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Nephron-specific expression of components of the renin–angiotensin–aldosterone system in the mouse kidney

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Abstract

Introduction: The renin–angiotensin–aldosterone system (RAAS) plays an integral role in the regulation of blood pressure, electrolyte and fluid homeostasis in mammals. The capability of the different nephron segments to form components of the RAAS is only partially known. This study therefore aimed to characterize the nephron-specific expression of RAAS components within the mouse kidney.

Materials and methods: Defined nephron segments of adult C57B/16 mice were microdissected after collagenase digestion. The gene expression of renin, angiotensinogen (AGT), angiotensin-converting enzyme (ACE), angiotensin II receptors 1a (AT1a), 1b (AT1b), and 2 (AT2) was assessed by reverse transcriptase polymerase chain reaction (RT-PCR).

Results: Renin mRNA was present in glomeruli, in proximal tubules, in distal convoluted tubules (DCT) and cortical collecting ducts (CCD). AGT mRNA was found in proximal tubules, descending thin limb of Henle's loop (dTL) and in the medullary part of the thick ascending limb (mTAL). ACE mRNA was not detectable in microdissected mouse nephron segments. AT1a, AT1b and AT2 mRNA was detected in glomeruli and proximal convoluted tubules.

Conclusions: Our data demonstrate a nephron-specific distribution of RAAS components. All components of the local RAAS – except ACE – are present in proximal convoluted tubules, emphasizing their involvement in sodium and water handling.

Keywords

Angiotensinogen, angiotensin, glomerulus, microdissection, mRNA, receptor, renin, RT-PCR, tubulus

Introduction

In the classical renin–angiotensin–aldosterone system (RAAS), traditionally viewed as a circulating endocrine system, renin produced by juxtaglomerular epithelioid cells cleaves hepatic-derived angiotensinogen (AGT) to form angiotensin I (ANG I), which is converted to the active angiotensin II (ANG II) by angiotensin-converting enzyme (ACE) within the pulmonary circulation. ANG II is the biological effector of the system and acts via cell surface receptors of the AT1 or AT2 type.[1] These angiotensin receptor subtypes were characterized using selective non-peptide ligands, such as losartan (AT1 selective) and PD123319 (AT2 selective).[2,3] AT1 and AT2 receptors share only a 32% homology on the amino acid level. In rodents two isoforms of the AT1 receptor, termed AT1a and AT1b exist. They share 94% identity in their amino acid sequences with only 60% identity in the 3′ and 5′ untranslated regions.[4] Besides this classical circulating RAAS, many of its prerequisite components such as renin, AGT, and ACE are localized in numerous tissues, including heart, brain and kidney, indicating the existence of a local tissue RAAS.[5] Moreover, ANG II can also be formed via other enzymes such as, for example, chymase, cathepsin G and kallikrein.[6]

AT1a receptors occur predominantly in vascular smooth muscle cells, liver, lung, and kidney, whereas AT1b can be found mainly in adrenal and anterior pituitary.[7] AT2 receptors are mainly expressed in the adrenal gland, brain and myocardium as well as in the vasculature in the adult.[8]

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By means of synthesis and the secretion of renin, the kidney plays a central role for the RAAS. It turns out that the kidney also produces other components of the RAAS such as AGT and ACE, albeit to a lesser extent than do other organs such as the liver (for AGT) or the lung (for ACE). The kidney, moreover, is also a major target for the RAAS, in the way that ANG II plays a major role for kidney development, for renal blood flow and glomerular filtration and for sodium reabsorption. The intrarenal expression sites of the different components have been analyzed in several species, and there is consensus in all species examined that renin is mainly expressed in the juxtaglomerular apparatus,[9] AGT in the proximal tubule,[10] mesangial cells and vasculature,[11] and ACE in proximal convoluted tubules (PCT) and vasculature.[12] The distribution of AT1 receptors in the kidney of adult mammals is highly conserved.[8] AT1 receptors are located in glomeruli and in high density in the innner stripe of the outer medulla. There is a moderate level of AT1 receptors in the proximal tubulus. In the outer stripe of the outer medulla and the inner medulla only a few AT1 receptors exist as demonstrated in receptor binding studies.[13] Electron microscopic autoradiography shows that AT1 receptors are present in glomerular mesangial cells, proximal tubular epithelia and type 1 renomedullary interstitial cells of the outer medulla.[14] AT1 receptor mRNA and protein have been demonstrated mainly at sites where AT1 receptor binding is present, but also in other localizations such as vascular smooth muscle cells, distal tubules and papilla. The expression of AT2 receptors is subject to controversial discussion.

Most of these data have been obtained with rats. Relatively little is known about the intrarenal expression sites for the components of the RAAS in mice. Since in view of the knockout methodology the normal mouse physiology is attracting increasing attention, it appeared of interest to us to learn more about the localization of the RAAS in this animal species. For this reason we aimed to localize the gene expressions of the different components in defined nephron segments of the mouse kidney.

