Intrarenal angiotensin II: Interstitial and cellular levels and site of production

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Background. Both local production and angiotensin II subtype 1 (AT1) receptor-mediated uptake from the circulation contribute to the high levels of angiotensin (Ang) II in the kidney. It is largely unknown where Ang II is produced in the kidney and how much of it originates from the circulation.

Methods. The concentrations of endogenous and 125I-labeled Ang I and II were measured in renal tissue and in blood from pigs receiving systemic infusions of 125I-Ang I. Pigs were either untreated or treated with the angiotensin converting enzyme (ACE) inhibitor captopril or the AT1 receptor antagonist eprosartan.

Results. 125I-Ang I was undetectable in renal tissue but the steady-state concentrations of 125I-Ang II in cortical and medullary tissue were four and two times the concentration in arterial blood plasma, respectively. The tissue concentrations of endogenous Ang II were 100 and 60 times higher than in arterial plasma. Eprosartan reduced 125I-Ang II accumulation by 90%, but did not lower tissue Ang II. Captopril did not alter either 125I-Ang II accumulation or tissue Ang II.

Conclusions. The bulk of Ang II in the kidney is cell-associated. The high tissue/blood concentration ratio of endogenous Ang II may depend on the same mechanism as demonstrated for 125I-Ang II, that is, AT1 receptor-mediated binding to cells and endocytosis. If so, the results indicate that most renal AT1 receptors are exposed to locally generated Ang II rather than Ang II from the circulation. We propose the existence of a low-Ang II vascular system-related interstitial compartment that is separate from tubular fluid, where, according to micropuncture studies, Ang II levels might be high.

Intrarenal angiotensin II (Ang II) has important effects on renal function and urinary sodium excretion and, via these local actions, on blood pressure regulation. The presence of Ang II in renal tissue depends on two mechanisms: uptake from the circulation and local production.

Uptake from the circulation has been demonstrated in heart, adrenal and kidney [1–3]. In rat and porcine kidney, blood-derived Ang II is accumulated to levels (per gram tissue) several times the level (per mL) in blood plasma [1–3]. This process depends on binding to cell membrane AT1 receptors, followed by receptor-mediated endocytosis [1]. Cell-associated blood-derived Ang II has a longer half-life than Ang II in the circulation [1] but the function of internalized Ang II is not known.

Chronic low-dose Ang II infusions in uninephrectomized rats increased the level of Ang II in renal tissue but not in heart and adrenal [2, 3]. This effect became apparent not earlier than after ten days and was prevented by the Ang II subtype 1 (AT1) receptor antagonist, losartan. It was therefore proposed that the AT1 receptor-dependent increase in renal Ang II during chronic Ang II infusion was caused not only by receptor-mediated endocytosis of blood-derived Ang II, but also by an enhancement of intrarenal Ang II formation [2, 3].

High concentrations of Ang II have been detected in renal interstitial fluid by some [4], but not all [5], investigators. High concentrations of Ang II also have been found in proximal tubular fluid [6, 7]. Measurements of renin in renal lymph indicated that renal interstitial fluid contains renin in high concentrations [8, 9], and the interstitial fluid compartment is thought to be the site of intrarenal Ang II production.

This concept needs some qualification, since in juxtaglomerular cells, renin, angiotensinogen and Ang I and Ang II are co-localized in secretory granules [10–12]. Ang I and probably also Ang II are co-secreted with renin by these cells [10–12]. The detection of high levels of Ang II in renal interstitial fluid may be viewed as evidence supporting the functional significance of locally produced Ang II. This finding, however, is difficult to reconcile with the fact that the kidney is very sensitive to systemically infused Ang II [13].

In this study we attempted (1) to estimate, how much Ang II in renal tissue is derived from blood and how
much is produced locally, and (2) to collect information on the site of intrarenal Ang II production and on its distribution over the interstitial fluid and cell compartments. Our approach was to measure the concentrations of endogenous Ang I and II in conjunction with measurements of $^{125}$I-Ang I and II in renal cortical and medullary tissue from pigs receiving a systemic infusion of $^{125}$I-Ang I. Studies were performed in untreated animals and after treatment with an ACE inhibitor or AT$_1$ receptor antagonist. The rationale for using these drugs as investigative tools lies in the fact that they act at different levels of the renin-angiotensin system, and may therefore differentially affect the uptake, production and compartmentalization of Ang II in the kidney.

