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Tonic stimulation of renin gene expression by nitric oxide is counteracted by tonic inhibition through angiotensin II

(nitric oxide synthase/juxtaglomerular cells/prostaglandins/ramipril/losartan)

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ABSTRACT **This study was designed to examine the possible involvement of prostaglandins and nitric oxide (NO) in the renin stimulatory effect of angiotensin II (AngII) antagonists. To this end, plasma renin activities (PRAs) and renal renin mRNA levels were assayed in rats that were treated with the Ang-converting enzyme inhibitor ramipril or with the AngII AT_1-receptor antagonist losartan. Ramipril and losartan increased PRA values from 7.5 ± 1.6 to 86 ± 6 and 78 ± 22 ng of AngI per h per ml and renin mRNA levels from 112 ± 9% to 391 ± 20% and 317 ± 10%, respectively. Inhibition of prostaglandin formation with indomethacin did not influence basal or ramipril-affected PRA. Basal renin mRNA levels also were unchanged by indomethacin, while increases in renin mRNA levels after ramipril treatment were slightly reduced by indomethacin. Inhibition of NO synthase by nitro-L-arginine methyl ester (L-NAME) reduced PRA values to 3.2 ± 0.9, 34 ± 13, and 12.1 ± 2.7 ng of AngI per h per ml in control, ramipril-treated, and losartan-treated animals, respectively. Renin mRNA levels were reduced to 77 ± 14% under basal conditions and ramipril- and losartan-induced increases in renin mRNA levels were completely blunted after addition of L-NAME. The AngII antagonists, furthermore, induced an upstream recruitment of renin-expressing cells in the renal afferent arterioles, which was also blunted by L-NAME. These findings suggest that renin mRNA levels are tonically increased by NO and that the action of NO is counteracted by AngII.**

One of the most prominent *in vivo* effects of angiotensin (Ang)-converting enzyme (ACE) inhibitors is a marked stimulation of renin secretion and renin gene expression in the kidneys (1–5). Since ANGII is known as a negative feedback regulator of renin secretion and of renin gene expression (6, 7), it is thought that the renin stimulatory effects of ACE inhibitors are related to the inhibition of AngII formation and consequently to the lowering of circulating and tissue AngII levels (8).

There is evidence that several *in vivo* effects of ACE inhibitors are not, as originally thought, due to the inhibition of AngII formation but to the inhibition of kinin degradation (9–12). Inhibition of kinin degradation results in elevated tissue levels of autacoids such as prostaglandins and nitric oxide (NO), the formation of which is stimulated by kinins (11–14). Although NO is reported to inhibit the renin system (15–17), contradictory findings suggest that NO stimulates the renin system (18–25) and also that prostaglandins stimulate the renin system (for review, see ref. 26). With such a stimulatory effect of NO and prostaglandins on the renin system, it is possible that the renin stimulatory effect of ACE inhibitors could be related to an enhanced formation of prostaglandins and NO.

To investigate the mode of action of ACE inhibitors on the renin system, we studied renin release and renin mRNA levels in the kidneys of conscious rats that were treated with the ACE inhibitor ramipril (27) or with the AngII AT_1-receptor antagonist losartan (28). To inhibit the formation of NO or prostaglandins, the animals were also treated with nitro-L-arginine methyl ester (L-NAME) (29) or with indomethacin (30), respectively. We found that inhibition of cyclooxygenase with indomethacin moderately diminished ACE-induced increases in renin mRNA levels without affecting the stimulation of renin secretion. Inhibition of NO synthase (NOS) by L-NAME significantly diminished the increases of renin secretion and blunted the increases of renin mRNA levels induced by ramipril. An increase of renin secretion and of renin mRNA levels similar to that obtained with the ACE inhibitor ramipril was achieved with the AngII AT_1-receptor antagonist losartan (28). Again the stimulation induced by losartan was blunted by L-NAME.

From these findings, we infer that NO is an important tonic stimulator for increases of renin mRNA levels, which under normal conditions, is counteracted by the inhibitory effects of AngII.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats were used for the experiments. Animals had free access to chow (Altromin, Feldkirchen-Heimstetten, Germany) and tap water.

