Developmental renin expression in mice with a defective renin-angiotensin system

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Submitted 6 July 2009; accepted in final form 20 August 2009

DURING MAMMALIAN KIDNEY development, renin expression undergoes major site changes by switching from expression in the walls of larger arteries in the early fetal kidney to the terminal parts of afferent arterioles in the mature kidney (1, 4, 8, 9, 19, 31, 32). As a consequence of this shift, the number of renin-expressing cells (RC) only increases moderately despite the fact that the postglomerular vascular tree develops almost exponentially until final maturation of the kidneys (31). The shift in renin expression from larger to newly developing smaller vessels requires regulatory mechanisms to activate and deactivate renin expression in a given vascular segment. We recently obtained evidence that the cAMP-signaling pathway is crucial for the activation of renin expression during kidney vessel development (2, 25). The mechanisms controlling the deactivation are still the subject of speculation. It is conceivable that increased activity of the renin-angiotensin system (RAS) itself downregulates renin expression during kidney development and thus essentially contributes to the shift in renin expression along the developing postglomerular vessel tree. A series of arguments mainly derived from findings obtained with adult kidneys would support such a concept. RC in the mature kidney, for instance, express ANG II (AT) receptors (6), which mediate a powerful inhibition of renin secretion (33). Infusion of ANG II in vivo reduces the number of RC in vivo (17, 39). Conversely, pharmacological inhibition of the RAS in the adult kidney increases the number of RC in postglomerular vessels and thus produces a distribution pattern of RC resembling that of the developing kidney (7). Correspondingly, mice genetically lacking defined components of the RAS such as angiotensinogen (Agt) (18, 24, 37), angiotensin-converting enzyme (ACE) (5, 20), or the ANG II type 1 (AT1) receptor (16, 23, 26, 27, 35, 38) display strong hyperplasia of RC in adult kidneys. However, since these mice also show major renal vascular abnormalities (10, 11, 15, 26–28), it is not yet clear to what extent RC hyperplasia is secondary to the vascular defects or if it really reflects the arrest of physiological renin downregulation during kidney development. The possibility of a potential physiological role for the RAS in developmental downregulation of renin expression further raises the question of whether such an effect would be mediated directly by ANG II receptors at the level of renin expression or more indirectly by other RAS-related mechanisms. In fact, doubts have already been raised that AT1 receptors may directly control the number of RC in the kidney (23). Since not only AT1 but also AT2 receptors may interfere with the function of RC (34), a possible role for AT2 receptors in the downregulation of renin expression during kidney development must also be considered.

In view of this background of findings and open questions, we were interested in defining the general role of ANG II in the developmental shift in renin expression more clearly. In particular, we aimed to obtain information about the relevance of ANG II AT1 receptors for the downregulation of renin expression during kidney development. For this purpose, we first studied the three-dimensional distribution patterns of RC and
renin mRNA levels in mice with genetic disruptions of the
main target of ANG II, AT1α receptors, at different stages
during kidney development, as well as in the mature kidney. To
further assess the role of RAS, we analyzed ACE−/− mice with deletions in the other subtypes of ANG II receptors,
AT1α(b), AT1b, and AT2.

We found that at birth, when the developmental shift in
wild-type mice had already taken place, the development of the
preglomerular vascular tree and the distribution of RC were
rather similar between the different genotypes. However, kid-
nies of adult ACE- and AT1-deficient mice displayed massive
hyperlplasia of RC, which were often ectopically located. The
renin cell hyperplasia was paralleled by strong morphological
alterations in the preglomerular vessels. From our results, we
infer that direct negative feedback via AT1 receptors at the
level of RC is not likely to explain the physiological shift in
intrarenal renin expression during kidney development.

MATERIALS AND METHODS

Animals. All animal experiments were conducted according to the
National Institutes of Health (NIH) guidelines for the care and use
of animals in research. The experiments were approved by a local ethics
committee.

For immunohistochemical analysis, the following genotypes were
analyzed: wild-type (129X1/SvJ), ACE−/− (20), AT1α(b)−/− (26),
AT1a−/− (16), AT1b−/− (26), and AT2−/− (14). Three male mice
of each genotype were analyzed 1 day after birth [postpartum
day 1 (pp1)] and at 12 wk of age (adult). Additionally, kidneys
were sampled from AT1α−/− and wild-type fetuses 18 days after formation
of the vaginal plug (embryonic day 18 (E18); plug considered as
E0) and 10 days after birth (pp10).

