of 20 hypertensive rats were studied 5–6 wk after the intervention, a period previously shown to be sufficient for development of hypertonation. Another group of uninephrectomized rats was implanted with silicone rubber without DOCA impregnation and served as controls. This group received tap water to drink. In an attempt to dissociate the role of DOCA, salt, and uninephrectomy, rats were submitted to procedures that were unlikely to allow the complete pattern of hypertonation to appear. Sixteen rats underwent sham uninephrectomy, which consisted of anesthetia and laparotomy without removal of kidney tissue. These rats did not receive DOCA and drank tap water. A second group of animals (n = 20) underwent uninephrectomy but were not exposed to DOCA or NaCl. DOCA was administered to 15 uninephrectomized rats that received regular tap water to drink. The last group of rats (n = 15) underwent uninephrectomy and drank water to which 1% NaCl was added, but they did not receive DOCA. The large number of animals studied was necessary to provide sufficient tissue samples for molecular biology studies. Animal experiments were reviewed and accepted by our institution’s animal experiments ethics board.

Blood pressure was measured 5–6 wk after the initial procedure. In our experience, this length of time is sufficient for hypertonation to develop in the vast majority of DOCA-salt-treated rats. The rats were anesthetized with isoflurane. A right carotid PE-50 catheter was inserted. The rats were allowed to recover, and blood pressure was measured the next morning using an American Optical blood pressure monitor (model 1331A). The rats were then killed, and tissues were quickly harvested and frozen in liquid nitrogen for Northern blot and ribosome-loading studies.

cGMP generation by renal papillae, mesenteric arteries, aorta, and adrenal cortex in response to ANP and CNP stimulation. Rat papillae from different experimental groups were dissected, minced, and subjected to digestion with collagenase (2 mg/ml). After 2 h of digestion, the papillae were totally disrupted, the cells were washed twice, and their viability was evaluated by trypan blue exclusion. The papillae were resuspended in Krebs buffer with the following composition (mmol/l): 118 NaCl, 1.18 MgSO₄, 1.18 KH₂PO₄, 5.5 dextrose, 25.0 NaHCO₃, 2.5 CaCl₂, and 4.7 KCl. The solution was bubbled with 95% O₂–5% CO₂. The cells were preincubated with 0.5 mmol/l L-isobutyl-3-methylxanthine for 10 min to inhibit phosphodiesterase activity, then stimulated with increasing concentrations of ANP (10⁻¹² to 10⁻⁶ mol/l) for 10 min. The reaction was quenched with 1 mmol/l trichloroacetic acid, and cGMP was determined by radiomunnoassay after acetylation. The adenylate cyclase was prepared from the renal medulla by removal of the medulla. Each tube, containing 50 mg of mesenteric arteries, aorta, and adrenal cortex slices of control and DOCA-salt-treated rats, was incubated with increasing concentrations of ANP and CNP. On the next day, the tissue slices were soaked in liquid nitrogen and quickly ground using Varimix (model 3, Caulk Dentsply Division International, Toronto, ON, Canada). The powdered tissues were resuspended in the same liquid used for stimulation, and cGMP generation was measured as previously described.

ANP receptor probe preparation. Polymerase chain reaction (PCR) analysis was performed on rat genomic DNA with primers designed in 3'-translated region in the primary structure of rat guanylyl cyclase A/ANP receptor gene to avoid cross hybridization between receptors of the same family. The sense and antisense primers were GCAAGG- GCAAGTGTCAGC/CGGTGCGTC and TGCTCTGCGTCG/ GTTGCGGCGTCA, respectively. PCR was performed on a Perkin-Elmer/Cetus DNA thermal cycler with Perkin-Elmer/ Cetus Taq DNA polymerase. Amplification was performed in 100-µl reactions with 100 ng of rat genomic DNA and 100 pmol each 27-mer oligonucleotide primer, 1 mmol/l each dGTP, dATP, dCTP, and dTTP, 10% dimethyl sulfoxide, 67 mmol/l Tris-HCl, pH 8.8, 6.7 mM MgCl₂, 6.7 mmol/l EDTA, 100 mmol/l β-mercaptoethanol, and 0.17 mg/ml bovine serum albumin, with 1 U of Taq DNA polymerase. The reaction was initially subjected to heating for 1 min at 94°C, then 30 cycles of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C. The PCR product yielded a fragment of 554 base pairs, which was cloned into the plasmid pSP72 and confirmed by sequencing. NPR-B was a gift from Garber Laboratory.