Application of Drugs. *Inhibition of ACE.* To inhibit ACE activity, animals were treated with ramipril (7.5 mg/kg). Ramipril was given to animals by gavage in the morning of each experimental day. *Blockade of AngII AT_1 receptors.* AngII AT_1 receptors were blocked by treatment of animals with losartan (40 mg/kg). Losartan was given by gavage in the morning of each experimental day. *Inhibition of endothelium-derived relaxing factor (EDRF) formation.* Formation of EDRF was inhibited by i.p. injections of L-NAME (40 mg/kg) twice the day. *Inhibition of cyclooxygenase activity.* Formation of prostaglandins was inhibited by i.p. injections of indomethacin (2 mg/kg) twice a day. Normally, animals were sacrificed by decapitation 50 h after the first application of drugs. Blood was collected for determination of plasma renin activity (PRA), and kidneys were extirpated, weighed, frozen in liquid nitrogen, and stored at −80°C until further processing.

Measurement of Blood Pressure. Systolic blood pressure was determined by using the tail-cuff method with a blood pressure recorder model 8005 (Rhema, Hofheim, Germany). Measurements were done before and 6, 24, 30, and 48 h after the first application of drugs.

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Abbreviations: NO, nitric oxide; NOS, NO synthase; PRA, plasma renin activity; Ang, angiotensin; ACE, Ang-converting enzyme; L-NAME, nitro-L-arginine methyl ester; EDRF, endothelium-derived relaxing factor.

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Renin Immunohistochemistry. Animals were anesthetized with methohexital (50 mg/kg) and perfusion-fixed as described by Dawson *et al.* (31).

For immunohistochemistry, at least five pieces of each kidney were shock-frozen in liquid propane. From each piece, five to seven serial sections 5 μm thick were cut with a cryostat. The sections were treated with a rabbit antiserum against rat renin, diluted 1:10,000. Binding sites of the primary antibodies were visualized by using a secondary fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin serum diluted 1:100 in phosphate-buffered saline. A total of five or six sections were evaluated from each animal.

The sections were analyzed microscopically (Polyvar, Reichert-Jung, Austria) and the renin-positive areas were quantified as described by Weibel (32). The sum of renin-positive areas measured per kidney, divided by the respective number of vascular poles, including those without visible immunoreactivity, was called the renin index.

Determination of Preprorenin mRNA. Total RNA was extracted from the kidneys stored at −70°C, as described by Chomczynski and Sacchi (33). Renin mRNA was measured by RNase protection as described (34), by using a preprorenin complementary RNA probe containing 296 bp of exons I and II, generated from a pGEM-4 vector carrying a *Pst* I–*Kpn* I restriction fragment of a rat preprorenin cDNA (35). Total RNA (20 μg) was assayed and the amount of renin mRNA was expressed as the percent of an external renin mRNA standard consisting of 20 μg of pooled RNA extracted from the 12 kidneys of six normal Sprague–Dawley rats.

Determination of Actin mRNA. The abundance of rat cytoplasmatic β-actin mRNA in total RNA isolated from the kidneys was determined by RNase protection assay as described (34). An actin complementary RNA probe containing the 76-nt first exon and ≈200 nt of surrounding sequence was generated from a pAM19 vector carrying an A*va* I–*Hin*dIII restriction fragment of actin cDNA. For one assay, 2.5 μg of total RNA was assayed from each sample.

PRA. PRA was determined by using a commercially available radioimmunoassay kit for AngI (Sorin Biomedica, Düsseldorf, Germany).

Statistics. Levels of significance were determined by ANOVA followed by Kruskal–Wallis test. $P < 0.05$ was considered significant.

RESULTS

This study was primarily designed to investigate the mode of action of ACE inhibitors on the renin system. In particular, we were interested in examining the involvement of prostaglandins and EDRF in the effect of ACE inhibitors on renin secretion and renin mRNA levels. For this purpose, male Sprague–Dawley rats were treated with the ACE inhibitor ramipril (7.5 mg/kg, daily) during simultaneous inhibition of EDRF or prostaglandin formation with injections of L-NAME (40 mg/kg, twice a day) or indomethacin (2 mg/kg, twice a day), respectively. To assess major adverse effects of the drugs employed, we determined body weights, kidney weights, and RNA levels of the animals in all experimental groups. None of the drugs applied caused significant changes in the following parameters compared with control animals that had body weights of 223 ± 4.1 g, kidney weights of 1.04 ± 0.011 g, and RNA contents of 764 ± 37 μg per kidney (mean ± SEM, $n = 5$).

