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Cellular and ultrastructural location of angiotensinogen in rat and sheep kidney

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Cellular and ultrastructural location of angiotensinogen in rat and sheep kidney. Recent evidence suggests the involvement of a local renin-angiotensin system in some renal actions of angiotensin II (Ang II). In this study the renal distribution of the precursor to angiotensin formation, angiotensinogen, was investigated in rats and sheep using immunohistochemistry, immunoelectron microscopy and non-isotopic hybridization histochemistry. Immunostaining for angiotensinogen was seen in proximal tubules (PCT) of both rat and sheep kidneys. In the rat the strongest immunostaining was found in the kidneys of neonatal (1 day old) rats. Staining declined after birth. Non-isotopic hybridization histochemistry using oligodeoxynucleotide probes labeled with biotin confirmed the presence of angiotensinogen mRNA expression in PCT of the rat renal cortex. Electron microscopic immunohistochemistry using antibodies raised against rat angiotensinogen showed weak staining in the adult of granule-like structures close to the apical membrane of PCT cells. In the neonatal rat kidney, angiotensinogen immunostaining was found throughout the PCT cells and was markedly stronger than that seen in adult rat kidney. In sheep, angiotensinogen immunostaining with an antibody raised against purified ovine angiotensinogen showed staining of PCT in fetal, newborn and adult sheep kidney. The strongest immunostaining seen was in fetal sheep kidney with a decline seen after birth. Reverse transcription polymerase chain reaction (RT-PCR) showed that angiotensinogen mRNA was expressed in the sheep kidney at all ages studied. Angiotensinogen expression was higher in fetal sheep kidneys (77 day and 141 day gestation) than in adult sheep kidney. In conclusion, angiotensinogen mRNA expression was detected in both rat and sheep kidneys. Immunostaining showed angiotensinogen protein in PCT cells of the renal cortex. Angiotensinogen staining and mRNA expression is highest during development and declines in the adult.

There is considerable evidence for an extravascular production of angiotensin II (Ang II) [1]. The existence of an intrarenal renin-angiotensin system is well documented. Ang II generated in the kidney may play a role in mediating several physiological functions including sodium reabsorption, tubuloglomerular feedback and inhibition of renin secretion [2–6]. Locally produced Ang II may also be important in some renal pathologies. The proximal tubule is a site of Ang II action and there is evidence that the PCT may also be a site of Ang II production. Ang II appears to be secreted into the PCT lumen and Ang II concentrations in luminal fluid are markedly higher than plasma levels [7, 8]. It is likely that Ang II in the kidney is generated from locally synthesized angiotensinogen. In the present study we have examined the renal location of angiotensinogen mRNA expression in the rat kidney and the location of angiotensinogen protein at the light and electron microscopic level. We also examined the presence of angiotensinogen protein and mRNA expression in the sheep kidney, including changes during ontogeny. In the sheep, previous reports have suggested that angiotensinogen mRNA is not expressed in the kidney either during fetal life or postnatally [9]. In the present study we have used RT-PCR to detect angiotensinogen mRNA expression in fetal and adult sheep kidney.

Methods

Tissue samples for light microscopic immunohistochemistry were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were incubated in polyclonal antibody raised against purified rat angiotensinogen [10] at a dilution of 1:2000. For ovine angiotensinogen, a polyclonal antibody raised against purified ovine angiotensinogen was used at a dilution of 1:100. Detection of immunostaining was performed using streptavidin-biotin and horseradish peroxidase detection system (Vectastain, Vector Laboratories, Inc. Burlingame, California, USA) and nickel-diaminobenzadine as chromogen. For immunoelectron microscopy, small blocks of cortical kidney tissue were fixed overnight in 2% paraformaldehyde and 0.1% glutaraldehyde then dehydrated through graded ethanols and embedded in LR White (London Resin Co. Ltd., Basingstoke, UK) embedding resin, polymerized for 48 hours at 40°C. The primary antibody was applied to sections at 1:500 and detection was with goat anti-rabbit IgG secondary antibody conjugated to 1 nm gold diluted 1:100. Silver enhancement of staining was performed with IntenseM (Amersham, Buckinghamshire, UK). Negative controls for both light microscopic and electron microscopic immunohistochemistry included incubation in pre-immune serum and preabsorption of the primary antibody with purified rat or ovine angiotensinogen.

