Role of renal nerves in stimulation of renin, COX-2, and nNOS in rat renal cortex during salt deficiency

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Höcherl, K., M. Kammerl, F. Kees, B. K. Krämer, H. F. Grobecker, and A. Kurtz. Role of renal nerves in stimulation of renin, COX-2, and nNOS in rat renal cortex during salt deficiency. Am J Physiol Renal Physiol 282: F478–F484, 2002. First published October 10, 2001; 10.1152/ajprenal.00209.2001—We investigated a possible involvement of the sympathetic nervous system in the parallel increase of renin, cyclooxygenase-2 (COX-2), and neuronal nitric oxide synthase (nNOS) gene expression in the juxtaglomerular apparatus of rat kidneys induced by salt deficiency. Therefore, we determined the effects of renal denervation and the β-adrenoceptor antagonist metoprolol (50 mg/kg body wt po, twice a day) on renocortical expression of renin, COX-2, and nNOS in rats fed a low-salt (0.02% wt/wt) diet or treated for 1 wk with ramipril (10 mg/kg body wt) in combination with a low-salt diet. We found that a low-salt diet in combination with ramipril strongly increased renocortical mRNA levels of renin, COX-2, and nNOS 9-, 7-, and 2.5-fold, respectively. Treatment with metoprolol did not change basal expression of the three genes or induction of renin and COX-2 gene expression, while induction of nNOS expression was clearly attenuated. Similarly, unilateral renal denervation attenuated induction of nNOS expression but had no effect on all other parameters. These findings suggest that β-adrenergic stimulation is not required for stimulation of renin and COX-2 gene expression in the juxtaglomerular apparatus during salt deficiency. However, β-adrenoceptor activity or renal nerve activity appears to be required for the full stimulation of nNOS expression by low salt intake or combined with angiotensin-converting enzyme inhibition.

cyclooxygenase-2; kidney; renal nerves; neuronal nitric oxide synthase

RECENTLY, IT HAS BEEN RECOGNIZED that expression of cyclooxygenase (COX)-2 and the neuronal isoform of nitric oxide synthase (nNOS) changes in renal macula densa cells in parallel with the expression of renin in the neighboring juxtaglomerular cells (3–5). Thus low salt intake (3, 4, 27), fall in renal perfusion pressure (3, 14, 22, 31), and angiotensin II (ANG II) antagonists (6, 19, 33) increase expression of these genes. Maximum effects have been observed with a low-salt diet in combination with ANG II antagonists (4, 6, 33). The pathways triggering the characteristic increase in expression of the three genes and the physiological implication of the increased expression are not yet clear. It has been suggested that the increase in renin expression may be causally related to a preceding increase in formation of COX-2-derived prostanooids in macula densa cells (1, 10, 18, 20). The increase in COX-2 expression in the macula densa, in turn, has been suggested to be dependent on nNOS activity in the macula densa (5), leading to the concept of a sequential stimulation: nNOS → COX-2 → renin. This concept, however, is questioned by other findings, which could not confirm a role for COX-2-derived prostanooids in expression of renin (22, 23) or confirm a functional interdependence of nNOS and COX-2 in macula densa cells (4). Therefore, we considered alternative pathways that could be relevant for the concerted induction of renin, COX-2, and nNOS in states of salt deficiency. We focused our interest on the sympathetic nervous system, which could be a mediator candidate, because the sympathetic nervous system will be activated by volume depletion and/or a fall in blood pressure during salt deficiency (8, 16, 29, 30) and because renin-producing juxtaglomerular cells (2, 15) and macula densa cells (2, 15) are equipped with β-adrenoceptor. Therefore, it appeared reasonable to determine the relevance of sympathetic outflow for stimulation of renin, COX-2, and nNOS gene expression by salt depletion. For this purpose, we performed experiments with β-adrenoceptor blockade and with unilateral renal denervation of rats fed a low-salt diet alone or in combination with treatment with an angiotensin-converting enzyme (ACE) inhibitor. A low-salt diet alone is known to induce only a minor stimulation of renin, COX-2, and nNOS gene expression (4, 12, 13, 28), whereas additional treatment with an ACE inhibitor strongly increases these three genes in the rat renal cortex (4, 28). Although the model of a low-salt diet in combination with ACE inhibition may have some limitations (stimulation of the renal baroreceptor, alterations in renal blood flow and glomerular filtration rate, interruption of the angiotensin-medi-
ated negative-feedback pathway on renin), a possible effect of β-adrenoreceptor blockade on induction of these genes may become more apparent in this experimental setting because of the strong stimulation of renin, COX-2, and nNOS gene expression.