METHODS

Animals

All experiments were carried out under the regulations of the Animal Care Committee of the Erasmus University Rotterdam, Rotterdam, The Netherlands, in accordance with the Guide for the Care and Use of Laboratory Animals as published by the U.S. National Institutes of Health. Twenty-one two- to three-month-old Yorkshire × Landrace pigs (weight 25 to 30 kg) of either sex were included in the study. In all animals a suture was placed around the left circumflex coronary artery (LCXCA) in order to examine the effect of coronary ligation on the cardiac levels of Ang II in these animals [14]. The LCXCA was permanently ligated in 16 animals, whereas the suture was removed in five animals (sham operation). Of the permanently ligated animals, five received captopril (25 mg b.i.d.), and five animals received the AT$_1$ receptor antagonist eprosartan (400 mg b.i.d.; a kind gift of Dr. P.K. Weck, SmithKline Beecham, Collegeville, PA, USA). This dose of eprosartan blocks Ang II-induced pressor responses by >95% [14]. Treatment was started 12 to 24 hours after ligation and was continued for three weeks.

Instrumentation and $^{125}$I-angiotensin infusion

Three weeks after ligation or sham operation, animals were prepared for administration of anesthetic and $^{125}$I-Ang I and for blood and tissue sampling as described previously [1]. After a stabilization period of 30 to 45 minutes following completion of instrumentation, animals were subjected to a one-hour infusion of $^{125}$I-Ang I ($\approx 1.5 \times 10^6$ cpm/min) into the left ventricle. $^{125}$I-Ang I and II reach steady-state levels in plasma within ten minutes, and in renal tissue within 60 minutes [1, 15].

Collection of blood and tissue samples

To measure the plasma levels of $^{125}$I-labeled and endogenous Ang I and II, blood samples (5 to 10 mL) were taken from the aorta and a renal vein during the infusion of $^{125}$I-Ang I as described before [15]. Plasma was stored at $-70^\circ$C and assayed within three days.

Renal tissue samples were collected as follows. The heart was stopped by fibrillation while the $^{125}$I-Ang I infusion was still running. The abdomen was opened by a longitudinal incision and both kidneys were rapidly dissected. Pieces of renal cortex and medulla (1 to 2 g) were immediately frozen in liquid nitrogen and stored at $-70^\circ$C.

Measurement of angiotensins

$^{125}$I-labeled and endogenous Ang I and Ang II were measured as described previously [1, 16, 17], using SepPak extraction and high-performance liquid chromatography (HPLC) separation. The concentrations of $^{125}$I-labeled and endogenous angiotensins were not corrected for losses occurring during extraction and separation (20 to 30% in whole tissue homogenates and <10% in plasma [1, 16, 17]). For Ang I, the lower limit of detection was 0.5 fmol/mL of plasma and 1 fmol/g of tissue. For Ang II, the lower limit of detection was 0.3 fmol/mL of plasma and 0.5 fmol/g of tissue.

Calculations

In a previous study [1] we observed that the renal tissue/arterial plasma concentration ratio of $^{125}$I-Ang II during $^{125}$I-Ang I infusion was not higher than during $^{125}$I-Ang II infusion. Thus, the $^{125}$I-Ang II that was present in renal tissue was derived from arterially delivered $^{125}$I-Ang II and not, via conversion, from arterially delivered $^{125}$I-Ang I. The renal tissue level of Ang II (fmol/g) that originates from angiotensin in the circulation was therefore calculated as follows:

\[
\text{[Ang II}_{\text{tissue}}\text{]} = \text{R} \times \text{[Ang II}_{\text{art}}\text{]} \tag{Eq. 1}
\]

in which [Ang II$_{\text{art}}$] is the steady-state concentration of Ang II in arterial plasma (fmol/mL), and R is defined by the equation:

\[
R = \frac{[^{125}\text{I-Ang II}_{\text{tissue}}]}{[^{125}\text{I-Ang II}_{\text{art}}]} \tag{Eq. 2}
\]

in which $[^{125}\text{I-Ang II}_{\text{tissue}}]$ and $[^{125}\text{I-Ang II}_{\text{art}}]$ are the steady-state concentrations of $^{125}$I-Ang II in tissue (cpm/g) and arterial plasma (cpm/mL), respectively.