Materials and methods

Animals

Animals were kept in accordance with the National Institute of Health (NIH) Guide for the care and use of laboratory animals and the German laws on the protection of animals. No experiments were performed. Male C57BL/6 mice (20–25 g), which had had free access to tap water and standard rodent chow were used (Charles River).

Preparation of kidneys and microdissection

The animals were anesthetized and decapitated. The kidneys were removed, decapsulated and separated into major regions by dissection on ice under a stereomicroscope. Regions were cut into approximately 1-mm thick coronar slices and incubated in a 20 ml scintillation vial with 2 ml of warm Eagle`s minimal essential medium (MEM) containing 0.5 mg/ml type 2 collagenase (Worthington), 5 mM glycine (7.5 mM glycine for inner medulla), 48 µg/ml soy bean trypsin inhibitor and 50 U/ml deoxyribonuclease (DNAse) at 37°C in a water bath according to a modified protocol from Schafer et al.[15] The supernatant was removed from the tissue slices and transferred into tubes containing 2 ml of ice-cold MEM containing 1% bovine serum albumin (BSA). Fresh collagenase–MEM medium was then added to the remaining tissue slices and digestion was continued with removal of the supernatant at 15-minute intervals for cortex and 25-minute intervals for outer and inner medulla until digestion of tissue pieces was completed. The transferred tubule segments sedimented in the tube. The overlying supernatant was removed with a Pasteur pipette and replaced by 2 ml ice-cold MEM containing 1% BSA to stop the digestion. For selection of nephron segments, the suspension was carefully pipetted with a large bore pipette into dissection dishes of 35 mm diameter. Glomeruli and nephron segments were sorted and measured individually with an ocular micrometer. The following nephron segments were collected: proximal convoluted tubule (PCT), proximal straight tubule (PST), descending thin limb (dTL) of Henle`s loop from outer medulla, thin limb of Henle`s loop from inner medulla (TL), medullary thick ascending limb of Henle`s loop (mTAL), cortical thick ascending limb of Henle`s loop (cTAL), distal convoluted tubules (DCT), cortical collecting ducts (CCD), outer medullary collecting ducts (OMCD), and inner medullary collecting ducts (IMCD). Twenty-two glomeruli with (Glo+) and without (Glo−) afferent arteriole as well as the selected tubules of a minimum length of 11 mm were lysed in 400 µl of solution D (guanidine thiocyanate [4 mol/L] containing 0.5% *N*-lauryl sarcosinate, 10 mmol/L EDTA, 25 mmol/L sodium citrate, 700 mmol/L β-mercaptoethanol) and stored at –80°C until RNA was extracted.

RNA isolation and reverse transcription

Total RNA was isolated according to the protocol of Chomczynski and Sacchi.[16] Samples were thawed on ice, 12 µg yeast tRNA was added as a carrier, then 0.1 volume 2 mol/L sodium acetate (pH 4), 1 volume phenol (water-saturated) and 0.2 volume chloroform were added sequentially to the homogenate and incubated for 15 minutes; samples were then centrifuged at 10,000 *g* for 15 minutes at 4°C. RNA in the supernatant was precipitated with an equal volume of isopropanol at 20°C for at least one hour. The RNA pellet was finally redissolved in 9 µl diethylpyrocarbonate-treated water. Synthesis of cDNA first strand was performed in a total reaction volume of 22 µl. Either 2 µg whole mouse kidney total RNA or 9 µl total RNA from microdissected glomeruli/tubules and 0.5 µg oligo(dT)$_{12–18}$ primer (Gibco Life Technologies) were heated at 65°C for

Table 1. Primer sequences used for reverse transcription polymerase chain reaction (RT-PCR)

RAAS component[a]	GeneBank accession no.	Upstream and downstream primer	Fragment size
Renin	X16642	5'-atg aag ggg gtg tct gtg ggg tc-3' 5'-atg tcg ggg agg gtg ggc acc tg-3'	194 bp
AT1a	S37484	5'-cat tcc tgg atg tgc tg-3' 5'-gaa caa gac gca ggc ttt-3'	289 bp
AT1b	S37491	5'-atg aat ctc aga act caa cac-3' 5'-aaa ctt gaa tat ttg gtg ggg a-3'	231 bp
AT2	NM_007429	5'-tcc ttt tga taa tct caa c-3' 5'-caa aca ctt tgc aca tca ca-3'	310 bp
AGT	NM_007428	5'-ctg gcc gcc gag aag cta g-3' 5'-ccc cac cat gat gga ctg ta-3'	299 bp
ACE	J04946	5'-ctg cgt aga ggt gcc aac c-3' 5'-acg gtg tca cgt ttg gga tg-3'	357 bp
β-Actin	M12481	5'-ccg ccc tag gca cca ggg tg-3' 5'-ggc tgg ggt gtt gaa ggt ctc aaa-3'	286 bp

[a]For key to abbreviations and symbols, see Materials and methods.