Treatment of animals with the ACE inhibitor ramipril (7.5 mg/kg, daily) for 2 days significantly reduced systolic blood pressure from initial values of 122 ± 8 mmHg (1 mmHg = 133 Pa) to 105 ± 5 mmHg (Fig. 1). Injections of indomethacin (2 mg/kg, twice a day) had no effect on blood pressure (data not shown). Application of L-NAME (40 mg/kg, twice a day) increased systolic blood pressure from 115 ± 3 mmHg to 138 ± 10 mmHg, and also application of ramipril increased blood pressure from 112 ± 5 mmHg to 126 ± 5 mmHg by L-NAME (Fig. 1).

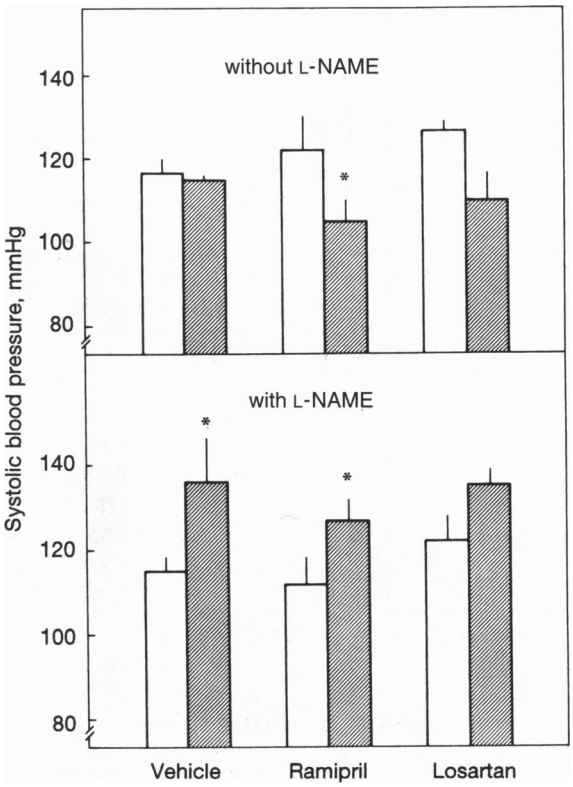

FIG. 1. Changes in systolic blood pressure after treatment of rats with ramipril and losartan during intact (*Upper*) and blocked (*Lower*) NOS activity. Open bars represent blood pressure before the start of the experiments, and hatched bars represent blood pressure averaged on the last two measurements before sacrifice of animals (30 and 48 h after first application of drugs). Data are the mean ± SEM of five animals in each experimental group, except the losartan-treated groups, which contained only three animals. $P < 0.05$ was considered significant.

Ramipril caused a strong stimulation of renin secretion. Thus PRA values increased from 7.5 ± 1.6 ng of AngI per h per ml to 86 ± 6 ng of AngI per h per ml after treatment with ramipril (7.5 mg/kg, daily). Injections of indomethacin (2 mg/kg, twice a day) had no effect on basal PRA values. After injection of L-NAME (40 mg/kg, twice a day), values for PRA decreased to 3.2 ± 0.9 ng of AngI per h per ml. The ramipril-induced increase in PRA was not affected by application of indomethacin but was significantly attenuated by injections of L-NAME to 34 ± 13 ng of AngI per h per ml (Fig. 2).

Treatment with the ACE inhibitor ramipril (7.5 mg/kg, daily) for 2 days increased renin mRNA levels from 112 ± 9% to 391 ± 20% of the RNA standard (Fig. 2). Inhibition of EDRF formation by injections with L-NAME (40 mg/kg, twice a day) decreased basal renin mRNA levels to 77 ± 14% of standard. Inhibition of prostaglandin formation with indomethacin (2 mg/kg, twice a day) did not change the basal content of renin mRNA. The combination of L-NAME and ramipril completely blunted the stimulatory effect of ramipril on renin mRNA levels. Inhibition of prostaglandin synthesis by indomethacin moderately reduced renin mRNA levels to 298 ± 21% of the standard value.