Data analysis

Data are expressed as mean ± SEM. Differences between treated and untreated animals were tested by ANOVA. Statistical significance was accepted at $P < 0.05$.

RESULTS

Uptake of angiotensin II from blood versus local production and contribution to tissue angiotensin II levels

Figure 1 shows an example of high pressure liquid chromatography (HPLC) separation patterns of $^{125}$I-labeled
and endogenous angiotensins in renal tissue from untreated animals and animals treated with captopril or eprosartan. The levels of endogenous Ang I and II in arterial and renal venous blood plasma and in renal cortical and medullary tissue are shown in Figures 2 and 3. In control animals (that is, animals that were not treated with captopril or eprosartan), coronary ligation did not affect plasma angiotensin levels [14]. Ligation also did not affect renal angiotensin levels. Ang I and II concentrations in renal cortex were $53.8 \pm 9.6$ and $109 \pm 39.2$ fmol/g in permanently ligated animals ($N = 6$) and $68.4 \pm 24.6$ and $115 \pm 10.4$ fmol/g in sham-operated animals ($N = 5$). Data for the two groups were therefore combined. In the combined control group, the levels of en-
than in arterial plasma. Thus, the tissue/blood plasma
concentration ratio of endogenous Ang II in medullary tissue was 60 times higher than the concentration in arterial plasma (Table 1). Therefore, it appears that 85 to 90% of renal venous Ang I originates from production within the kidney. No such release from renal tissue sites could be demonstrated for Ang II, since the renal venous/arterial plasma concentration ratio of endogenous Ang II was as low as that of Ang II (Table 2).

Effects of captopril and eprosartan

Captopril lowered the 125I-Ang II/I ratio in arterial plasma. It was 0.17 ± 0.03 (N = 5) in the captopril-treated animals, as compared to 0.53 ± 0.05 (N = 11) in controls (P < 0.01). The endogenous Ang II/I ratio in arterial plasma also was lower in the captopril-treated animals than in controls (0.17 ± 0.04 vs. 0.54 ± 0.08; P < 0.01). 125I-Ang I was undetectable in tissue after captopril, as was the case in controls.

The Ang II/I concentration ratios in cortical tissue were not significantly different in captopril-treated animals and controls (1.80 ± 0.19 and 2.27 ± 0.47, respectively). This was also true for the Ang II/I concentration ratios in medullary tissue (1.29 ± 0.18 and 2.00 ± 0.66, respectively). In the captopril group, as in controls, most tissue Ang II appears to be derived from local production, and not from the circulation (Fig. 3).

The 125I-Ang II/I concentration ratio in arterial plasma was 0.73 ± 0.09 in the eprosartan-treated animals (N = 5), which was not different from the ratio in controls. The endogenous Ang II/I concentration ratio in arterial plasma in the eprosartan-treated animals was 0.92 ± 0.09, which was also not different from the ratio in controls. 125I-Ang I was undetectable both in cortical and medullary tissue in the eprosartan-treated animals, as was the case in the captopril group and in controls. In the eprosartan group, as in the captopril group and in controls, most tissue Ang II appears to be derived from local production and not from the circulation (Fig. 3).

In the captopril group, the cortical tissue concentration of 125I-Ang II was about five times the concentration in arterial plasma, which was not significantly different from the cortical tissue/arterial plasma 125I-Ang II concentration ratio in controls (Table 2). In the eprosartan group, the cortical tissue concentration of 125I-Ang II was only 0.4 times the concentration in arterial plasma. A similar difference between the eprosartan group on the one hand and the captopril and control groups on the other was seen for the medullary tissue concentrations of 125I-Ang II. These results demonstrate that the accumulation

