Fetal rat kidney contains renin in renal microvasculature, whereas adult rat kidney contains renin predominantly in juxtaglomerular cells. It is hypothesized that renin isoforms stored within these renal tissues may differ chemically and functionally. To test this hypothesis, stored renin isoforms in fetal and adult rat kidney were compared by isolating renin from adult and fetal kidney homogenate with pepstatin agarose. Pepstatin-eluted renin isoforms were separated by relative molecular size using one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE), or by isoelectric point (pI) and size using two-dimensional (2D) gel electrophoresis. Isoforms were identified either by silver staining or immunoblotting. One-dimensional polyacrylamide gel electrophoresis of pepstatin-treated kidney homogenates showed a silver-stained band in the range of ~45 kDa, which corresponded to a silver-stained spot consistently seen on 2D gels. In fetal kidney homogenate, the ~45 kDa band had a pI of 5.3 ± 0.1, whereas the corresponding band in adult samples had a basic pI of 6.0 ± 0.05. Angiotensin I generation was measured to assess renin enzymatic activity. There was significantly more inactive renin in fetal kidney homogenate than in adult kidney homogenate (60.2 ± 22.4 vs. 9.6 ± 4.0 ng AI/mg protein/h, P < .05). There was significantly less active renin in fetal kidney homogenate than in adult kidney homogenate (5.4 ± 0.4 vs. 36.5 ± 14.2 ng AI/mg protein/h, P < .05). The average total renin activity in fetal kidney homogenate was significantly higher than in adult kidney homogenate (65.6 ± 22.3 vs. 46.0 ± 15.2, P < .05). These results demonstrate major differences in the physical and enzymatic forms of stored renin found in fetal and adult kidney. It is speculated that these variations in stored renin isoforms play a role in the developmental differential regulation of the intrarenal renin angiotensin system. Am J Hypertens 1998;11:213–218 © 1998 American Journal of Hypertension, Ltd.

KEY WORDS: Renin angiotensin system, fetal, renin, isoforms, rat kidney.
Biochemical and molecular evidence suggest that renin is heterogeneous, existing in multiple forms.7–10 Studies of this heterogeneous enzyme have resulted in inconsistencies in reported renin isoform numbers. For example, up to six renin isoforms and three to five renin glycoforms have been defined biochemically by differing isoelectric points and lectin binding affinities, respectively.8,11,12 These differences in reported renin isoform number may result from variations in intracellular processing of the enzyme and other extracellular factors. Upon reviewing such extracellular factors as source, species, treatment, and isolation technique, which may affect reported isoform numbers, it appears that isolation technique is the single most significant extracellular factor responsible for variations in reported isoform number.7,12–22

Site-directed mutagenesis of renin glycosylation suggests that the carbohydrate composition of renin influences release and secretion of this enzyme in transfected cells.10 Accumulating evidence supports the concept that pathophysiologic and pharmacologic changes in the renin angiotensin system are accompanied by changes in expression of the renin isoforms,7,23,24 suggesting that renin isoforms may play a role in the pathogenesis of hypertension and other diseases.

Increases in renin mRNA and protein levels, plus the greater distribution of renin in the fetal kidney, raise the possibility that the expression and function of renal renin isoforms might be influenced by development. To address this issue, renal renin isoforms were characterized in fetal and adult Sprague-Dawley rat kidney.

**METHODS**

**Animals** Timed-gestation pregnant female or adult male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Kingston, NY) and received a diet of Purina Rat Chow 5001 ad libitum plus unrestricted access to water. Kidneys were removed from 20-day gestation fetuses or adult male rats under anesthesia with intraperitoneal sodium pentobarbital, 50 mg/kg. Studies were conducted according to NIH guidelines for the care and use of laboratory animals under protocols approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital.

**Tissue Preparation** Immediately after nephrectomy, kidneys were placed in phosphate buffered saline (PBS), pH 7.4, at 4°C. Phosphate buffered saline was decanted from kidneys, which were then rinsed in ice-cold homogenization solution (5% glycerol, vol: vol, 10 mM ethyleneaminediethaetraacetic acid (EDTA), 0.1 mM phenylsulfonyl fluoride (PMSF), 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF)). Adult kidneys were homogenized at 4°C for 30 sec in 5 volumes (wt:vol) of homogenization solution (Polytron, setting 6; Brinkmann, Lucerne, Switzerland). Fetal kidneys, pooled from 10 to 15 fetuses, were suspended in 1 mL homogenization buffer and homogenized identically as for adult rat kidneys.