5 minutes in a volume of 10 µl. Then samples were cooled on ice, and the following components were added for reverse transcription: 4 µl deoxynucleoside triphosphates (2.5 mM; Sigma), 4 µl RT buffer (5× concentrate; Gibco Life Technologies), 0.5 µl RNasin (40 IU/µl; Promega), 2 µl RNAse free water and 1 µl murine Molony leukemia virus (M-MLV) reverse transcriptase (RT) (20 U/µl; Gibco Life Technologies). Samples were incubated at 37°C for 60 minutes and then heated to 94°C for 2 minutes to stop the reaction. Aliquots of 2 µl from the respective cDNA samples were used for the polymerase chain reaction (PCR).

PCR analysis

Block cycler PCRs were performed for marker tests and primer tests as described before.[17,18]

Primers were based on available cDNA sequences for mouse renin, angiotensin II type 1 subtype a and b receptors (AT1a, AT1b), angiotensin II type 2 receptor (AT2), angiotensinogen (AGT), angiotensin-converting enzyme (ACE), and β-actin in the Gene Bank and are summarized in Table 1. With the exception of renin, all primers were generated with *Bam* H1 and *Eco* R5 restriction sites in the 5′-direction; therefore the amplified fragments had the indicated size plus an additional 15 bp. Renin, ACE and ß-actin primers were intron-spanning to avoid possible coamplification of genomic DNA coding for renin. The remaining primers were not intron-spanning, but because of widely different, including absent, expression of these four genes throughout the nephron, contamination with genomic DNA basically could be ruled out. PCRs were run at 32 cycles.

Results

Renin, AGT and ACE, AT1a, AT1b and AT2 receptor expression along the murine nephron was determined by RT-PCR. The mRNA content of each collection of nephron segments was confirmed by a β-actin PCR. If there was no clear β-actin signal, the collection was discarded. Each figure represents the results of at least three different collections of microdissected nephron segments. To confirm the findings and exclude contamination, samples of nephron segments were analyzed for the expression or absence of marker primers (Figure 1). Contamination with vasa recta cannot be excluded, which we consider a limitation of this study.

Renin mRNA was expressed in glomeruli with and without afferent arteriole, PCT, PST, DCT and CCD (Figure 2, Figure 8). A weak signal was seen in OMCD in one collection of nephron segments and in IMCD in another one, which we consider a chance finding as it was not reproducible in the other collections.

We found AGT mRNA in PCT, PST, dTL and in the mTAL (Figure3, Figure 8). In one collection of nephron segments, we detected AGT mRNA also in TL, which might point to an expression in the inner medulla.

ACE mRNA was not found at all in microdissected nephron segments, but there was always strong expression in whole kidney homogenate (Figure 4, Figure 8).

AT1a, AT1b and AT2 mRNAs were found in glomeruli with and without afferent arteriole and PCT (Figures 5–8). AT1a mRNA was also found in DCT, but this expression was not always reproducible.

Discussion

The present study using microdissection of mouse kidneys confirmed the main renal localization of renin to be in glomeruli with and without attached afferent arterioles. In addition clear signals were obtained from proximal (PCT, PST), and distal tubule segments (DCT) and from cortical collecting ducts (CCD). The renin expression in glomeruli, especially with adherent afferent arterioles and proximal