To distinguish between AngII-dependent and AngII-independent mechanisms of ramipril, we compared changes of renin secretion and renin mRNA levels induced by ramipril treatment with respective changes evoked by treatment with losartan. Application of losartan (40 mg/kg, daily), similar to that of ramipril, decreased systolic blood

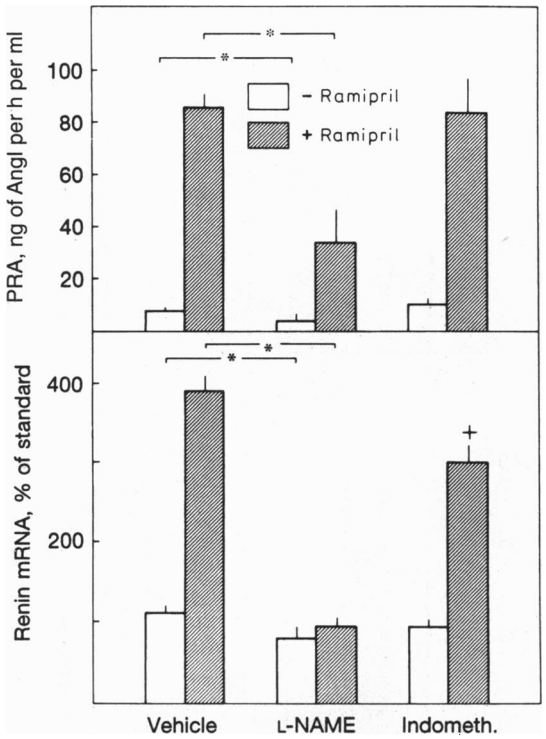

FIG. 2. Effect of blockade of NO and prostaglandin formation on ramipril-induced increases in PRA (*Upper*) and renin mRNA levels (*Lower*). Data are the mean ± SEM of five animals in each experimental group, with both kidneys of each animal measured. *, $P < 0.05$; +, $P < 0.05$ vs. ramipril-treated animals.

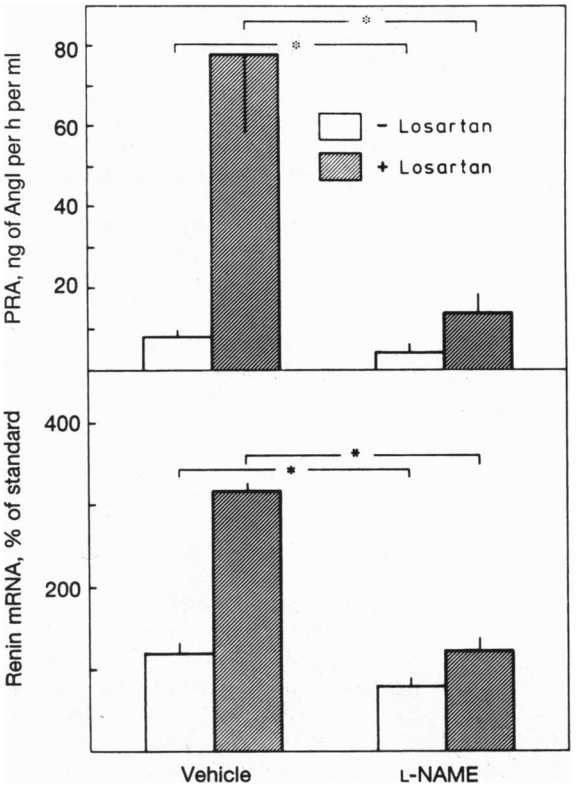

FIG. 3. Reduction of losartan-induced increases in PRA (*Upper*) and renin mRNA levels (*Lower*) by inhibition of NOS. Data are the mean ± SEM of three animals in each experimental group, with both kidneys of each animal examined. *, $P < 0.05$.