Non-radioactive hybridization histochemistry was performed on sections from rat kidneys embedded in paraffin after fixation in 4% paraformaldehyde. Sections were pre-treated with 0.1 N HCl for 15 minutes, proteinase K 25 μg/ml for 30 minutes at 37°C (Sigma, St. Louis, Missouri, USA). Sections were hybridized overnight at 40°C with two oligonucleotide probes internally labeled with four biotins at a concentration of 500 ng/ml. Detection of hybrids was performed with streptavidin biotin alkaline phosphatase and NBT/BCIP. As a negative control, some sections were pretreated with RNase A (Sigma) at a concentration of 1 mg/ml for 30 minutes prior to hybridization with the oligonucleotide probes.

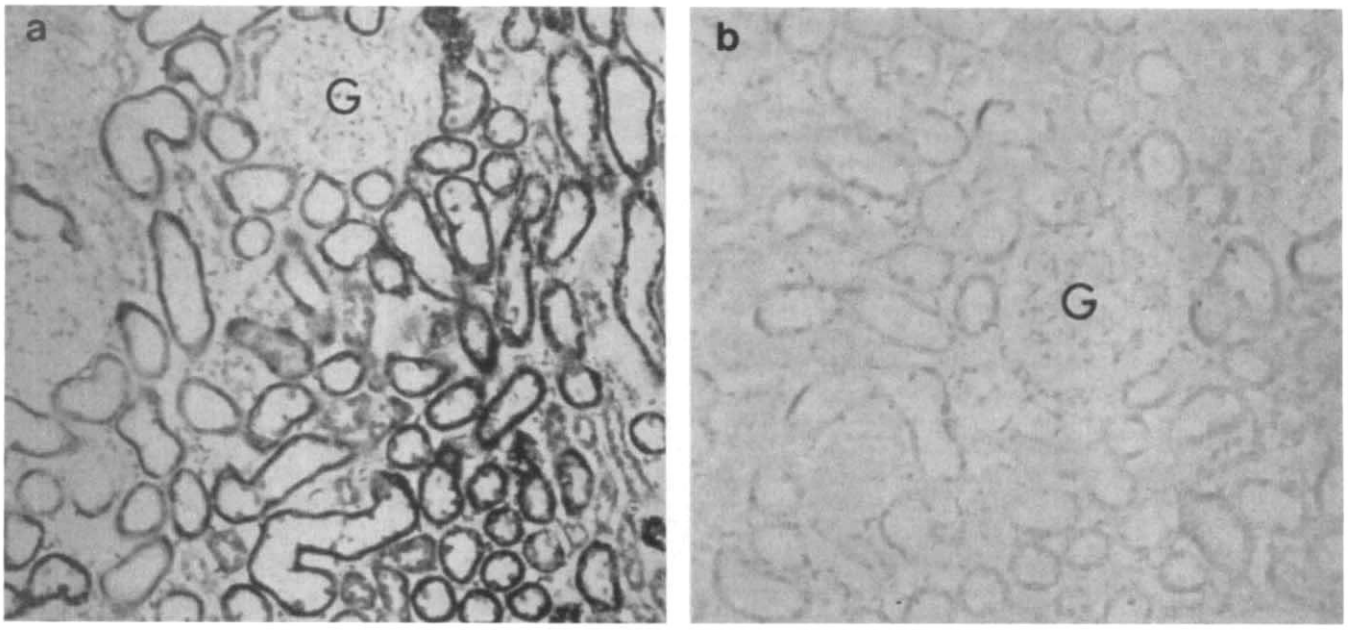

Fig. 1A. *Non-radioactive hybridization histochemistry showing rat renal proximal tubules hybridize with biotinylated oligonucleotide probes for angiotensinogen.* Glomeruli (G) are not labeled. **B**: A section of adult rat kidney pre-treated with RNase A before hybridization shows no hybridization with the angiotensinogen probes. (Magnification ×300).