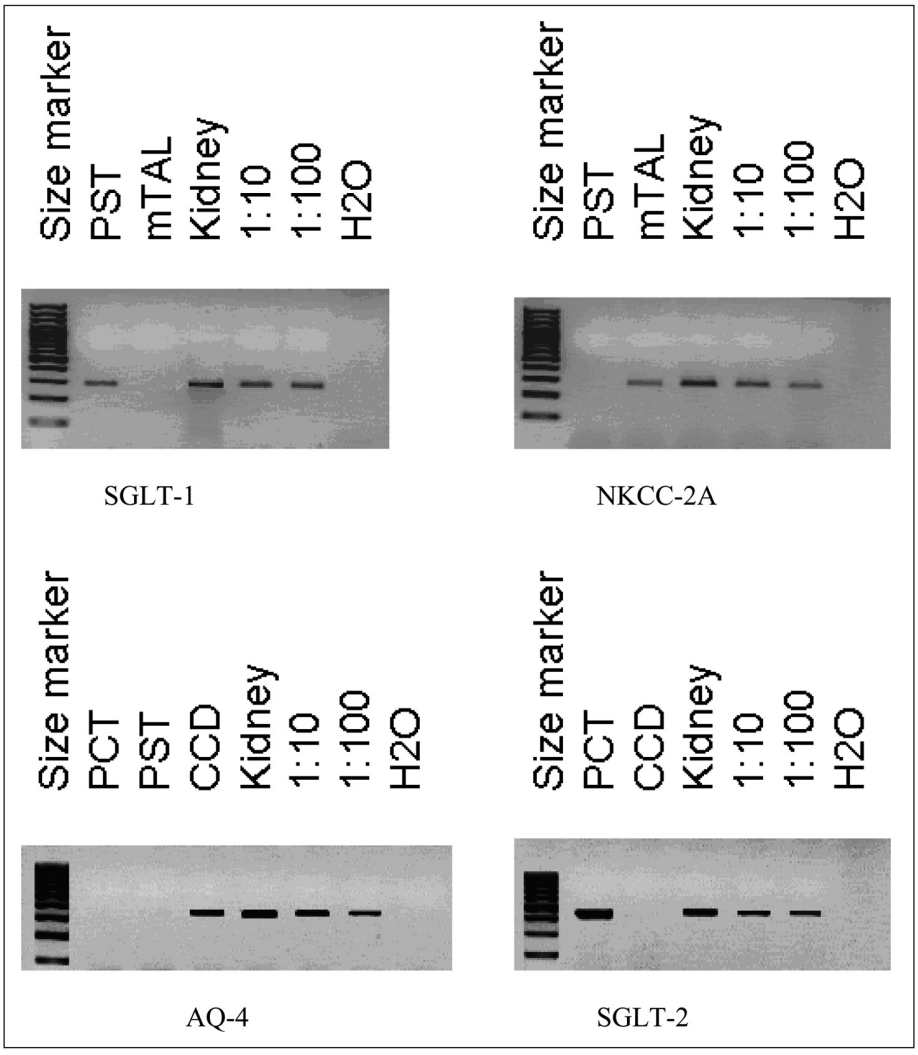

Figure 1. Marker primer expression in microdissected nephron segments in proximal convoluted tubules (PCT), connecting and cortical collecting ducts (CCD), proximal straight tubules (PST), medullary thick ascending limbs (mTAL). SGLT1, Sodium Glucose Transporter Types 1 and 2 respectively. NKCC2A, Na-K-2Cl cotransporter subtype A; AQ-4, Aquaporin-4.

tubules, is very well known and was demonstrated in several studies for rats and mice already a long time ago.[19–22] More recently, renin was localized also along the distal nephron with a focus on the cortical collecting duct, again in rats[23] and mice,[24] extending down to the rat medullary collecting duct.[23] Data on renin expression in the DCT are scarce, as are data for connecting tubules. Renin was co-localized with kallikrein in murine connecting tubules[25] and this renin expression was confirmed in a murine collecting duct cell line in vitro[26] and especially in vivo in mice,[24] nicely matching with our results. The old hypothesis that filtered renin is the source of tubular renin stresses the importance of our results, obtained by analysis of mRNA expression in comparison to localization by immunohistochemical methods or western blot analysis. Renin mRNA expression should be similar in mice and rats and thus we would have expected to find renin mRNA along the whole collecting duct as suggested, for example, by the results by Prieto-Carrasquero et al.,[23] but the expression of renin in the OMCD and IMCD was in our experiments not reproducible and, again, even Prieto-Carrasquero and coworkers discuss the tubular uptake of filtered renin and the collecting duct cell line used by Kang et al.[26] was a cortical collecting duct cell line. The clear and reproducible expression in the DCT in our study points to an expression already in the DCT and not only in the connecting tubules, though a contamination by cells of the juxtaglomerular apparatus cannot be completely ruled out. So we asserted and extended the existing data on renin mRNA expression, reinforcing the importance of the local RAAS.

We found AGT mRNAs in microdissected nephron segments in PCT, PST, dTL and mTAL. This is somewhat at variance with data showing AGT predominantly in rat proximal tubules[10,27] and demonstrating AGT only or predominantly

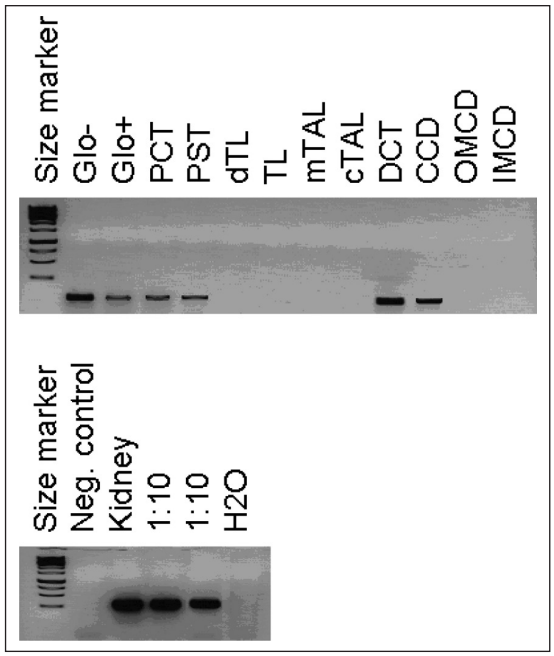

Figure 2. Renin mRNA expression along the mouse nephron. Glomeruli without (Glo-) and with arterioles (Glo+), proximal and distal convoluted tubules (PCT/DCT), proximal straight tubules (PST), descending thin limbs (DTL), descending/ascending thin limbs of Henle (TL), medullary thick ascending limbs (mTAL), cortical thick ascending limbs (cTAL), connecting and cortical collecting ducts (CCD), outer medullary collecting ducts (OMCD), inner medullary collecting ducts (IMCD).