| Table 1. Renal tissue/arterial blood plasma concentration ratios and renal venous/arterial blood plasma concentration ratios of 125I-angiotensin I and endogenous angiotensin I |
| Control | Captopril | Eprosartan |
| Cortex/artery | Medulla/artery | Vein/artery | Cortex/artery | Medulla/artery | Vein/artery | Cortex/artery | Medulla/artery | Vein/artery |
| P | 0.05 | 0.01 | 0.05 | 0.01 | 0.05 | 0.01 |

| Endogenous angiotensin I | Control | Captopril | Eprosartan |
| Cortex/artery | Medulla/artery | Vein/artery | Cortex/artery | Medulla/artery | Vein/artery | Cortex/artery | Medulla/artery | Vein/artery |
| P | 0.05 | 0.01 | 0.05 | 0.01 | 0.05 | 0.01 |

Steady-state levels of 125I-angiotensin I in arterial plasma ranged from 308 to 519 cpm/mL in the untreated animals, and from 448 to 828 cpm/mL in the untreated animals, and from 49 to 158 cpm/mL and 323 to 405 cpm/mL in the captopril-treated and eprosartan-treated animals, respectively. Medullary tissue and venous blood were not available in 4 of the 11 control animals. Data are mean ± SEM.

*P < 0.05 for difference from control

| Table 2. Renal tissue/arterial blood plasma concentration ratios and renal venous/arterial blood plasma concentration ratios of 125I-angiotensin II and endogenous angiotensin II |
| Control | Captopril | Eprosartan |
| Cortex/artery | Medulla/artery | Vein/artery | Cortex/artery | Medulla/artery | Vein/artery | Cortex/artery | Medulla/artery | Vein/artery |
| P | 0.05 | 0.01 | 0.05 | 0.01 | 0.05 | 0.01 |

Steady-state levels of 125I-angiotensin II in arterial plasma ranged from 161 to 530 cpm/mL in the untreated animals, and from 19 to 158 cpm/mL and 323 to 405 cpm/mL in the captopril-treated and eprosartan-treated animals, respectively. Medullary tissue and venous blood were not available in 4 of the 11 control animals. Data are mean ± SEM.

*P < 0.05 for difference from control

*P < 0.05 vs. cortex/artery ratio

Endogenous Ang II was not detectable (<0.3 fmol/mL) in renal venous plasma from 5 animals.
of blood-derived Ang II in tissue is mediated by AT₁ receptors.

The renal venous/arterial plasma concentration ratios of both ¹²⁵I-Ang I and ¹²⁵I-Ang II in the animals treated with captopril and eprosartan were comparable to the ratios in controls, and the same was true for endogenous Ang I (Tables 1 and 2). The renal venous/arterial plasma concentration ratio of endogenous Ang II in the captopril group tended to be higher than in the other groups, but the difference did not reach statistical significance. Our results in the captopril and eprosartan groups were therefore similar to those in controls, in that these results provided evidence for the release of Ang I, but not Ang II, from renal tissue into the circulation.

DISCUSSION

Intrarenal Ang II has important functions in regulating urine flow, urinary sodium excretion and glomerular filtration rate. It has a major role in the pathogenesis of 2-kidney, 1 clip (2K1C) hypertension and possibly also in the hypertension of the spontaneously hypertensive rat [2, 3, 18, 19]. Although renin is suppressed in the contralateral non-clipped kidney of 2K1C hypertensive rats and in the remaining kidney of uninephrectomized rats during chronic low dose Ang II infusion, Ang II in the kidneys is increased to levels that are too high to be explained by passive diffusion from circulation to tissue [18]. Studies using AT₁ receptor antagonists provided strong evidence to support that tissue Ang II in the non-clipped kidney contributes to the pathogenesis of systemic hypertension by reducing renal function and sodium excretion [19]. AT₁ receptor-mediated uptake and accumulation of Ang II from the circulation could be one of the mechanisms by which intrarenal Ang II is maintained even when renin is suppressed [1–3]. Another possibility is the existence of a positive feedback loop by which intrarenal Ang II formation is enhanced after AT₁ receptor stimulation [2, 3].