Reverse transcription-polymerase chain reaction (RT-PCR) was performed on mRNA samples from sheep kidneys and livers of various ages using primers from the ovine angiotensinogen gene sequence. After 40 cycles, reaction products were run on 0.8% agarose gels, Southern blotted and probed with an oligonucleotide probe, specific for ovine angiotensinogen, labeled with γ^{32}P-ATP (Bresatec, Adelaide, Australia).

Results

Rat kidney studies

Non-radioactive hybridization histochemistry using biotinylated oligonucleotide probes showed positive hybridization in the proximal convoluted tubules of the renal cortex. Adjacent sections which were pre-treated with ribonuclease (RNase A) showed no specific hybridization (Fig. 1).

Light microscopic immunohistochemistry showed immunostaining for angiotensinogen in the PCT cells of the renal cortex in neonatal and adult rat kidney. The neonatal rat kidney was more strongly immunostained than the adult kidney (Fig. 2). This difference was reflected in measurements of angiotensinogen protein in kidney homogenates from rats at various ages which showed the highest level of angiotensinogen protein in the neonatal kidney. Angiotensinogen was detectable in the kidney from day 16 of embryonic development and was highest at one day post-natal development (8.49 ± 0.45 μg/mg protein compared to 1.5 ± 0.045 μg/mg protein at embryonic day 20). Angiotensinogen protein content in the kidney declined after birth to 1.0 ± 0.2 μg/mg protein in the adult male rat kidney.

Electron microscopic immunohistochemistry showed few weakly angiotensinogen-immunostained electron dense granules close to the apical membrane of the PCT cells in the adult rat kidney (Fig. 3A). No other structures stained positively for angiotensinogen. In the neonatal rat kidney, immunostaining for angiotensinogen in PCT cells was stronger and more widely distributed, with angiotensinogen-positive granules staining both in the apical part of the cell (Fig. 3B) and close to the basal membrane (Fig. 3C). Pre-absorption of the antibody with purified rat angiotensinogen abolished staining.

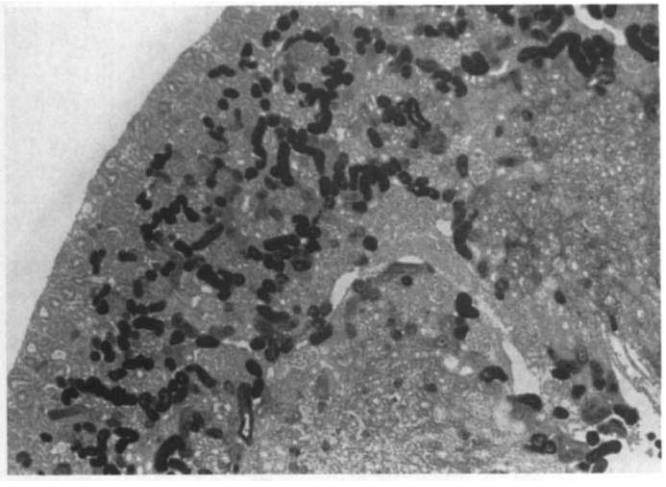

Fig. 2. *Immunohistochemical detection of angiotensinogen in neonatal rat kidney, showing strong staining of proximal tubules in the cortex of the developing kidney.* (Magnification ×40).

Ovine kidney studies

In the ovine kidney, angiotensinogen immunostaining was present in the kidney at all ages which were studied. Fetal kidneys were stained specifically for angiotensinogen from 70 days gestation to 140 days gestation (term = 145–150 days). Angiotensinogen immunoreactivity was confined to tubules of the kidney cortex