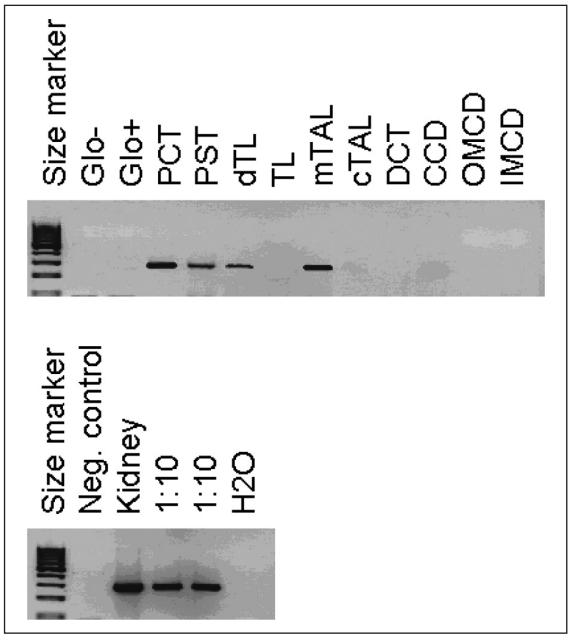

Figure 3. Angiotensinogen mRNA expression along the mouse nephron. Glomeruli without (Glo-) and with arterioles (Glo+), proximal and distal convoluted tubules (PCT/DCT), proximal straight tubules (PST), descending thin limbs (DTL), descending/ascending thin limbs of Henle (TL), medullary thick ascending limbs (mTAL), cortical thick ascending limbs (cTAL), connecting and cortical collecting ducts (CCD), outer medullary collecting ducts (OMCD), inner medullary collecting ducts (IMCD).

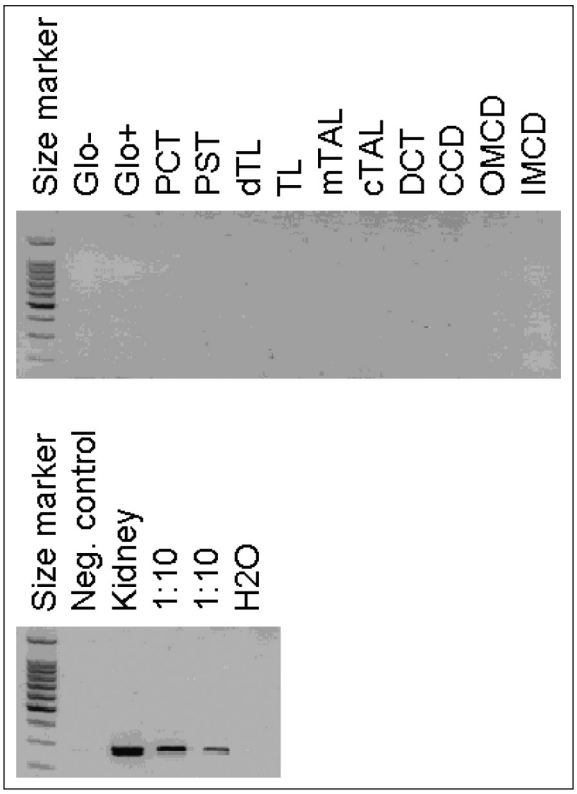

Figure 4. Angiotensin-converting enzyme mRNA expression along the mouse nephron. Glomeruli without (Glo-) and with arterioles (Glo+), proximal and distal convoluted tubules (PCT/DCT), proximal straight tubules (PST), descending thin limbs (DTL), descending/ascending thin limbs of Henle (TL), medullary thick ascending limbs (mTAL), cortical thick ascending limbs (cTAL), connecting and cortical collecting ducts (CCD), outer medullary collecting ducts (OMCD), inner medullary collecting ducts (IMCD).

in proximal tubules of mice, using immunohistochemistry.[21,24] Furthermore AGT mRNA has been demonstrated to be expressed to a large extent in PCT and PST and to a small amount in glomeruli and vasa recta in rat kidneys in one study,[28] and to a small extent in mesangial cells, vasculature and PCT in another study.[11] To confirm the mRNA expression in dTL and mTAL, we tested the nephron segments for their marker primers and thus we can rule out a contamination by other nephron segments. Yet we cannot completely exclude a contamination by vasa recta, which could in principle as well account for the strong mRNA signals, as for the expression in TL, which we detected once.

