Angiotensin II feedback is a regulator of renocortical renin, COX-2, and nNOS expression

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During the last years, it has been recognized that the expressions of cyclooxygenase-2 (COX-2) and of the neuronal isoform of nitric oxide synthase (nNOS) in renal macula densa cells change parallel with the expression of renin in the neighboring juxtaglomerular cells. Low-salt diet causes a concerted increase of the expression of the three genes. The pathways triggering this characteristic increase in the expression of the three genes as well as the physiological meaning of the increased expression are not yet clear. It has been suggested that the increase of renin expression may be causally related to a preceding increase in the formation of COX-2-derived prostanooids in the macula densa cells (7, 8, 10, 13). The increase of COX-2 expression in the macula densa, in turn, has been suggested to be dependent on nNOS activity in the macula (12), leading to the concept of a sequential expression in the order of nNOS-COX-2-renin. This concept, however, is questioned by other findings that could not confirm a role of COX-2-derived prostanooids for the expression of renin (15, 19) or confirm a functional interdependence of nNOS and COX-2 in macula densa cells (4).

Another striking phenomenon in the juxtaglomerular control of renin, COX-2, and nNOS expression is the observation that the stimulatory effect of low-salt intake on these genes is strongly enhanced if the renin-angiotensin system is inhibited. This suggests a direct or indirect negative feedback function of ANG II on renin, COX-2, and nNOS expression during low-salt intake. In fact, both renin-producing juxtaglomerular cells and COX-2- and nNOS-expressing macula densa cells are equipped with ANG II receptors that could mediate a possible direct effect of ANG II on these cells (7, 16, 25). Cell culture studies provided preliminary evidence that ANG II is capable of inhibiting COX-2 expression at the cellular level (7). Whether a direct negative feedback by ANG II in the control of renin, COX-2, and nNOS also exists in vivo is less clear, because inhibition of the renin-angiotensin-aldosterone system (RAAS) during low-salt intake not only prevents the formation of ANG II but also the stimulation of aldosterone production. This change in al-
dosterone levels may affect juxtaglomerular gene expression by disturbing the control of the sodium homeostasis. To elucidate the contribution of a direct negative feedback of ANG II on the juxtaglomerular expression of renin, COX-2, and nNOS and to exclude secondary effects of insufficient aldosterone production, we assessed the effect of RAAS inhibition in the presence of mineralocorticoid excess. Animals were treated with the mineralocorticoid receptor agonist fludrocortisone (2, 26) to blunt endogenous aldosterone formation and to maintain constant levels of mineralocorticoids. This maneuver was effective as indicated by the findings that fludrocortisone prevented the fall of systolic blood pressure and of glomerular filtration in animals on low-salt diet treated with an ANG I-converting enzyme (ACE) inhibitor.

The sum of our findings suggests the existence of a strong mineralocorticoid-independent negative feedback effect of ANG II on the expression of renin, COX-2, and nNOS in the juxtaglomerular region.

MATERIALS AND METHODS

In vivo experiments. Male Sprague-Dawley rats weighing 160–175 g were treated with low-salt diet or a combination of low-salt diet (0.02% NaCl wt/wt; smiff Spezialdienen, Soest, Germany) with the ACE inhibitor ramipril (10 mg·kg body wt⁻¹·day⁻¹ orally via drinking water) for 7 days. Parallel groups were additionally treated with fludrocortisone for 5 days (6 mg·kg body wt⁻¹·day⁻¹ orally via gavage, dissolved in 1% methyl cellulose; Sigma, Deisenhofen, Germany). Controls received standard rat chow (0.5% NaCl wt/wt; Trouw Nutrition, Burgheim, Germany) and were treated with vehicle. The animals had free access to tap water and were housed individually in metabolic cages with a 12:12-h light-dark cycle and a 3-day equilibration period before drug administration. Measurements of urine volumes were performed for 24 h during the last 2 days.

Furthermore, the effect of fludrocortisone on blood pressure was assessed in controls, in animals on low-salt diet, and in animals treated with the combination of low-sodium diet and ramipril (as described above). For measurements of blood pressure, the animals had to be removed from their cages. To avoid falsification of 24-h urine volume, these animals were excluded from urine collections.

After 7 days of treatment, the animals were killed by decapitation. Blood was collected from the cervical vessels in EDTA-pretreated tubes. Kidneys were removed rapidly, and the cortices were dissected with a surgical blade and frozen in liquid nitrogen. Until further processing, the tissues were stored at −80°C.