Northern blot analysis of NPR-A and NPR-B mRNA. Total RNA was extracted by guanidine and phenol methods, as previously described (9). Total RNA (10 µg), denatured with formaldehyde and electrophoresed in 1% agarose gels (35% formaldehyde in 3-(N-morpholino)propanesulfonic acid buffer, was transferred to a Zetaphore membrane. The RNA was cross-linked onto the membrane using the ultraviolet cross-linker FB-UVXL-1000. The membrane was prehydrated for 2 h at 69°C in a sodium phosphate-SDS buffer (400 mmol/l) containing 60% formamide and hybridized overnight in the same buffer to a high-specific activity cRNA probe (3 x 10⁴ counts·min⁻¹·µg⁻¹·RNA). The filters were washed in 0.1% SDS-1 mmol/l.
Comparisons between two groups of rats were made by Friedman's test. Differences were considered significant at \( P < 0.05 \).

**RESULTS**

Ninety percent of DOCA-salt-treated rats were hypertensive (systolic blood pressure > 160 mmHg). Only hypertensive rats were studied. The mean arterial blood pressure was 220 ± 2 mmHg (n = 20). All other groups of rats had significantly lower blood pressures (Table 1). The group of rats exposed to DOCA without saline was borderline hypertensive, with a mean systolic blood pressure of 133 ± 4 mmHg.

Expression of NPR-A mRNA in tissues from DOCA-salt-treated rats. In a first series of experiments, we measured NPR-A mRNA levels by Northern blot analysis in vascular tissues, lungs, adrenal cortex, and renal papillae of DOCA-salt-treated and control rats. The levels were dramatically increased in mesenteric arteries (to 520%) and in aorta (to 160%) in DOCA-salt-treated rats compared with controls. Total RNAs (10 µg) from each tissue were used for analysis. Data represent a mean of 4 different experiments. Note significant increases in mRNA in aorta (A) and mesenteric arteries (MA) and significant decrease in mRNA in renal papillae (RP) and adrenal cortex (AdC). No significant differences were observed in lung (L) kb. Kilobase.

**Table 1. Blood pressure and PRA in sham-operated and uninephrectomized rats treated with salt and/or DOCA**

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Mean BP, mmHg</th>
<th>PRA, ng·ml⁻¹·h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2K sham operated</td>
<td>16</td>
<td>99 ± 3</td>
<td>2.13 ± 0.23</td>
</tr>
<tr>
<td>Uni-Nx</td>
<td>20</td>
<td>103 ± 2</td>
<td>1.3 ± 0.20</td>
</tr>
<tr>
<td>Uni-Nx-salt</td>
<td>16</td>
<td>111 ± 4</td>
<td>1.4 ± 0.13</td>
</tr>
<tr>
<td>Uni-Nx-DOCA</td>
<td>15</td>
<td>133 ± 12</td>
<td>ND</td>
</tr>
<tr>
<td>Uni-Nx-DOCA-salt</td>
<td>20</td>
<td>220 ± 2</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. PRA, plasma renin activity; ND, not detectable; Uni-Nx, uninephrectomized; 2K sham operated, sham operated with 2 kidneys; Uni-Nx-salt, uninephrectomized and given NaCl in drinking water; Uni-Nx-DOCA, uninephrectomized and impregnated with deoxycorticosterone acetate; Uni-Nx-DOCA-salt, uninephrectomized, given NaCl in drinking water, and impregnated with DOCA. *P < 0.01 vs. all. †P < 0.05 vs. 2K sham operated, Uni-Nx, and Uni-Nx-salt. ‡Systolic blood pressure.
hypertensive rats. In mesenteric arteries, NPR-B mRNA was four times NPR-A mRNA. Hypertension reversed this ratio, with the level of NPR-A mRNA being threefold higher. In the adrenal cortex, as in the aorta, NPR-A mRNA was about four times higher than NPR-B mRNA; this ratio was not significantly changed by hypertension. In the lungs the ratio of the two receptors was nearly identical in normal and hypertensive rats.