In the present study ACE mRNA could not be detected in any microdissected mouse nephron segments, but the presence of ACE was clearly demonstrated in the whole kidney homogenate. ACE had already been detected in the PCT and vascular endothelial cells in adult human kidneys,[12] as well as in human kidney cell lines.[29] High concentrations of ACE have been found in tubular fluid from proximal tubules in rats, but much lower concentrations in late proximal and early distal tubules, and absence of ACE in distal tubules.[30] High concentrations of ACE in urine suggested a high ACE

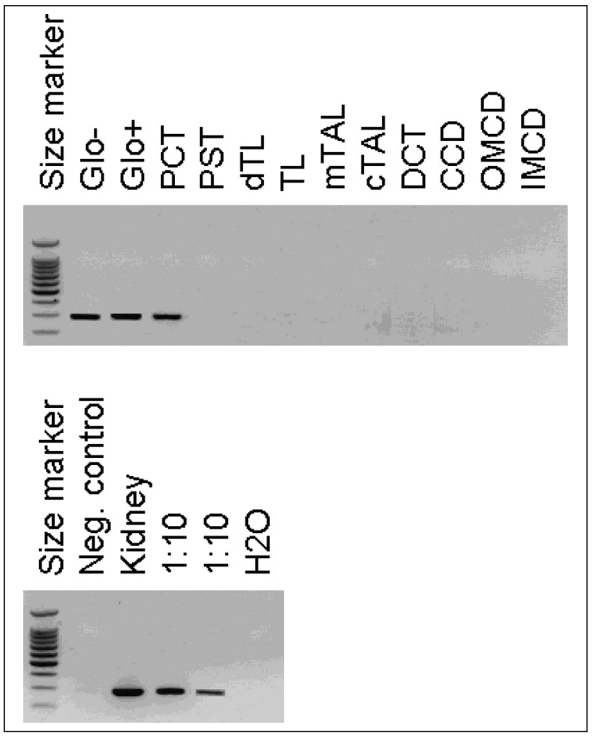

Figure 5. Angiotensin type 1a (AT1a) receptor mRNA expression along the mouse nephron. Glomeruli without (Glo-) and with arterioles (Glo+), proximal and distal convoluted tubules (PCT/DCT), proximal straight tubules (PST), descending thin limbs (DTL), descending/ascending thin limbs of Henle (TL), medullary thick ascending limbs (mTAL), cortical thick ascending limbs (cTAL), connecting and cortical collecting ducts (CCD), outer medullary collecting ducts (OMCD), inner medullary collecting ducts (IMCD).

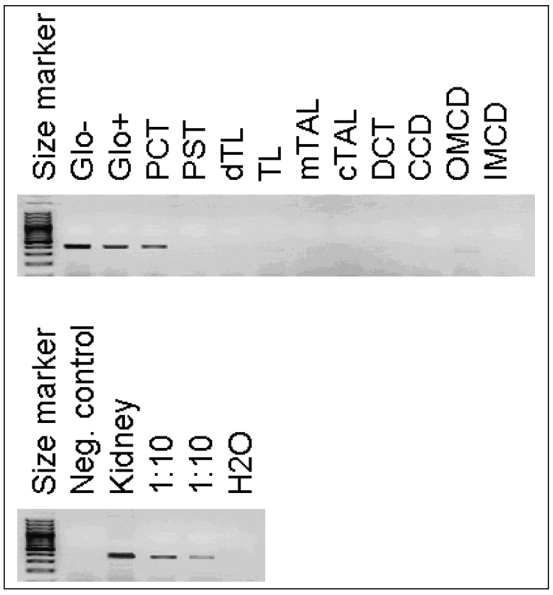

Figure 6. Angiotensin type 1b (AT1b) receptor mRNA expression along the mouse nephron. Glomeruli without (Glo-) and with arterioles (Glo+), proximal and distal convoluted tubules (PCT/DCT), proximal straight tubules (PST), descending thin limbs (DTL), descending/ascending thin limbs of Henle (TL), medullary thick ascending limbs (mTAL), cortical thick ascending limbs (cTAL), connecting and cortical collecting ducts (CCD), outer medullary collecting ducts (OMCD), inner medullary collecting ducts (IMCD).

production also in the collecting duct, but the predominant expression of ACE in proximal tubules was confirmed in rat kidneys using autoradiography[31] and recently in murine glomeruli and the apical brush border of proximal tubuli, podocyte foot processes and the slit diaphragm using immunofluorescence and immunogold electron microscopy.[32] In a recent study ACE was immunolocalized in the murine renal vasculature.[33] This matches our results as our collection of nephron segments did not include any blood vessels. Taking a close look at our results, one might ask why the glomeruli with arterioles also showed no ACE expression compared to glomeruli without arterioles, but our results still match with the immunolocalization,[33] as ACE was immunolocalized in larger arterioles only. A weak ACE staining was also shown in the glomerular tuft and in proximal tubules of db/m mice, but this may be ACE taken up by glomerular cells and filtered in Bowman´s space, consequently being present in proximal tubules as it was once also suggested for renin.[19] In another recent study in mouse kidney, ACE was localized using immunochemistry to brush borders of proximal tubules and the endothelium of arterioles, however no mRNA data to support these tubular