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Extraction of total RNA. Total cortical RNA was extracted according to the acid-guanidinium-phenol-chloroform protocol described in detail by Chomczynski and Sacchi (9). RNA pellets were dissolved in diethylpyrocarbonate-treated water, the yield of RNA was quantified by spectrophotometry at 260 nm, and samples were divided into aliquots and stored at −80°C until assaying.

RNAse protection assay for determination of β-actin, renin, COX-2, and nNOS mRNA. β-Actin, renin, COX-2, and nNOS mRNA levels were measured by RNase protection assay as described previously (5, 22, 27). In brief, after linearization and purification with phenol/chloroform, the plasmids yielded radiolabeled antisense cRNA transcripts by incubation with SP6 polymerase (Promega, Mannheim, Germany) and α-32-P-GTP (Amersham Pharmacia Biotech, Freiburg, Germany) according to the Promega riboprobe in vitro transcription protocol. Five micrograms of total RNA for β-actin, 40 μg for renin, 100 μg for COX-2, and 100 μg for nNOS were hybridized with 500,000 counts/min of the cRNA probes at 60°C overnight; thereafter RNase A/T1 (RT/30 min) and proteinase K (37°C/30 min) were used for digestion. After phenol/chloroform extraction and ethanol precipitation, protected fragments were separated on a 8% polyacrylamide gel. The gel was dried for 2 h, and bands were quantitated in a Phosphoimager (InstantImager 2024, Packard BioScience, Meriden, CT). Autoradiography was performed at −80°C for 1 day. t-RNA served as negative control.

Fig. 1. Effect of 5-day fludrocortisone administration on plasma aldosterone concentrations (A), plasma renin activity (PRA; B), and renin mRNA (C) levels in control animals (n = 8) and in rats on low-salt diet (n = 5) and low-salt diet with concomitant ramipril treatment (n = 10). Data are means ± SE. *P < 0.05 compared with parallel group, $P < 0.05 compared with controls. cpm, Counts/min.
Determination of plasma renin activity and plasma aldosterone concentrations. Plasma renin activity and plasma aldosterone concentrations were determined by a commercially available radioimmunoassay (Byk & Diasorin Diagnostics, Dietzenbach, Germany).

Creatinine clearance. For calculation of creatinine clearance, the formula

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\text{clearance} = \frac{c_{\text{urine}} \times \text{urineflow}[\text{ml/min}]}{c_{\text{plasma}}}
\]

was used with \(c_{\text{plasma}}\) = plasma creatinine concentration (\(\mu\text{mol/l}\)) and \(c_{\text{urine}}\) = urine creatinine concentration (\(\mu\text{mol/l}\)). Plasma and urinary concentrations of creatinine were measured with the Jaffé reaction as described previously (20).

Blood pressure measurements. Blood pressure was measured with the tail cuff method using a TSE 2100 000–2T BP monitor (Technical and Scientific Equipment, Bad Homburg, Germany).

Statistical analysis. Differences between the groups were analyzed by ANOVA for multiple comparisons. \(P\) values <0.05 were considered statistically significant.

RESULTS

Low-salt diet increased plasma renin activity (2-fold) (Fig. 1), renocortical renin mRNA (3-fold) (Fig. 1), and COX-2 mRNA (3-fold) (Fig. 2), but it did not change nNOS mRNA (Fig. 2). Systolic blood pressure remained normal (Fig. 3), but creatinine clearance as a measure for glomerular filtration tended to decrease during low-salt diet (Fig. 4).

To interrupt a possible feedback control of juxtaglomerular gene expression by ANG II, we used the ACE inhibitor ramipril. Additional treatment of the animals on low-salt diet with ramipril further enhanced renin mRNA (9-fold) (Fig. 1), COX-2 (4-fold) (Fig. 2), and nNOS mRNA (2.5-fold) (Fig. 2). Systolic blood pressure fell from 110 to 75 mmHg (Fig. 3) and creatinine clearance from 0.75 to 0.3 ml/min (Fig. 4).

To clamp endogenous levels of mineralocorticoids, the animals were treated with the aldosterone agonist fludrocortisone, which abrogated endogeneous aldosterone production as indicated by the unmeasurable low levels of plasma aldosterone (Fig. 1). In animals on normal-salt diet, fludrocortisone further lowered plasma renin activity (Fig. 1), but it left renocortical levels of renin (Fig. 1), COX-2 (Fig. 2), and nNOS...
mRNA (Fig. 2) unchanged. In these animals, fludrocortisone increased systolic blood pressure from 110 to 140 mmHg and tended to increase creatinine clearance (Figs. 3 and 4). In animals on low-salt diet, fludrocortisone lowered plasma renin activity, renin mRNA (Fig. 1), and COX-2 mRNA (Fig. 2). It increased systolic blood pressure and tended to increase creatinine clearance (Figs. 3 and 4). Additional treatment of these animals with ramipril in the presence of fludrocortisone increased renin mRNA (9-fold) (Fig. 1), COX-2 mRNA (2.5-fold) (Fig. 2), and nNOS mRNA (2.5-fold) (Fig. 2). The relative increases were comparable to the increases observed without fludrocortisone treatment.