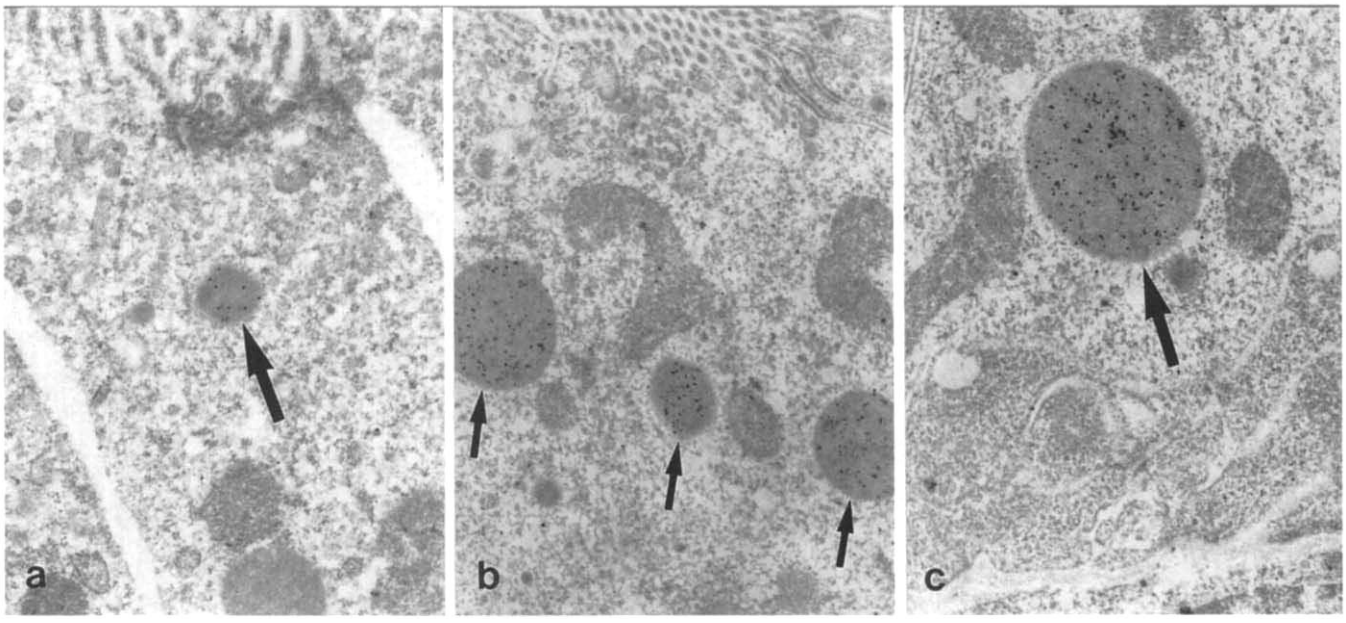

Fig. 3A. *Immunoelectron microscopy showing angiotensinogen staining in electron dense granule (arrowed) close to the apical membrane in adult rat kidney proximal tubule cell.* (Magnification ×20,000). **B.** Immunoelectron microscopy showing strong angiotensinogen staining of electron dense granules (arrowed) in proximal tubule cells of neonatal rat kidney close to the apical membrane (Magnification ×12,700). **C.** Angiotensinogen immunostaining in neonatal rat kidney was also located close to the basal cell membrane. (Magnification ×14,600).