The present study in pigs shows that, normally, most Ang II in the kidney is not derived from the circulation, but is produced locally. We found that AT₁ receptor-mediated binding and accumulation of ¹²⁵I-Ang II from the circulation led to a tissue level four times the level in arterial plasma, whereas the tissue level of endogenous Ang II was 100 times the level in arterial plasma. ¹²⁵I-Ang I from the circulation was not accumulated. Its tissue level was at or below the detection limit, whereas endogenous Ang I in tissue was much higher than in arterial plasma. A previous study, in which we compared the results after ¹²⁵I-Ang I infusion with those after ¹²⁵I-Ang II infusion, has shown that the ¹²⁵I-Ang II that was found in renal tissue was derived from arterially delivered ¹²⁵I-Ang II and not from arterially delivered ¹²⁵I-Ang I [1]. Taken together, our results lead to the conclusion that the bulk of Ang II in the kidney is formed locally by conversion of locally produced Ang I and is not derived from Ang I or II in the circulation.

Our results provide some information on the distribution of Ang II in the extracellular and cell compartments of renal tissue. In this context, one has to consider the possibility that blood-derived Ang II does not enter all regions of the kidney. For the sake of simplicity, we consider a region that is reached by blood-derived Ang II and a region that is not reached by blood-derived Ang II. The present study showed a 90% reduction of the tissue/arterial plasma ¹²⁵I-Ang II concentration ratio after eprosartan. If it is assumed that after the AT₁ receptors had been blocked by eprosartan, all ¹²⁵I-Ang II in tissue was localized in extracellular fluid, our results would imply that, in the absence of AT₁ receptor blockade, 10% of ¹²⁵I-Ang II in the kidney, in the region in which blood-derived Ang II is distributed, is localized in extracellular fluid and 90% is cell-associated. Most likely, however, AT₁ receptor blockade by eprosartan was not complete. If our finding that the tissue/arterial plasma ¹²⁵I-Ang II concentration ratio was reduced by 90% after eprosartan would be the consequence of 90% AT₁ receptor occupancy by this drug, then 100% occupancy would result in a tissue/arterial plasma ¹²⁵I-Ang II concentration ratio of zero, which would mean that, in the absence of AT₁ receptor blockade, virtually all ¹²⁵I-Ang II in the kidney is cell-associated.

That AT₁ receptor blockade after eprosartan was indeed not complete, was indicated by earlier experiments in which we measured the steady-state levels of ¹²⁵I-Ang II in subcellular fractions of renal cortical tissue from pigs receiving systemic infusions of ¹²⁵I-Ang I or II [20]. Not only in control animals, but also in animals treated with the same doses of eprosartan as used in the present study, ¹²⁵I-Ang II was found to be concentrated in the nuclear, lysosomal and microsomal cell fractions. In the eprosartan-treated animals, 70% of the total amount of ¹²⁵I-Ang II in the combined cell fractions was found in the nuclear, lysosomal and microsomal fractions with less than 20% loss of ¹²⁵I-Ang II during preparation of these fractions. These results indicate that AT₁ receptor blockade was not complete and that indeed nearly all blood-derived Ang II in renal cortical tissue is cell-associated.