For mRNA measurements, kidneys (5–7 of the individual stages)
were sampled from E18, 1–3, 5–7, and 10-day-old pups, and
adult AT1α−/− and their corresponding wild-type (129X1/SvJ) mice.

Immunohistochemistry for renin and α-smooth muscle actin. After
the death of the animals, kidneys from each E18 and pp1 subject
were removed and fixed in methyl Carnoy’s solution (60% methanol, 30%
chloroform, and 10% glacial acetic acid) at 4°C for 24 h for the
histological preparation. Kidneys from pp10 and adult mice were
perfusion-fixed with 4% paraformaldehyde dissolved in PBS.
The fixed kidneys were dehydrated by a graded series of alcohol solutions
(2 times in 70, 80, 90, and 100% methanol), followed by two times
in 100% isopropanol for 0.5 h each and embedded in paraffin.

Immunolabeling was performed on 300 consecutive 5-μm sagittal
paraffin sections. After blocking with 10% horse serum/1% BSA in
PBS for 0.5 h at room temperature, sections were incubated with
chicken anti-renin IgG (diluted 1:400, Davids Biotechnologie) and
mouse anti-α-smooth muscle actin (α-SMA) IgG (diluted 1:100,
Beckman Coulter, Immunotech) overnight at 4°C. Following several
washing steps, the sections were incubated with Cy2-conjugated
donkey anti-chicken IgG and rhodamine (TRITC)-conjugated donkey
anti-mouse IgG fluorescent antibodies (diluted 1:400, Dianova)
for 1.5 h and mounted with glycerol (DakoCytomation).

Three-dimensional reconstruction. Serial sections of kidney spec-
imens were fixed and stained for renin and for α-SMA as described
above. Digitalization of the serial slices was performed using an
Axiocam MRm camera (Zeiss) mounted on an Axiosvert 200M
microscope (Zeiss) with fluorescence filters for Cy2 (filter set 38 HE,
Zeiss) and TRITC (filter set 43, Zeiss). After acquisition, a stack
of equal-size images was built using the graphic tool ImageJ (Wayne
Rasband, NIH, Bethesda, MD). The equalized data were then im-
ported into Amira 4.1 visualization software (Visage Imaging Carls-
bad, CA) on a Dell Precision 690 computer system (Dell, Frankfurt,
Germany) and subsequently split into Cy2 (renin) and TRITC (α-
SMA) channels. After this step, the renin and α-SMA channels were
aligned. In the segmentation step, the renin and α-SMA data sets
served as a scaffold, and the fluorescent signals were spanned manually
or automatically using grayscale values. The glomeruli and the cap-
sules were reconstructed using the fluorescence of the background
staining. Matrices and volume surfaces were generated from these
segments.

Semiquantification of renin mRNA by real-time PCR. Total RNA
was isolated from the frozen kidneys as described by Chomczynski
and Sacchi (3) and quantified by a photometer. One microgram of the
resulting RNA was used for RT-PCR. The cDNA was synthesized by
Moloney murine leukemia virus reverse transcriptase (Superscript,
Invitrogen). For quantification of renin mRNA expression (sense:
5'-ATG AAG GGG GTG TCT GTG GGG TC-3', antisense: 5'-ATG
CGG GGA GGG TGG GCA CCT G-3'), real-time RT-PCR was
performed using a Light Cycler Instrument (Roche Diagnostics) and a
Quantitect SYBR Green PCR kit (Qiagen), with GAPDH (sense:
5'-TTC ATT GAC CTC AAC TAC AT-3', antisense: 5'-GAG GGG
CCA TCC ACA GTC TT-3') as a control. PCR was run for 30 cycles
with 15 s/95°C denaturation, 20 s/60°C annealing, and 20 s/72°C
elongation. The accuracy of the amplicon was verified by conducting
a melting-curve analysis after amplification. Samples were run in
duplicate.

Statistics. Levels of significance were calculated by unpaired t-test.
A p value <0.05 was considered significant.

RESULTS

As ANG II AT1α receptors are the main biological targets of
ANG II in mice, we first analyzed the development of renin
expression in kidneys lacking AT1α receptors. In wild-type
kidneys at E18, the main trunks of arcuate arteries together
with arcuate side branches developed within the vascular tree.
Juxtamedullary afferent arterioles became visible, and cortical
interlobular arterioles began to spread. RC were found along the
entire length of the arcuate arteries (Fig. 1a). At that develop-
mental stage, we found no differences in both the distribution
of RC and renin mRNA levels between wild-type and
AT1α−/− (Figs. 1b and 2).