Polysomal and monosomal profile analysis of rat renal papilla mRNA. To determine the translational status of NPR-A mRNA, polysomes and monosomes from Nonidet P-40-lysed tissues were isolated, and their RNA was analyzed by Northern blot. The experiment revealed an important reduction in full-length NPR-A mRNA molecules in DOCA-salt-treated rats, as demonstrated by the degradation smear with the monosomal and polysomal fractions that are between and beyond the two subunits of the ribosomes (Fig. 4).

cGMP generation by rat isolated tissues. NPR-A and NPR-B are guanylyl cyclases. In all the tissues studied, including renal papillae, mesenteric arteries, aorta, and adrenal cortex, cGMP generation in response to stimulation with ANP and CNP was significantly higher in DOCA-salt-treated rats than in controls (Figs. 5–7). This increase occurred at ANP and CNP concentrations endogenously observed in the DOCA-salt hypertensive animals (22).

DISCUSSION

The initial purpose of this study was to understand the molecular mechanisms responsible for the upregulation of NPR-A in the renal papillae of DOCA-salt-treated rats observed in our previous studies. The results appear to contradict our initial findings: NPR-A mRNA is decreased in renal papillae in this model
compared with control animals (Fig. 1). In addition, whereas NPR-A binding appeared suppressed in vascular tissues in DOCA-salt-treated rats (27), NPR-A mRNA is increased in these tissues. To further our understanding of this paradox, we undertook transcription and translation studies. We were unable to determine any variation at the level of transcription, since no transcription of the NPR-A gene was detectable by run-on assay conducted with renal papilla nuclei of control as well as DOCA-salt-treated rats (data not shown). The translation study demonstrates a decrease in NPR-A mRNA levels in polysomal as well as monosomal fractions (Fig. 4), an indication that this mRNA becomes susceptible to degradation in DOCA-salt hypertension.

Fig. 4. A representative Northern blot analysis of polysomal and monosomal NPR-A mRNA in rat renal papillae of DOCA-salt hypertensive rats compared with control rats. Polysome and monosome separation was performed by centrifugation on sucrose gradient. Note significant decrease in NPR-A mRNA in polysomal and monosomal fractions in DOCA-salt hypertension.

Fig. 5. Dose-response curve of cGMP production by renal papilla in response to increasing concentrations of atrial natriuretic peptide (ANP, 10^{-12}–10^{-6} mol/l) in DOCA-salt hypertensive and uninephrectomized (control) rats. Values are means ± SE of 2 different experiments. Statistical differences between 2 curves for each organ were evaluated by comparing goodness of fit when maximal response was allowed to iterate freely. F values for curves were 21.47, 12, and 14.94 for A, B, and C, respectively (P < 0.01).

Fig. 6. Dose-response curve of cGMP production by mesenteric arteries (A), aorta (B), and adrenal cortex (C) in response to increasing concentration of ANP (10^{-12}–10^{-6} mol/l) in DOCA-salt hypertensive and uninephrectomized (control) rats. Values are means ± SE of 2 different experiments. Statistical differences between 2 curves for each organ were evaluated by comparing goodness of fit when maximal response was allowed to iterate freely. F values for curves were 21.47, 12, and 14.94 for A, B, and C, respectively (P < 0.01).
The results of Northern blot studies are in total agreement with the ribosome-loading studies. How can the upregulation of NPR-A in the renal papillae in DOCA-salt-treated rats be explained in light of these transcription and translation studies? To explain the paradox between the lower levels of NPR-A mRNA as demonstrated by Northern blot analysis and the ribosome-loading studies and our previous studies in which we showed that the binding and cGMP production were higher in renal papillae of DOCA-salt-treated rats than control rats, one must postulate that although the biosynthesis of NPR-A may be decreased, the half-life and activity of these receptors may be prolonged. Indeed, Hughes et al. (15) demonstrated that ANP receptor recycling occurred at 37°C in a rabbit carotid cell line but not at 4°C. Subsequently, Pandey (24, 25) conducted elegant studies on the kinetics and stoichiometry of internalization and recycling of ANP receptors in smooth muscle cells and murine Leydig tumor cells. This author demonstrated that a population of ANP receptors rapidly recycled (half-life = 5 min) from intracellular compartments to the plasma membrane. The apparent upregulation of ANP binding we observed in renal papillae of DOCA-salt hypertensive rats could be due to receptor recycling; i.e., even if the overall levels of translatable mRNA are decreased, the protein produced may be significantly less catabolized and is constantly mobilized through the recycling process. The factors that may induce this putative increase in NPR-A recycling in DOCA-salt-treated rats compared with controls remain unknown. In vitro, the mechanisms of recycling are influenced by temperature, the energy-depleting dinitrophenol and lysosomotropic agents like chloroquine (6), which are known to interfere with the intracellular routing, and the redistribution of the ligand-receptor complex (29). In vivo, in DOCA-salt-treated rats, there may be induction of some protein factors that can direct NPRs through the recycling machinery.