METHODS

Male Sprague-Dawley rats (150–180 g; Charles River, Sulzfeld, Germany) were habituated for 5 days and had access to tap water ad libitum. Body weight and systolic blood pressure (tail cuff method) were monitored daily.

Rats were divided into 9 groups of 10 rats each and treated for 1 wk as follows: 1) normal diet [0.6% (wt/wt) NaCl; Altromin, Lage, Germany] and vehicle; 2) normal diet and metoprolol tartrate (50 mg/kg twice a day); 3) low-salt diet [0.02% (wt/wt) NaCl; Ssniff Special Diets, Soest, Germany] and vehicle; 4) low-salt diet and metoprolol tartrate (50 mg/kg twice a day); 5) low-salt diet and ramipril (10 mg·kg⁻¹·day⁻¹) in drinking water; 6) low-salt diet and ramipril (10 mg·kg⁻¹·day⁻¹) in drinking water and metoprolol tartrate (50 mg·kg⁻¹·day⁻¹) in drinking water; 7) left-side renal denervation by a combination of mechanical and chemical methods, as described previously (16), and, after 3 days, a normal-salt diet for 1 wk; 8) left-side renal denervation followed by a low-salt diet; and 9) left-side renal denervation followed by a low-salt diet in combination with ramipril (10 mg·kg⁻¹·day⁻¹) in drinking water.

Rats were killed by decapitation during anesthesia with sevoflurane. Ramipril and metoprolol were gifts from AstraZeneca (Möln达尔, Sweden).

Samples. Blood was collected into EDTA tubes. The kidneys were quickly removed and cut into longitudinal halves. Cortices were dissected with a scalpel blade under a stereomicroscope, frozen in liquid nitrogen, and stored at −80°C until extraction of total RNA (7).

Ribonuclease protection assays for β-actin, renin, COX-1, COX-2, nNOS, and endothelial NOS (eNOS) mRNA levels were measured by ribonuclease protection assays, as described elsewhere (4, 26). Briefly, cRNA probes (5 × 10⁵ cpm) were hybridized at 60°C overnight with 40 μg of total RNA for COX-1 and COX-2, 100 μg of total RNA for nNOS and eNOS, 20 μg of total RNA for renin, 1 μg of total RNA for β-actin, and 20 μg of total RNA for negative control. Then they were digested with ribonuclease A/T₁ (room temperature for 30 min) and proteinase K (37°C for 30 min). After phenol-chloroform extraction and ethanol precipitation, protected fragments were separated on an 8% polyacrylamide gel. The gel was dried for 2 h, and bands were quantitated by phosphorimaging (Instant Imager 2024, Packard). Autoradiography was performed at −80°C for 1–3 days.

Figure 1 shows typical autoradiographs of gels using renocortical total RNA (20 μg of total RNA for renin mRNA, 40 μg for COX-2, and 100 μg for nNOS) of six rats: two fed a normal-salt diet, two treated with ramipril in combination with a low-salt diet, and two were treated with ramipril and metoprolol in combination with a low-salt diet.

Immunoblotting for nNOS, eNOS, and COX-2 protein in the rat renal cortex. One hundred micrograms of total renocortical protein were loaded per lane, separated by an 8% SDS-polyacrylamide gel (10% for COX-2), and transferred onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked overnight at 4°C and incubated with the following antibodies for 2 h at room temperature: nNOS (diluted 1:500; Transduction Laboratories), eNOS (diluted 1:500; Transduction Laboratories), COX-2 (diluted 1:500; Santa Cruz), and a horseradish peroxidase-labeled secondary antibody (goat anti-mouse IgG, diluted 1:500). Detection was achieved by enhanced chemiluminescence (Amersham). The band intensities were quantified by densitometry.

Determination of tissue catecholamines. Catecholamines were determined by reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection, as previously described in detail (9). Renal cortex samples were frozen in liquid nitrogen and pulverized with a mortar. Catecholamines were extracted from 200-mg tissue samples in 10 volumes of 0.2 M perchloric acid (wt/vol) containing 3,4-dihydroxybenzylamine as internal standard and further treated as described elsewhere (9).