Shortly after birth (pp1), arcuate arteries, arcuate side
branches, and juxtamedullary afferent arterioles were fully
developed in wild-type mice. The renocortical vascular tree
developed further with growing interlobular arteries and mid-
cortical afferent arterioles (Fig. 1c). Renin expression had
already ceased in larger arcuate arteries and was now found in
a spotted pattern in thicker arcuate side branches, growing
interlobular arteries, and juxtamedullary afferent arterioles.
Developing afferent arterioles in the subcapsular space were
free of renin (Fig. 1c). In AT1α−/− mice, there were no marked
differences in both the apparent vascular anatomy and
in the general distribution pattern of RC compared with wild-
type mice: only the number of RC in the developing cortex
appeared to be increased (Fig. 1d). Renin mRNA levels were
about fourfold increased in AT1α−/− kidneys at this stage
(Fig. 2).

Ten days after birth (pp10), cortical interlobular and mid-
cortical afferent arterioles were fully developed, while subco-
cortical afferent arterioles were still in the process of maturation.
RC in the cortex were mainly restricted to the terminal (jux-
taglomerular) ends of afferent arterioles in wild-type kidneys
(Fig. 1e). Juxtamedullary afferent arterioles occasionally
showed RC along their entire length in wild-type kidneys. In
AT1α−/− kidneys, RC were found along almost the entire
length of all afferent arterioles. In addition, they were found in

AFIP-Renal Physiol • VOL 297 • NOVEMBER 2009 • www.afiprenal.org
Fig. 1. Three-dimensional (3D) reconstruction of α-smooth muscle actin (α-SMA)-immunoreactive vascular structures (red) and of renin-immunoreactive areas (green) in isolated arcuate artery trunks (embryonic day 18; E18) or side branches with associated interlobular arteries in mouse kidney at E18 (a and b), postpartum day 1 (pp1; c and d), and pp10 (e and f) from wild-type (a, c, and e) and ANG II type 1a receptor-deficient (AT1a/−−; b, d, and f) mice. Selected glomeruli and a section of the capsule are also shown. Bars = 100 µm in a, b, e, f and 200 µm in c and d.
interlobular arteries and also in arcuate side arteries in a more discontinuous pattern (Fig. 1f). In parallel, renin mRNA levels were increased about sixfold relative to wild-type kidneys (Fig. 2). A more detailed analysis revealed that at this stage of development in AT1a−/− kidneys, a substantial number of RC were ectopically located in a way that they were associated with the outer circumference of the vessels and not integrated into the vessel wall. In wild-type kidneys, RC were typically restricted to the vessel wall (Fig. 3).

In adult wild-type kidneys, RC were localized at the classic juxtaglomerular position at the terminal ends of afferent arterioles and very rarely also at the origin of efferent arterioles (Fig. 4a). Virtually every afferent arteriole contained RC in its juxtaglomerular portion. Imaging at higher resolution revealed that RC visibly formed the media layer of the terminal part of the afferent arterioles, i.e., they replaced the smooth muscle cell monolayer (Fig. 5a). In adult AT1a−/− kidneys, the number of RC was increased relative to wild-type kidneys. RC were mainly associated with juxtaglomerular apparatuses, afferent arterioles, and to a lesser degree also with interlobular arteries (Fig. 4b). Few RC were seen in association with arcuate side arteries. A more detailed analysis revealed localization of RC within the arteriolar vessel wall. Additional RC were attached to the outer circumference of the vessels (Fig. 5b). Aberrant renin expression outside the juxtaglomerular apparatus and vessel walls was not noted. Renin mRNA levels in AT1a−/− kidneys were increased about fourfold relative to wild-type kidneys (Fig. 2). In adult AT1a−/− kidneys, significant changes in the vessel architecture also became apparent, as evidenced by the appearance of multilayered, thicker, and shorter arcuate and interlobular arteries (Fig. 6b).

As AT1a receptors only mediate parts of the biological effects of ANG II, we also considered the effects of a more complete depletion of circulating ANG II by analyzing mice lacking ACE (Figs. 4c and 7a). In adult ACE−/− kidneys, the number of RC was clearly increased relative to wild-type kidneys but also compared with AT1a−/− kidneys (Fig. 4c). In parallel, the vascular changes already noticed in AT1a−/− kidneys were markedly exaggerated in ACE−/− kidneys (Fig. 6c). The kidney cortex was thinner, and the packing density of glomeruli was increased. Many of the superficial glomeruli showed signs of sclerosis. ACE−/− kidneys displayed strong hyperplasia of RC, which were found in the walls of afferent and occasionally of efferent arterioles, interlobular arteries, and to a lesser degree of arcuate side arteries. Preglomerular vessels were thickened due to a multilayered vessel wall. Numerous RC were attached to the outer circumference of the thickened vessel (Fig. 5c). Ectopic renin expression occurred within many glomeruli and in the periglomerular interstitium.