The regulation of NPR-A in DOCA-salt-treated rats is tissue specific. The mRNA levels are increased in the aorta and mesenteric arteries, whereas they are decreased in renal papillae and adrenal cortex. In mesenteric arteries the increase in mRNA correlated with an increase in cGMP generation, despite decreased NPR-A binding (22). This discrepancy may reflect a distribution and an activity of the receptor particular to this tissue and remains to be clarified. For example, in human umbilical vein endothelial cells, the constitutive form of nitric oxide synthase activity is increased, whereas its mRNA is decreased, suggesting that enzyme-specific activity, rather than enzyme quantity, was increased (26).

This differential regulation of the same mRNA in DOCA-salt hypertension is probably due to one or several factors differentially induced in tissues. It is possible that regulatory factors induced in renal papillae and the adrenal cortex at the onset of the hypertension are not expressed in the aorta and mesenteric arteries or vice versa. For example, it has been shown that, in some pathological states, RNA-binding proteins are expressed that trigger a rapid degradation of specific mRNA (34). Conversely, mRNA-stabilizing proteins may be produced that induce a net increase in the steady state of some mRNA. This concept of differential regulation of mRNA stability in DOCA-salt-treated rats may best explain our observations in the various tissues examined in this study.

Fig. 7. Dose-response curve of cGMP production by mesenteric arteries (A), aorta (B), and adrenal cortex (C) in response to increasing concentration of C-type natriuretic peptide (CNP, 10^{-12}-10^{-6} mol/l) in DOCA-salt hypertensive and uninephrectomized (control) rats. Values are means ± SE of 2 different experiments. Statistical differences between 2 curves for each organ were evaluated by comparing goodness of fit when maximal response was allowed to iterate freely. F values for curves were 12.13, 11.9, and 14.9 for A, B, and C, respectively (P < 0.01).
Unlike NPR-A mRNA, NPR-B mRNA seems to be downregulated in all tissues examined, including the aorta, mesenteric arteries, adrenal cortex, and lungs (Fig. 2). Whether transcriptional or translational regulations are involved in these changes could not be verified in this study because of difficulties in obtaining good-quality nuclei and polysomes from these tissues. The NPR-B mRNA was undetectable in renal papillae in DOCA-salt-treated and control rats, obviating the ability to perform transcriptional and translational studies. However, on a molecular basis, NPR-B mRNA is intrinsically unstable because of the presence of two canonic sequences AUUUA in its 3’-untranslated region between nucleotides 3230 and 3235 as well as nucleotides 3237 and 3242 that induce destabilization and degradation in mRNA molecules (28). This may explain the decrease in NPR-B mRNA levels in DOCA-salt-treated rats (Fig. 2). The fact that the NPR-B mRNA levels are decreased in the vascular tissues (aorta and mesenteric arteries) and adrenal cortex of DOCA-salt-treated rats leads to a second paradox. Whereas tissues from control rats, which contain more NPR-B mRNA, have low guanyloyl cyclase activity, tissues from DOCA-salt-treated rats, which contain less mRNA, have a higher level of the enzymatic activity (cf. Figs. 2 and 7). In the aorta and mesenteric arteries, NPR-A and NPR-B are regulated in opposite directions. This may be linked to different regulatory cis elements present in the genes or their mRNA. The trans factors expressed in these tissues during hypertension might selectively or differentially affect the regulation of the receptor genes, leading to downregulation of the NPR-B mRNA and upregulation of NPR-A mRNA.

In conclusion, the present study demonstrates that, in DOCA-salt-treated rats, despite an upregulation of guanylyl cyclase activity of the NPR-A in renal papillae and NPR-B in the aorta and mesenteric arteries, the steady-state level of their mRNA is decreased. In the case of the NPR-A mRNA, this is probably due to destabilization by factors induced during the hypertension. To explain our findings, we postulate that NPR-A in renal papillae of DOCA-salt-treated rats are recycled, as are NPR-B in vascular tissues. However, further studies are necessary for molecular identification of these factors and, therefore, their functional significance in the pathophysiology of DOCA-salt hypertension.

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