pressure to 110 ± 7 mmHg. This reduction was counteracted by additional injections of L-NAME (40 mg/kg, twice a day) (Fig. 1). Values of PRA (78 ± 22 ng of AngI per h per ml) and renin mRNA levels (317 ± 10%) increased after treatment with losartan to levels similar to those after treatment with ramipril (Fig. 3). Treatment with L-NAME blunted the increase in PRA to 12.1 ± 2.7 ng of AngI per h per ml (Fig. 3). The losartan-induced increase in renin mRNA levels was also abolished by L-NAME (Fig. 3) and by *N*-monomethylarginine (80 mg/kg) (Fig. 4). To further characterize the time course of the effect of NOS inhibition, we also determined renin mRNA levels after 1 day and 4 days of treatment. After 1 day of treatment with losartan, renin mRNA levels had increased to 151 ± 34% of the standard ($n = 3$) but did not change in animals receiving losartan plus L-NAME (105 ± 6% of standard, $n = 3$). After 4 days of treatment, renin mRNA levels had increased with losartan to 318 ± 68% ($n = 3$) of the standard, whereas losartan plus L-NAME produced only a small increase to 130 ± 15% ($n = 3$) of the standard renin mRNA value.

For control of general transcription, we also measured mRNA levels of the housekeeping gene β-actin. The β-actin mRNA level was not significantly altered by ramipril, losartan, or an additional treatment with L-NAME (data not shown).

In view of the blood pressure changes evoked by the drugs and the well-established influence of blood pressure on renin secretion and gene expression, we analyzed the relationship between changes of renal renin mRNA levels and changes of blood pressure. To this end, we determined the relationship of renal renin mRNA levels and systolic blood pressure at the end of the experiments for individual animals. As shown in Fig. 4, there was an overlap of blood pressure values in animals treated with AngII antagonists with and without additional treatment with NOS inhibitors, but there was no overlap of renin mRNA levels between these groups of animals. As a consequence, it appears less likely that changes of blood pressure are the primary event by which NOS inhibitors blunted the increase of renin mRNA levels induced by AngII antagonists.

To obtain information about the influence of AngII antagonists on the distribution of renin-expressing cells in the

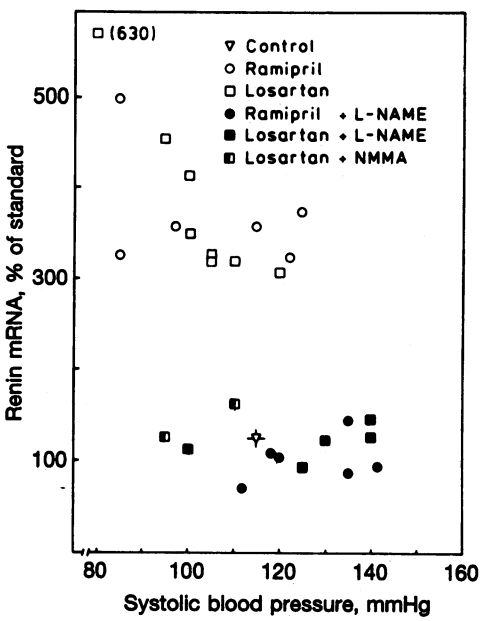

FIG. 4. Lack of correlation between renin mRNA levels and systolic blood pressure in the individual animals after inhibition of NOS. Values of renin mRNA content represent the average between both kidneys of each animal and values of blood pressure are averaged on the last two measurements before sacrifice of the animals (30 and 48 h after first application of drugs).

kidney, we performed immunohistochemical investigations on kidney sections from animals receiving losartan and/or the NOS inhibitor L-NAME. Fig. 5 shows sections treated with renin antisera from a control rat, a rat treated with losartan, and a rat treated with losartan plus L-NAME. The renin-positive areas in the rat receiving losartan are markedly enlarged upstream of the afferent arteriole. Also, renin immunoreactivity appears in the efferent arteriole in the losartan-treated animal (data not shown). Quantitative morphometry revealed a 100% increase in the area of renin immunoreactive per vascular pole in five rats treated with losartan compared with five control rats (Fig. 6). Fig. 5 suggests that in animals treated with losartan plus L-NAME, renin immunoreactivity was not changed compared with the control animals. This observation was confirmed by the morphometric analysis indicating that renin-immunoreactive areas were the same in control rats and rats treated with losartan plus L-NAME (Fig. 6).