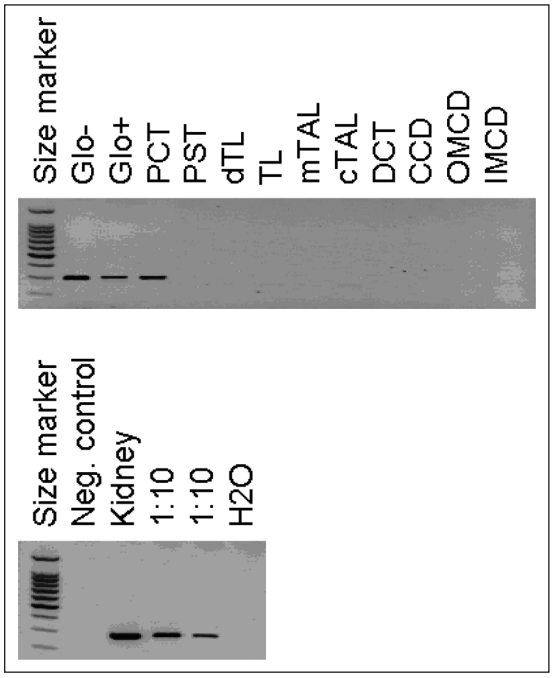

Figure 7. Angiotensin type 2 (AT2) receptor mRNA expression along the mouse nephron. Glomeruli without (Glo-) and with arterioles (Glo+), proximal and distal convoluted tubules (PCT/DCT), proximal straight tubules (PST), descending thin limbs (DTL), descending/ascending thin limbs of Henle (TL), medullary thick ascending limbs (mTAL), cortical thick ascending limbs (cTAL), connecting and cortical collecting ducts (CCD), outer medullary collecting ducts (OMCD), inner medullary collecting ducts (IMCD).