Determination of plasma renin activity. Plasma renin activity (PRA) was determined using an commercially available radioimmunoassay kit (Sorin Biomedica, Düsseldorf, Germany).

Statistical analysis. Values are means ± SE. Levels of significance were calculated by ANOVA followed by Bonferroni’s test for multiple comparisons. P < 0.05 was considered statistically significant.

RESULTS

Systolic blood pressure and heart rate. Systolic blood pressure was 120–130 mmHg for control rats fed a normal-salt diet and 115–125 mmHg for rats fed a low-salt diet. Treatment with metoprolol did not change systolic blood pressure. Ramipril in combination with a low-salt diet lowered systolic blood pressure significantly to 90–100 mmHg. Additional treatment with metoprolol did not further decrease systolic blood pressure (Fig. 2).

Heart rate was ~390–410 beats/min for control animals fed a normal-salt diet and ~380–410 beats/min for animals fed a low-salt diet. A combination of ramipril and a low-salt diet increased heart rate significantly to 420–440 beats/min. Treatment with metoprolol decreased heart rate significantly to 330–365 beats/min for all groups (Fig. 2).
**PRA and renin mRNA.** A low-salt diet alone increased PRA and renocortical renin mRNA abundance about twofold. The combination of a low-salt diet and ACE inhibitor strongly increased PRA about eightfold and renal renin mRNA abundance about ninefold. Metoprolol did not change basal PRA or renin mRNA. Moreover, metoprolol attenuated the rise of PRA and renin mRNA in response to a low-salt diet only marginally. Furthermore, metoprolol did not change the rise of PRA and renin mRNA in response to salt depletion and ramipril (Fig. 3). Left-side renal denervation did not change PRA during basal conditions but attenuated nNOS mRNA during low salt intake and low salt intake combined with ACE inhibition.

Renocortical nNOS and eNOS mRNA. A moderate increase of ~1.7-fold for nNOS mRNA was observed during low salt intake. A low-salt diet in combination with ACE inhibitor treatment increased nNOS mRNA ~2.5-fold. Metoprolol did not change basal nNOS mRNA abundance but clearly attenuated the rise of nNOS mRNA in rats fed a low-salt diet and in rats fed a low-salt diet and treated with ramipril (Fig. 5). Left-side renal denervation did not change nNOS mRNA during basal conditions but attenuated nNOS mRNA during low salt intake and low salt intake combined with ACE inhibition.

eNOS mRNA was not changed during any of these experimental maneuvers (Fig. 5).

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**Renocortical COX-2 and COX-1 mRNA.** A low-salt diet alone increased renocortical COX-2 mRNA abundance about twofold, whereas additional treatment with ramipril strongly increased COX-2 mRNA abundance about sevenfold. Metoprolol did not change basal COX-2 mRNA. Moreover, metoprolol did not attenuate the rise of COX-2 mRNA in response to a low-salt diet or low salt intake and ramipril treatment (Fig. 4). Left-side renal denervation did not affect COX-2 mRNA during basal conditions or during low salt intake or low salt intake and ramipril treatment. Denervation significantly lowered basal expression of renin mRNA, but the increase during low salt intake or during salt depletion and ramipril treatment was not attenuated (Fig. 3).

COX-1 mRNA was not changed during any of these experimental maneuvers (Fig. 4).

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**Fig. 2.** Systolic blood pressure and heart rate in vehicle- or metoprolol (50 mg/kg twice a day)-treated rats, fed a normal-salt diet, a low-salt diet or a low-salt diet combined with ramipril (10 mg·kg⁻¹·day⁻¹) treatment. Values are means ± SE of 10 animals. P < 0.05 vs. control rats fed a normal diet.

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**Fig. 3.** A: plasma renin activity (PRA) and renocortical renin mRNA levels in vehicle- or metoprolol (50 mg/kg twice a day)-treated rats, fed a normal-salt diet, a low-salt diet or a low-salt diet combined with ramipril (10 mg·kg⁻¹·day⁻¹) treatment. B: effect of normal salt intake, low salt intake or low salt diet combined with ramipril (10 mg·kg⁻¹·day⁻¹) treatment on PRA and renin mRNA levels in the denervated or innervated kidney of rats subjected to left-side renal denervation. Values are means ± SE of 10 animals. P < 0.05 vs. control rats fed a normal diet.
Effect of salt depletion on renal cortical catecholamine concentration. Norepinephrine and dopamine concentrations remained unchanged during low salt intake and low salt intake in combination with ramipril treatment. Epinephrine content was at the limit of determination (Table 1). Metoprolol treatment had no influence on catecholamine concentration in any of the experimental maneuvers (data not shown). Catecholamine content of innervated kidneys from untreated and treated rats showed no difference compared with kidneys from vehicle-treated rats, rats fed a low-salt diet, or rats treated with ramipril and fed a low-salt diet. In the denervated kidneys of rats fed a low-salt diet or rats treated with ramipril and fed a low-salt diet, norepinephrine content dropped to ~35% of that in innervated kidneys. These results did not differ from untreated rats (Table 1).