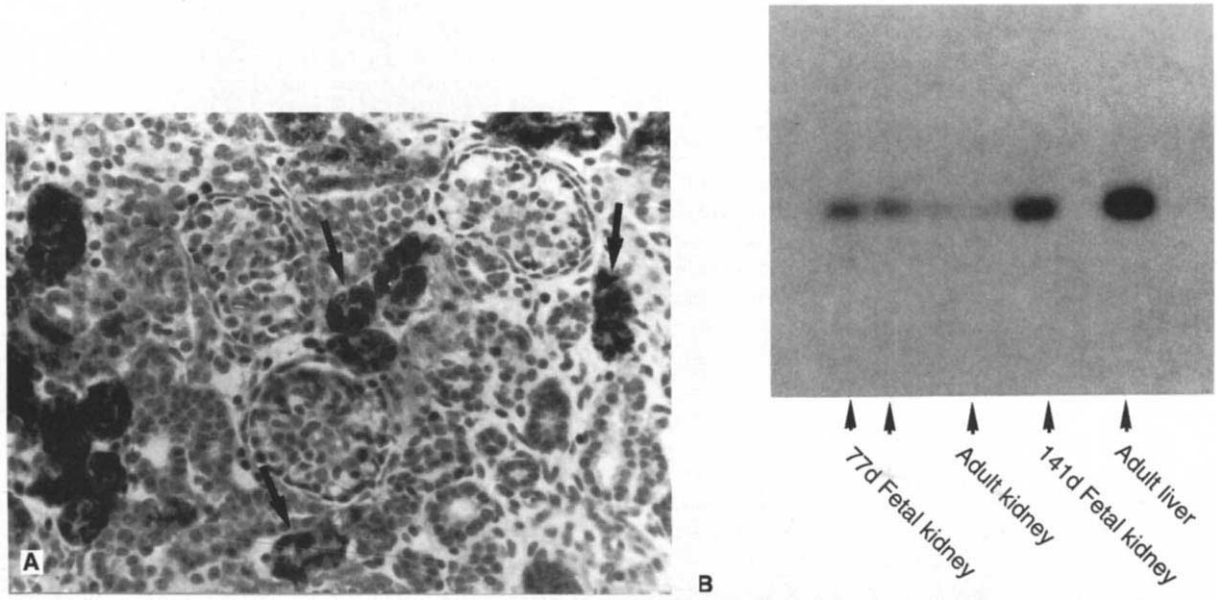

Fig. 4A. *Angiotensinogen immunostaining of a section of 77-day-old fetal sheep kidney, showing strong immunostaining of cortical proximal tubules.* (Magnification ×200). **B.** Reverse transcription PCR (40 cycles) probed with an angiotensinogen specific oligonucleotide probe after Southern blotting, shows visible bands in kidneys from all ages studied and from adult sheep liver. The mRNA levels for angiotensinogen appear strongest during fetal development in the kidney.

in fetal sheep kidney sections (Fig. 4B). The strongest immunostaining in the kidney was seen during fetal development and staining declined after birth, with the adult kidney staining weakly for angiotensinogen. RT-PCR studies showed that angiotensinogen mRNA was expressed in the kidney at all stages studied. The level of angiotensinogen mRNA expression in the kidney appeared to be less in the adult compared to fetal ages 77 days or 141 days (Fig. 4A).

Discussion

Intrarenally generated Ang II may play a role in multiple functions in the kidney [2–6]. The PCT is a site of Ang II action

and a probable site of local production of Ang II. Angiotensinogen protein and mRNA expression have been reported in PCT [11, 12], and may be regulated by Na [13] and by Ang II [14]. ACE is present on the brush border of PCT cells [15] and there is evidence of release of Ang II into PCT fluid as concentrations of Ang II in tubular fluid are markedly higher than plasma levels [7, 8]. There have also been reports of local expression of renin mRNA [16] and release of renin like activity by PCT cells in culture [17]. It has also been reported that angiotensinogen and renin immunoreactivity are co-localized in PCT cells of rat kidney [18]. However, the presence of renin mRNA expression in PCT needs to be confirmed by *in situ* hybridization histochemistry.

In the present study we have shown mRNA expression and immunostaining for angiotensinogen in both the rat and sheep kidney. It is possible that in the adult kidney, presence of angiotensinogen protein in PCT cells results at least partially from uptake of the protein from the tubule lumen. In the neonatal rat and fetal sheep kidney, there was stronger cytoplasmic staining for angiotensinogen. In the rat, renal development is still occurring in early neonatal life [19]. In the sheep, renal development is largely complete at birth. It has been reported previously that angiotensinogen mRNA expression was not found in the sheep kidney during development or postnatally [9]. The present findings may differ from this previous study because of the increased sensitivity of the RT-PCR technique compared to Northern blotting. In both species we saw more abundant mRNA expression and stronger staining for angiotensinogen in the kidney during development. The presence of increased angiotensinogen mRNA expression and protein during development may reflect a role for locally generated Ang II in growth and differentiation of the developing kidney. Ang II is mitogenic to several cell types and has been shown to promote hypertrophy of PCT cells in culture [20]. In addition, the blockade of Ang II by ACE inhibition or angiotensin receptor antagonists during early postnatal development in the rat results in renal malformations including abnormal tubule development and impairment of renal function [21, 22]. In conclusion, circumstantial evidence would support a role for Ang II in renal development. Locally synthesized angiotensinogen and the regulation of this synthesis may be an important factor in regulating development.

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