However, in animals receiving the combination of low-salt diet with ramipril, fludrocortisone lowered the abundance of renin mRNA (Fig. 1) and of COX-2 mRNA but not of nNOS mRNA (Fig. 2). In these animals, fludrocortisone kept both systolic blood pressure (120 mmHg) and creatinine clearance (1.0 ml/min) in the normal range (Figs. 3 and 4).

DISCUSSION

On the basis of observations that the stimulations of renin, COX-2, and nNOS expression in the renal juxtaglomerular regions in response to low-salt intake are strongly enhanced if the formation of ANG II is concomitantly inhibited (4), our study aimed to identify possible pathways along which ANG II could be involved in the regulation of renin, COX-2, and nNOS expression. In particular, we aimed to distinguish between more direct effects of ANG II on juxtaglomerular epithelioid and macula densa cells (7, 16, 25) and more indirect effects mediated by ANG II-dependent aldosterone formation such as salt balance, extracellular volume, or blood pressure (3, 11, 28).

For our experiments, we used a mineralocorticoid clamp by administration of the potent mineralocorticoid fludrocortisone (2, 26) to achieve a constant blood level of mineralocorticoids and to abrogate influences of ANG II on aldosterone formation. The efficacy of the maneuver is indicated by the fact that in accordance with previous reports (17), fludrocortisone administration strongly suppressed plasma aldosterone concentrations under all experimental maneuvers applied in this study.

Fludrocortisone treatment also prevented the marked fall of blood pressure and of glomerular filtration in response to the combined treatment with low-salt diet and the ACE inhibitor, suggesting that the mineralocorticoid increased the salt balance and the extracellular volume under all experimental maneuvers (3). Salt retention and volume expansion could also be a likely explanation for the attenuation of plasma renin activity and of renin and COX-2 mRNA by fludrocortisone during normal- and low-salt diet (Figs. 1 and 2). This observation is in accordance with previous reports that mineralocorticoids exert a suppressing effect on renin in juxtaglomerular epithelioid cells (1) and on COX-2 in macula densa cells (24, 28).

Our data now indicate that even at constant levels of mineralocorticoids, inhibition of ANG II formation is still capable to enhance the expression of renin, COX-2, and nNOS in the kidney cortex in animals kept on low-salt diet. Because in these animals blood pressure and glomerular filtration were quite normal, it appears unlikely that the strong stimulation of renin, COX-2, and nNOS expression normally seen in salt-deficient animals treated with an ACE inhibitor is mediated by a fall of blood pressure or by a fall of glomerular filtration rate, both of which have been previously considered as potential regulators of renin and COX-2 expression (14, 21). It appears more reasonable to assume that the strong stimulatory effect of the ACE inhibitor on juxtaglomerular gene expression reflects the interruption of a more direct negative effect of ANG II on gene expression. Such an effect is generally assumed for renin in juxtaglomerular epithelioid cells (16), but it also appears to hold for COX-2 and nNOS expression in the macula densa cells. Compatible with this conclusion is the demonstration of ANG II-AT1 receptors on macula densa cells (25). This conclusion would also be consistent with the demonstration of a direct inhibitory effect of ANG II on COX-2 expression in cultured cells of the thick ascending limb of Henle, which are closely related to macula densa cells (7). The intracellular signaling pathways along which ANG II could suppress COX-2 gene expression remain to be elucidated (6).

Notably, nNOS expression in the macula densa, which we indirectly assayed by the abundance of renocortical nNOS mRNA (18), appears to be differently regulated from COX-2 and renin expression. In accordance with previous data, we found that only severe maneuvers to induce salt deficiency such as the combination of low-salt diet with ACE inhibition, but not low-salt diet alone, produced clear elevations of nNOS expression (4, 22, 23). Because fludrocortisone did not change nNOS expression under any experimental condition applied in our study, we infer that the stimulation of nNOS was not related to the fall of glomerular filtration or clearly related to sodium content of the organism but that it results from negative feedback control by ANG II. Moreover, nNOS expression in the macula densa appears not to be directly regulated by steroids.

In conclusion, our data suggest that the renocortical expression of renin, COX-2, and nNOS in states of sodium deficiency is subject to a direct negative feedback control by ANG II.

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