Renocortical nNOS, eNOS, and COX-2 protein expression. Renocortical immunoreactivity for COX-2 showed a significant threefold increase in rats fed a low-salt diet and treated with ramipril compared with vehicle-treated rats, whereas additional feeding with metoprolol had no further influence (Fig. 6, top). Renocortical immunoreactivity for nNOS showed a 1.8-fold significant increase in rats fed a low-salt diet and treated with ramipril compared with vehicle-treated rats. Additional feeding with metoprolol clearly attenuated the rise of nNOS protein in response to a low-salt diet and ramipril treatment (Fig. 6, middle). eNOS
immunoreactivity was not changed during any of these experimental maneuvers (Fig. 6, bottom).

**DISCUSSION**

Our study aimed to assess the relevance of renal nerve activity and the involvement of β-adrenoreceptor activity in the well-known upregulation of renin, COX-2, and nNOS gene expression in the juxtaglomerular apparatus during low salt intake and low salt intake in combination with ACE inhibition. In confirmation of previous data, we found that a low-salt diet moderately upregulated renin, COX-2, and nNOS mRNAs and proteins and that a low-salt diet in combination with blockade of the renin-angiotensin system by ACE inhibition strongly upregulated renin, COX-2, and nNOS mRNAs and proteins in the kidney cortex (4). Because renin, COX-2, and nNOS mRNA levels in the cortex correlate well with the expression of the respective encoded proteins in juxtaglomerular cells (renin) (14) and macula densa cells (COX-2 and nNOS) (14, 32, 33), we considered the mRNA abundance to be a quantifiable measure for expression of these genes in the juxtaglomerular apparatus. Our findings now show that salt depletion plus ramipril treatment lowered blood pressure and increased heart rate, suggesting sympathetic activation of the cardiovascular system. This assumption is supported by the finding that inhibition of β-adrenoreceptors lowered heart rate of the hypotensive animals into the normal range. Renocortical catecholamines, however, showed only minor changes. Nonetheless, the increase of nNOS induced by the combination of low salt intake and ACE inhibition was markedly attenuated by β-adrenoreceptor blockade as well as the more moderate increase induced by low salt intake alone. Expression of the renin gene was only marginally affected by β-adrenoreceptor blockade, while COX-2 mRNA and protein were not attenuated. Data similar to those obtained with β-adrenoreceptor blockade were obtained with renal denervation, suggesting that it is likely that renal nerves influence expression of juxtaglomerular genes via β-adrenoreceptors. A possible effect of β-adrenoreceptor

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**Table 1. Catecholamine concentration in the rat renal cortex**

<table>
<thead>
<tr>
<th></th>
<th>Epinephrine</th>
<th>Norepinephrine</th>
<th>Dopamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal salt</td>
<td>1.0 ± 0.7</td>
<td>152.6 ± 17.9</td>
<td>3.8 ± 0.7</td>
</tr>
<tr>
<td>Low salt</td>
<td>1.1 ± 0.4</td>
<td>129.3 ± 15.4</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>Low salt + ramipril</td>
<td>1.7 ± 0.5</td>
<td>123.3 ± 16.4</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td>Normal salt</td>
<td>1.3 ± 0.3</td>
<td>165.4 ± 14.9</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Innervated kidney</td>
<td>1.2 ± 0.5</td>
<td>6.8 ± 2.0*</td>
<td>2.6 ± 0.5*</td>
</tr>
<tr>
<td>Denervated kidney</td>
<td>1.1 ± 0.4</td>
<td>148.3 ± 15.7</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>Low salt</td>
<td>1.2 ± 0.5</td>
<td>7.9 ± 1.6*</td>
<td>3.1 ± 0.4*</td>
</tr>
<tr>
<td>Innervated kidney</td>
<td>1.3 ± 0.7</td>
<td>154.4 ± 13.9</td>
<td>4.6 ± 1.1</td>
</tr>
<tr>
<td>Denervated kidney</td>
<td>1.3 ± 0.6</td>
<td>6.1 ± 1.5*</td>
<td>2.9 ± 0.6*</td>
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Values are means ± SE expressed in ng/g wet wt; n = 10. *P < 0.05 vs. innervated kidney. Epinephrine content in renal cortex was at the limit of quantification.