One day after birth, the distribution pattern of RC in ACE−/− kidneys was not significantly different between that seen in wild-type or AT1a−/− kidneys (Fig. 7a). RC were found in interlobular arteries and afferent arterioles, while arcuate arteries were mainly free of RC.

The severe phenotype of ACE−/− kidneys prompted us to also consider the role of other defined receptors for ANG II such as AT1b and AT2 receptors.

Fig. 2. Renin mRNA abundance expression during kidney development of wild-type and AT1a−/− mice. Data are given relative to the value obtained for pooled RNA of adult AT1a−/− kidneys. Values are means ± SE of 5–7 kidneys/developmental stage. *P < 0.05 AT1a−/− vs. wild-type.

Fig. 3. Immunohistochemistry for α-SMA (red) and renin (green) on kidney sections of pp10 mice. Magnification ×400. Bars = 50 μm. G, glomerulus. a: pp10 wild-type (129X1/SvJ). b: pp10 AT1a−/−.
Fig. 4. 3D reconstruction of α-SMA-immunoreactive vascular structures (red) and of renin-immunoreactive areas (green) in an arcuate side branch with associated interlobular arteries and afferent arterioles in mouse kidneys at 12 wk of age (adult). Glomeruli and a section of the capsule are also shown. Bars = 100 μm. a: Wild-type (129X1/SvJ). b: AT1a−/−. c: angiotensin-converting enzyme-deficient (ACE−/−). d: AT1a+b−/−. e: AT1b−/−. f: AT2−/−.
We found out that the architecture of the preglomerular vessel tree and the number and distribution pattern of RC in AT1b/H11002 and AT2/H11002 kidneys were very similar to those of wild-type kidneys both 1 day after birth (Fig. 7, c and d) and in the adult animals (Figs. 4, e and f, 5, e and f, and 6, e and f).

Mice lacking both isoforms of the AT1 receptor (i.e., complete AT1 deficiency) developed a renal phenotype that was very similar to that of wild-type kidneys both 1 day after birth (Fig. 7, c and d) and in the adult animals (Figs. 4, e and f, 5, e and f, and 6, e and f).

One day after birth, the number of RC in AT1(a/b)/H11002 appeared to be increased relative to that in wild-type kidneys (Fig. 7b). However, similar to wild-type kidneys, RC were confined to the walls of afferent arterioles and interlobular arteries. Arcuate side branches were mainly free of RC.

The vessel walls but to a major extent also in association with the outer circumference of the vessels (Fig. 5d, *). Ectopic localization of RC was seen within glomeruli and in the periglomerular interstitium (Fig. 5d, arrows). Similar to ACE−/− kidneys, the architecture of the preglomerular tree was also strongly altered in AT1(a+b)/−/− kidneys (Fig. 6d). A number of sclerotic glomeruli with direct contact to the kidney capsule became visible.

We found that the architecture of the preglomerular vessel tree and the number and distribution pattern of RC in AT1b−/− and AT2−/− kidneys were very similar to those of wild-type kidneys both 1 day after birth (Fig. 7, c and d) and in the adult animals (Figs. 4, e and f, 5, e and f, and 6, e and f).

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One day after birth, the number of RC in AT1(a/b)/H11002 appeared to be increased relative to that in wild-type kidneys (Fig. 7b). However, similar to wild-type kidneys, RC were confined to the walls of afferent arterioles and interlobular arteries. Arcuate side branches were mainly free of RC.
Fig. 6. Reconstruction of α-SMA-immunoreactive vascular structures (red) in an isolated arcuate side branch with associated interlobular arteries and afferent arterioles in mouse kidneys at 12 wk of age (adult). Bars = 100 μm. a: Wild-type (129X1/SvJ). b: AT₁a−/−. c: ACE−/−. d: AT₁(α+β)−/−. e: AT₁b−/−. f: AT₂−/−. The figure was generated by removing the renin labels from Fig. 4.
DISCUSSION