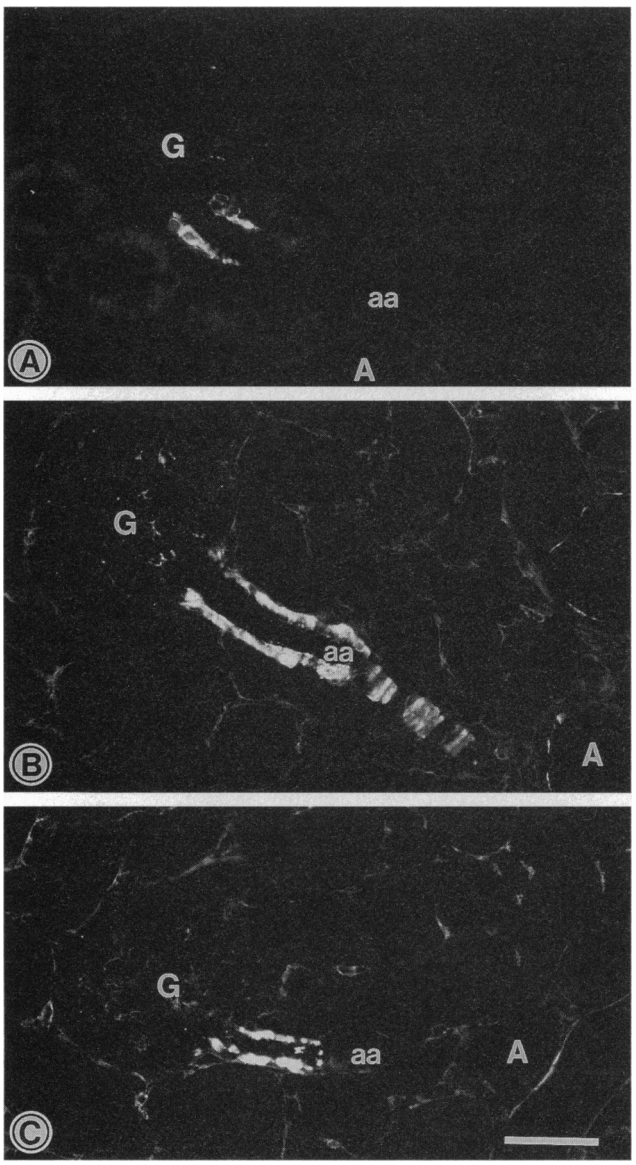

FIG. 5. Juxtamedullary afferent glomerular arterioles in cryostat sections of rat kidneys, immunostained with a renin antiserum and a fluorescein isothiocyanate-conjugated secondary antibody. (*A*) Control animal. (*B*) Animal treated with losartan. (*C*) Animal treated with losartan and L-NAME. A, artery; aa, afferent arteriole; G, glomerulus. (×245.) (Bar = 50 μm.)

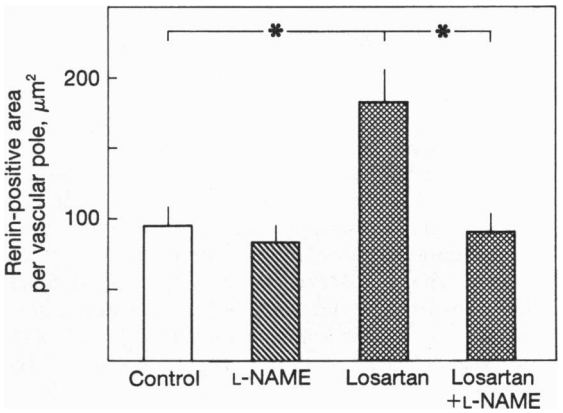

FIG. 6. Prevention of losartan-induced recruitment of renin-positive cells by blockade of NOS. Data are the mean ± SEM of five animals in each experimental group. *, $P < 0.05$.

DISCUSSION

Our results suggest that NO and to a minor extent prostaglandins tonically increase levels of renal renin mRNA and that this stimulation of renin system is counteracted by normal levels of AngII.

We observed that inhibition of prostaglandin synthesis during ramipril treatment produced a tendency to counteract ramipril effects by significant reduction of renin mRNA levels, albeit without changes in values of PRA (Fig. 2). More striking, however, was the effect of NOS inhibition, which blunted ramipril-induced increases in renin mRNA levels and significantly diminished values for PRA (Fig. 2). This blockade of ramipril effects by inhibition of NO and in part by inhibition of prostaglandin formation would be in accordance with a ramipril effect via kinin degradation and resulting increases in prostaglandin and NO formation.