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**Fig. 6.** Effect of treatment with vehicle, low salt intake and ramipril (10 mg·kg⁻¹·day⁻¹) treatment, and low salt intake and ramipril and metoprolol (50 mg/kg twice a day) treatment on renocortical COX-2, nNOS, and eNOS protein levels. Representative experiments for COX-2, nNOS, and eNOS immunoreactivity (ir) are presented with 3 animals per group. Values are means ± SE of 6 animals. *P < 0.05 vs. control. **P < 0.05 vs. low salt intake and ramipril treatment.
relevance for the expression of COX-2 gene and protein in the macula densa. Moreover, the different behavior of nNOS and COX-2 expression does not support the concept that COX-2 expression is secondary to nNOS expression (5).

**Renin gene expression.** The cAMP pathway is the best-established stimulatory pathway for renin secretion and renin gene expression (19), and renal nerve activity has been found to be important not only for basal expression of renin (16), but also for stimulation of renin secretion and renin gene expression in response to a fall of renal perfusion pressure (29). However, in this context, the effects of β-adrenoreceptor blockade and renal denervation on the strong stimulation of renin secretion and renin gene expression by the combination of low salt intake and ACE inhibition and also on stimulation by low salt intake were rather marginal, indicating that renal nerve activity plays only a minor role in stimulation of the renin system during salt depletion (8). It is well known that ACE inhibitors and ANG II type 1 receptor antagonists markedly stimulate renin secretion and renin gene expression (6, 33), but to a much lower extent than the combination of ACE inhibition and low salt intake (4, 33). Because low salt intake per se stimulates renin gene expression only moderately (4, 33), stimulation of the renin system by a low-salt diet in combination with ACE inhibition is therefore clearly overadditive relative to the individual effects of ACE inhibition and a low-salt diet. It is conceivable that stimulation of the renin system by a low-salt diet is already limited by ANG II negative-feedback inhibition. Interruption of this feedback mechanism by ACE inhibition during low salt intake will therefore strongly enhance stimulation of the renin system to an extent seen in this and previous studies (4, 33). Such a disinhibition of the renin system by ACE inhibition raises the question about the factors causing the “background” stimulation of the renin system under basal conditions and, in particular, during low salt intake. Apparently, renal nerve activity is not essentially required as a stimulatory signal in this context. Therefore, identification of the underlying mechanisms remains a task for future work.

**COX-2 gene expression.** COX-2 gene expression in the cortical thick ascending limb of Henle, including the macula densa region, shows a striking parallelism to expression of renin in the neighboring juxtaglomerular epithelioid cells (6, 14, 22, 33), which has led to the concept that expression of renin is essentially regulated by macula densa-derived prostanoids triggered by the expression of COX-2 (5, 6, 31). Because this intriguing concept is still a matter of controversy (4, 21–24), it appears not unlikely that renin and COX-2 gene expression could alternatively be regulated in parallel by a common yet unknown denominator. If this is the case, strong stimulation of COX-2 expression by the combination of low salt intake and an ACE inhibitor may reflect the interruption of a negative control of COX-2 expression by ANG II, as assumed for stimulation of the renin system. The existence of ANG II type 1 receptors on the surface of macula densa cells would be compatible with such an idea. Again the identity of the background stimulator of COX-2 in the macula densa remains an open question.

**nNOS gene expression.** nNOS expression in the macula densa appears to be regulated by pathways that are somewhat different from those that regulate renin and COX-2. Such a difference is already suggested by the smaller amplitude of stimulation than that caused by renin and COX-2 and is confirmed by the dependency on renal nerve activity.

Neither renal activity nor catecholamines appear to be of major relevance for stimulation of renin and COX-2 expression in the juxtaglomerular apparatus during low salt intake or low salt intake in combination with ACE inhibition. Renal nerve activity, however, appears to be required for full stimulation of nNOS expression in the macula densa in this situation, an effect being mediated by β-adrenoreceptors.

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**REFERENCES**