Our study was designed to define a possible counterregulatory role of the RAS itself in the development of RC in the developing kidney. It is a striking phenomenon in all mammalian kidneys that renin expression initially starts in the walls of larger preglomerular vessels and then shifts to afferent arterioles with ongoing differentiation of the kidney. A major shift in renin expression from the larger vessels to interlobular arteries and afferent arterioles has already occurred in mouse kidneys at the date of birth (31). In contrast to humans, kidney development in mice is not finished at birth but takes a period of postnatal maturation. During this time, the majority of corticoradial interlobular arteries and superficial afferent arterioles develop. In parallel, renin expression disappears from interlobular arteries and proximal afferent arterioles and becomes finally restricted to the juxtaglomerular position in the distal parts of the afferent arterioles (31). Throughout the whole process of shifting, RC are integral components of the vascular walls (31). Mesangial cells and efferent arterioles normally do not express renin. Our present findings on developmental renin expression in wild-type mice are in full agreement with our previous observations.

Our new data show that in kidneys lacking ANG II AT1 receptors, the shift in renin expression from larger vessels to afferent arterioles is not prevented, at least in mice. At pp1, the normal pattern of renin expression is observed in the absence of the AT1 receptor. Apparently between pp1 and pp10, the renin expression pattern reverts to the embryonic pattern in the absence of the AT1 receptor, with an exaggerated appearance of RC during the development of interlobular arteries and cortical afferent arterioles. In contrast to wild-type kidneys, RC were not restricted to the walls of the vessels but were also found attached to the outer layer of the vessels. Within ongoing maturation, those extravasal RC had disappeared in part, as also indicated by the fall of renin mRNA levels during the final maturation period of the kidneys. Nonetheless, the number of RC in adult AT1-deficient kidneys was still increased relative to wild-type kidneys, as described by others (27, 35). Complete abrogation of ANG II plasma levels by ACE deletion also did not prevent the shift in renin expression from the larger to the
smaller vessels. In adult ACE-deficient kidneys, the number of extravasally and ectopically localized RC remained markedly increased, as already noticed by others (12). This strong phenotype of adult ACE-deficient kidneys was mimicked by deletion of both AT1 receptor isoforms, while the effect of deletion of AT1a alone was much more moderate, and deletion of AT1b alone did not at all produce visible changes in renin expression. These observations are also in accordance with previous studies (26, 29). This striking overadditive effect of combined AT1a and AT1b receptor deletion on renin expression in the adult kidney could be explained by a minor rest function of AT1b receptors in RC, which is strong enough to provide some residual AT1 receptor function in AT1a-deficient kidneys, but is small enough to be fully compensated by the normal AT1a receptor function in AT1b receptor-deficient kidneys. Such a scenario has been experimentally supported by the role of ANG II AT1 receptor isoforms in renovascular contractility (10, 30). An alternative explanation for the overadditive effect of complete AT1 receptor deletion would be that the renin phenotype of ACE- or AT1-deficient mice results from extrarenal events rather than from a direct interaction of ANG II with RC. A candidate for such extrarenal processes could be the function of the adrenal gland, which expresses higher levels of AT1b than the kidney (6). Evidence for such an extrarenal modulation of RC by components of the RAS would be the observations that genetic deletion of the aldosterone synthase (22) and the mineralocorticoid receptor (13) probably produces similar renal phenotypes, as does complete deletion of AT1 receptors. Together with the observation that rescue of AT1 receptor expression in the proximal tubulus of AT1a-receptor-deficient mice also rescues the renin phenotype (21), we speculate that the effects of disruption of the RAS on the number and distribution of RC in the adult kidney probably do not result from the interruption of ANG II signaling in RC themselves, but are rather related to an indirect, perhaps volume-related effect. Despite the fact that RC in the adult mouse kidney express the AT1a receptor gene (6), doubts have already arisen as to whether this receptor really essentially controls renin expression and in consequence RC formation at the level of the RC themselves (23).

Our findings also confirm previous studies reporting strong alterations of the renovascular architecture in those genotypes with severe RC hyperplasia (35, 37). It remains to be clarified in future experiments as to whether RC hyperplasia is a trigger for the development of vessel abnormality such as thickening of vessel walls or shortening of the vessels, or if it is a parallel phenomenon. Initial reports that mice lacking renin also develop hypertrophy of renal vessels (36) would argue in favor of the latter explanation.

ACKNOWLEDGMENTS

The expert technical assistance provided by Anna M’Bangui is gratefully acknowledged.

GRANTS

This study was supported by the Deutsche Forschungsgemeinschaft (SFB 699).

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