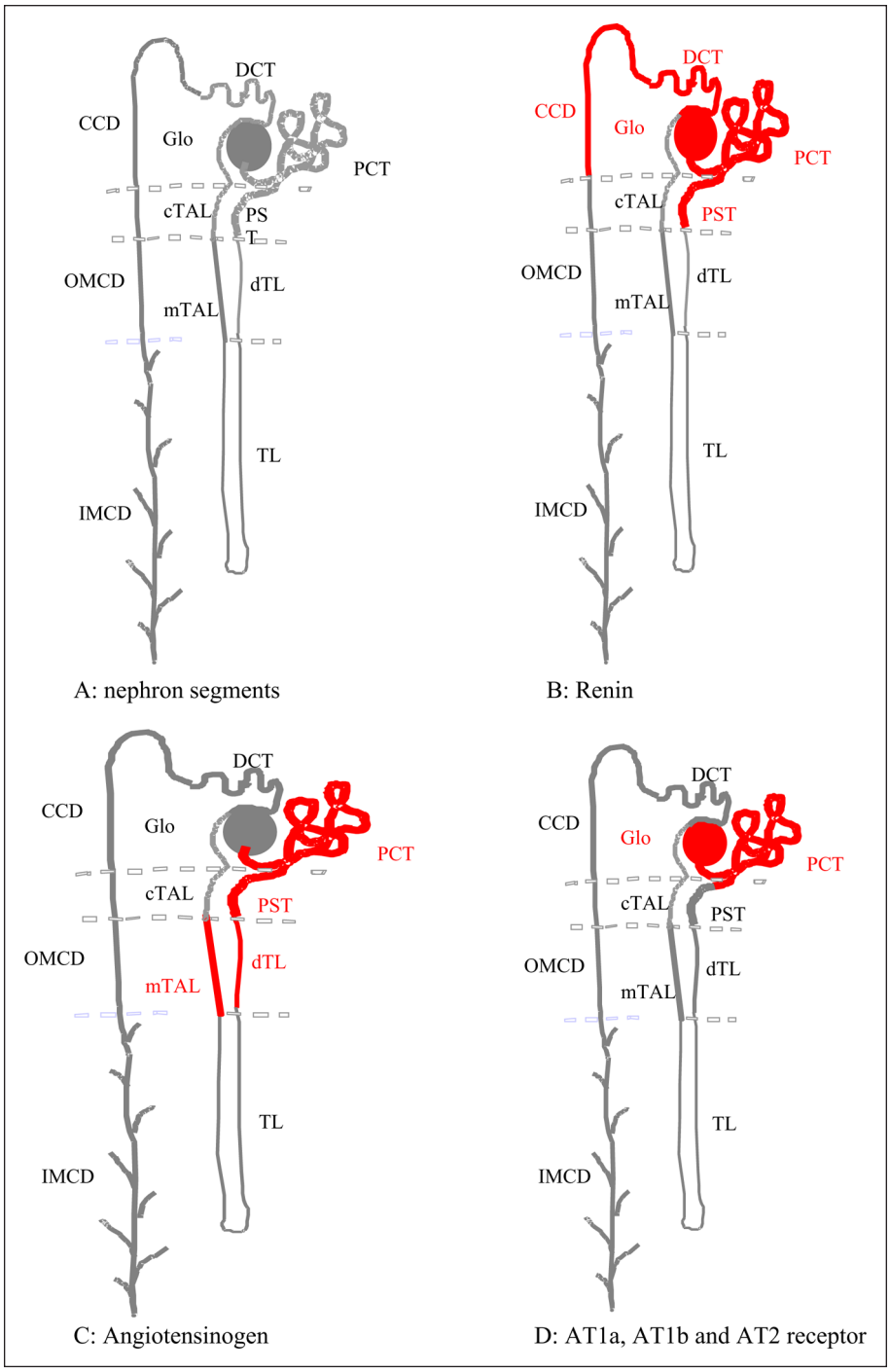

Figure 8. Expression of the renin–angiotensin–aldosterone system along the mouse nephron. A: Localization of the collected nephron segments. B: Renin mRNA was detected in the glomerulus, in proximal convoluted tubules (PCT) and proximal straight tubules (PST) and in the distal nephron (distal convoluted tubules [DCT] and cortical collecting ducts [CCD]). C: Angiotensinogen mRNA was present in PCT, PST, descending thin limb (dTL) and medullary thick ascending limb of Henle's loop (mTAL). D: mRNA of both angiotensin II type AT1 receptors (a and b) and of AT2 receptors was found in glomeruli and PCT.

findings were provided.[24] ANG II levels are high in proximal tubules of rats and mice.[34] If ACE mRNA is really absent in PCT as suggested by our results, the question then may be raised where the origin of this proximal tubular ANG II is located. A conclusive answer was given recently by the verification of AT1 receptor-mediated endocytosis of ANG II in vitro and in vivo using autoradiography in mice.[35,36] Furthermore, differences in animal subspecies

and experimental methods used may contribute to the above discrepancies regarding ACE expression in mouse kidney.

AT1a and AT1b mRNAs were found mainly in glomeruli and PCT in our study, though AT1b was expressed at a very low abundance. This is in accordance with several studies from rat kidneys providing data for the prevalence of AT1 receptors in glomeruli and proximal tubules, [28,37–40] but also in distal tubules,[39] collecting ducts[28,38-40] and in (renal) vasculature.[39,41] In a recent study, AT1R was localized by immunohistochemistry in mouse kidney to glomeruli and proximal tubules, but also faintly to distal tubules and collecting ducts, however no mRNA data to support these tubular findings were provided.[24] Our study revealed a much more restricted expression of AT1a/AT1b gene expression in mouse kidney, but there was also a weak AT1a expression in the DCT. The AT1a gene may be induced, for example, by glucocorticoids[42]. Also age influences RAAS gene expression and localization.[43] Thus the above described differences are possibly due to species differences when comparing mice with rats, but more likely to the specific methodology used, and also the age of the animals may play a role, especially when a RAAS component is expressed at low abundance. The exact localization of angiotensin receptors in the kidney is nevertheless of utmost importance also for the concept where the local RAAS might exert its renal effects.

AT2 receptor mRNA was present in glomeruli and PCT of adult mouse kidneys according to our results. In adult rats, AT2 was immunolocalized under physiological conditions at low level in glomeruli,[38,44] and after sodium depletion, an additional localization in interstitial cells could be shown in association with a more intense staining in glomeruli.[44] An additional staining was present in tubular epithelium in the contralateral (non-ischemic) kidney in the model of 2-kidney 1-clip hypertension.[45] Further insight into AT2 receptor localization was added by AT2 receptor radioligand binding, which was reported in glomeruli and proximal tubules of adult rat kidneys,[46] while AT2 mRNA was detected during nephrogenesis in nephrogenic mesenchyma, but not at all in adult rat kidneys by in situ hybridisation.[47]

Northern blot analysis revealed no AT2 receptor expression in adult mouse kidney under physiological conditions,[48] while mRNA of AT2 was detected in mouse kidneys at a low level using RT-PCR[46] and renal cortical AT2 receptor gene expression has been shown to be induced in the cortex of mouse kidney by ANG II infusions.[49] This finding was further clarified by AT2 radioligand binding in murine cortex mainly related to vasculature[47] and mRNA detection in a murine podocyte cell line,[50] what is in accordance with our results. AT2 receptor knockout mice were reported to lack a diuretic response to an AT2 blocker,[51] also strongly suggesting a tubular expression, as shown by us.

In conclusion, the present study confirmed and extended data of nephron-specific distribution of components of the RAAS derived from studies in rats and mice. All components of the local RAAS – except for ACE – are present in PCT, suggesting an involvement in handling of sodium and water, and presumably also of hydrogen ion and bicarbonate in proximal and / or distal tubule segments.[52–57] AGT secreted from PCT thereby could locally (or in the collecting duct) be converted to ANG I, and further to ANG II via ACE originating from the renal vasculature (or the systemic circulation). ANG II itself affects sodium and volume reabsorption in both proximal and distal tubules and regulates H+-ATPase activity in intercalated cells of the collecting duct.[58]

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Conflict of interest

All authors declare that they have no potential conflict of interest with regard to the present manuscript.

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