It is commonly held that tissue Ang II is produced in the extracellular interstitial fluid compartment. Extracellular Ang II is then bound to cell surface AT₁ receptors and internalized. If this view is correct, our results imply that most renal AT₁ receptors are exposed to locally produced Ang II rather than Ang II from the circulation. Following this line of reasoning, we propose that the high tissue/arterial plasma concentration ratio of endogenous Ang II, as observed in the present study, depends on the same mechanism as has been demonstrated for ¹²⁵I-
in plasma. How can this discrepancy be explained? Siragy et al. measured so-called immunoreactive Ang II-like AT1 receptor-mediated endocytosis. However, because of the microdialysis technique itself. In order to perform microdialysis experiments, a dialysis membrane probe has to be inserted into the tissue. The probe used by Siragy et al. had an internal diameter of 0.3 mm [4]. The presence of this probe alters the micro-environment of the surrounding cells. Normally, the water space between the cells is very small. Considering a model of two parallel cell layers 1 μm apart, insertion of a cylindrical probe of 0.3 mm diameter between the two layers will increase the extracellular space separating the cells by a factor of about 150 (cross-sectional area of cylinder divided by the product of half cylinder circumference and normal distance between cell layers). As a consequence, the cell surface receptors facing the probe are bathed in a much larger volume of water than normal. The binding of Ang II to its receptors, therefore, takes place at an artificially low receptor concentration. If normally the in vivo binding of Ang II takes place at receptor concentrations that are higher than the equilibrium dissociation constant (Kd), so that most Ang II is complexed with the receptors, a 150-fold reduction in receptor density may lead to a new steady state with a big increase in free Ang II. It is therefore possible that the high Ang II level, as measured with the microdialysis technique, is an artifact. A model of two parallel cell layers 1 μm apart, insertion of a cylindrical probe of 0.3 mm diameter between the two layers will increase the extracellular space separating the cells by a factor of about 150 (cross-sectional area of cylinder divided by the product of half cylinder circumference and normal distance between cell layers). As a consequence, the cell surface receptors facing the probe are bathed in a much larger volume of water than normal. The binding of Ang II to its receptors, therefore, takes place at an artificially low receptor concentration. If normally the in vivo binding of Ang II takes place at receptor concentrations that are higher than the equilibrium dissociation constant (Kd), so that most Ang II is complexed with the receptors, a 150-fold reduction in receptor density may lead to a new steady state with a big increase in free Ang II. It is therefore possible that the high Ang II level, as measured with the microdialysis technique, is an artifact.

Our proposal that the high AT1 receptor density in the kidney is responsible for a low Ang II concentration in interstitial fluid has important implications. It means there is no need for postulating a blood/interstitium ‘barrier’ preventing in situ produced Ang II from leaking into the blood. It also resolves the conflict between the allegedly high concentration of Ang II in renal interstitial fluid and the observed high responsiveness of the kidney to arterially delivered Ang II [13].

Although in our study the level of 125I-Ang II in renal tissue was effectively suppressed by the AT1 receptor antagonist eprosartan, endogenous Ang II in tissue was not decreased by this drug. Thus, neither the decrease in AT1 receptor-mediated uptake nor a negative effect on intrarenal Ang II formation caused by interruption of a supposedly positive AT1 receptor-dependent feedback loop [2, 3] had led to a decrease in tissue Ang II after eprosartan administration. This might be explained, at least in part, by the stimulation of renin following AT1 receptor blockade. Such stimulation causes enhanced Ang II generation in extracellular fluid, as reflected by increased plasma Ang II (Fig. 3). The decrease in the tissue/arterial plasma concentration ratio of endogenous Ang II after eprosartan was similar to the decrease in the tissue/arterial plasma concentration ratio of 125I-Ang II (Table 2), which is in agreement with the concept that endogenous Ang II, like 125I-Ang II, enters the cells via AT1 receptor-mediated endocytosis. However, because of the eprosartan-induced rise in extracellular Ang II, the kidney is capable of maintaining its high intracellular Ang II levels despite the AT1 receptor blockade. An additional explanation for the absence of eprosartan-induced reductions in renal Ang II could be that changes in Ang II concentration in whole tissue do not reliably reflect the changes that might occur at the level of AT1 receptors in the tissue region in which blood-derived Ang II is distributed. In our study renal tissue Ang II also was not reduced by treatment with the ACE inhibitor captopril in doses that were high enough to suppress the Ang II/I concentration ratio in blood. It is tempting to invoke alternative pathways of Ang I-II conversion. Other explanations however, like those mentioned above with respect to the effects of eprosartan, cannot be excluded.

Micropuncture experiments showed high levels of Ang II in proximal tubular fluid from rats [6, 7]. There is further evidence in the rat that interstitial cells, located in the inner stripe of the outer renal medulla, are exposed to high interstitial Ang II concentrations [23, 24]. When these observations are incorporated into our results, a picture emerges in which the renal interstitium contains a low-Ang II vascular system-related compartment, and a high-Ang II tubular system-related compartment. The juxtaglomerular cells, as part of the vascular compartment, are in close anatomical relationship with the macula densa cells that are part of the tubular compartment. These cells are therefore strategically situated to serve communication between the two compartments.

ACKNOWLEDGMENT

This study was supported by the Dutch Kidney Foundation, grant no. NSN C96.1585. No portion of the manuscript has been published elsewhere except in abstract form.

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