To test such an AngII-independent action of ramipril, we directly inhibited AngII AT_1 receptors with the specific antagonist losartan (28). Losartan treatment resulted in increases in PRA and renin mRNA levels similar to those observed after treatment with the ACE inhibitor ramipril (Fig. 3), and notably the increases of renin secretion and renin mRNA levels were blunted by two inhibitors of NOS activity, namely, L-NAME and *N*-monomethylarginine (Figs. 3 and 4). It has been reported (5) that ACE inhibitors induce an upstream recruitment of renin-expressing cells in afferent arterioles. Our findings show that AngII AT_1-receptor antagonists have the same effect and that this recruitment is abolished if NOS activity is inhibited (Figs. 5 and 6). These findings suggest (*i*) that the inhibition of AngII formation mediates the stimulation of the renin system induced by ACE inhibitors and (*ii*) that intact NOS activity is required for the disinhibition of the renin system by AngII antagonists.

Considering the effects of NOS inhibition on elevated renin mRNA levels raises the question of whether the observed effect of NO on renin mRNA levels is a direct effect or is indirectly mediated by changes of intrarenal hemodynamics or by increases of systemic blood pressure. Inhibition of NOS decreases renal perfusion primarily by an increase of preglomerular resistance (36). An increase of preglomerular resistance, however, should decrease rather than increase the blood pressure in the juxtaglomerular cells, which are located in the most distal part of afferent arterioles and would be expected to increase rather than to decrease renin gene expression.

A more evident mediator of the inhibitory effect of NOS blockade on renin mRNA levels could be the increase of systemic blood pressure, because renin gene expression has been found to be inversely related to the renal perfusion pressure (37–40). Systolic blood pressure decreased after

ramipril and losartan treatment, while additional injections of the NOS inhibitor L-NAME counteracted this decrease in blood pressure (Fig. 1). The relationship between renin mRNA levels and systolic blood pressure (Fig. 4), however, does not suggest that the increase of blood pressure was the singular mechanism by which NOS inhibition blunted the increase of renin mRNA levels in response to losartan and ramipril. This hypothesis is supported by recent findings of Johnson and Freeman (41), who demonstrated that the NO-induced stimulation of the renin system is independent of blood pressure changes. Moreover, inhibition of NOS activity also inhibits the renin system in isolated rat kidneys in which the perfusion pressure is kept constant (18, 19, 21, 24). We therefore consider the possibility less likely that changes in blood pressure primarily account for the changes in the renin system in our experiments.

We also exclude the possibility that general alterations of transcription rates are responsible for changes in renin mRNA levels since none of the applied drugs caused significant changes in levels of the housekeeping gene β-actin (data not shown). We therefore favor the idea of a local stimulatory effect of NO on the renin system in the environment of juxtaglomerular cells, which was reported for the isolated perfused kidney (18, 19, 21, 24) and in cell culture experiments (20, 25). Given a direct stimulatory effect of NO on the renin system, there would be two possibilities as to how AngII could counteract this stimulatory effect of NO. One would be a possible inhibition of NOS activity by AngII, thus, lowering levels of available NO. Such an effect would be compatible with the observations that juxtaglomerular cells *in situ* contain the inducible form of NOS (42) and that AngII inhibits the expression of the inducible NOS in vascular smooth muscle cells (43), to which juxtaglomerular cells are directly related.

A second possible interpretation for an AngII-related NO-dependent action of ramipril and losartan could be the tonical stimulation of the renin system by continuously released NO from the endothelium, macula densa (44–46), and also the juxtaglomerula cells themselves (42), the stimulatory effect of which is counteracted in juxtaglomerular cells by local levels of AngII. Such a tonical stimulation of renin secretion by NO has been suggested by experiments with isolated perfused kidneys (18, 24) that concluded that NO is a tonical stimulator of renin secretion that is counteracted by the renal perfusion pressure.

Thus, we infer from our experiments that NO increases renin mRNA levels and in part renin secretion in the kidneys. In adult rats this stimulatory effect of NO is almost completely counteracted by AngII, but any relief of AngII unlocks the stimulatory effect of NO. Since AngII antagonists influence not only the renal renin mRNA levels but also the recruitment of renin-producing cells (Fig. 6 and ref. 47), our findings suggest that NO could play a role in the reversible metaplastic transformation of vascular smooth muscle cells into renin-producing cells.

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