The kidney homogenates were centrifuged for 5 min at 4°C at 14,000 g (Microcentrifuge, model 235A; Fisher Scientific, Pittsburgh, PA). The supernatant of kidney homogenate was used for isolation and characterization of renal renin isoforms.

**Renin Isolation** Aliquots of supernatant from kidney homogenate were analyzed for protein concentration using the BioRad protein assay system (BioRad Laboratories, Hercules, CA). Equal amounts of supernatant protein (generally 200–400 mg) were combined with 100 μL of pepstatin agarose suspension (Sigma Chemical Co. Inc., St. Louis, MO) in PBS, pH 7.4, containing 10 mM EDTA, 1% Triton×100 (vol:vol), 0.1 mM AEBSF, and 60 mg/mL PMSF (Triton-PBS buffer) in a total volume of 500 mL, which was then incubated with shaking for 1 h at 4°C. After incubation, the pepstatin agarose was washed twice with Triton-PBS buffer and once with distilled water. As previously described, renin was eluted from the pepstatin agarose in a denaturing STOP solution, followed by heating to 95°C for 5 min.24

**Identification of Renin Isoforms** Renin was separated by relative molecular size in 10% SDS-PAGE gels.24 The protein was identified by silver staining or by immunoblotting with a synthetic peptide antibody recognizing rat renin (1:100 dilution), as previously described.24,25 Renin isoforms were identified by 2D gel electrophoresis under denaturing conditions, as previously described.24 Briefly, pepstatin-bound proteins eluted in Anderson’s buffer (2.7% SDS [wt/vol], 6.7% 2-mercaptoethanol [vol/vol], and 13.4% glycerol [vol/vol]) were combined with urea (final concentration 3% [wt/vol]) and ampholines (final concentration 2% [vol/vol]) and applied to isoelectric focusing tube gels. The gels contained ampholines of pH 4 to 6.5 (the isoelectric point range in which renin isoforms were expected to be identified), or pH 2 to 5 and pH 7 to 9 (isolectric ranges below and above the expected isoelectric point range for renin isoforms). After focusing overnight at 15°C and 750 V, samples were focused for 1 h at 15°C and 1000 V, which separated protein in the samples by differences in isoelectric point. Tube gels were then applied to 10% polyacrylamide slab gels, where samples were further separated by relative molecular size. Renin isoforms of 2D gels were identified by silver staining (BioRad Laboratories, Hercules, CA).

**Renin Enzymatic Activity** Renin enzymatic activity was measured in samples of kidney homogenate, as
previously described.²⁴ Briefly, kidney homogenate was combined with an excess of partially purified rat angiotensinogen at pH 6.2 and incubated for 1 h at 37°C. Total renal renin content was determined by incubating some samples with trypsin crosslinked to agarose for 30 min at room temperature to activate inactive renin, before incubation with angiotensinogen. Trypsin agarose was removed by centrifugation. Angiotensin I was measured using a commercial radioimmunoassay kit for Angiotensin I (New England Nuclear, Boston MA).

Data Analysis Data represent the mean plus or minus SEM. Analysis of variance and z test for comparison of the mean were used for statistical comparison of renin enzymatic activity. Statistical significance was defined as $P < .05$. In renin enzymatic assays, $n = 4$ for both adult and fetal samples, which were tested in duplicate. For 2D gel electrophoresis pH gradient 4 to 6.5, 6 adult samples and 5 fetal samples were analyzed. For pH gradient 2 to 5 studies, three fetal and two adult samples were analyzed. All 2D gels were run in duplicate. For SDS-PAGE gels, four to six adult and fetal samples were tested.

RESULTS

Fetal and Adult Rat Kidney Renin When equal concentrations of fetal and adult kidney homogenate protein were incubated with pepstatin agarose to bind renin, differences in the amount of eluted proteins were evident (Figure 1). Silver-stained, SDS-PAGE gels of the pepstatin-eluted proteins showed that fetal rat kidney contained significantly more of an ~45-kDa protein than adult rat kidney samples ($19 \pm 4 \times 5 \pm 1$ arbitrary densitometric units, respectively, $P < .01$) (Figure 1A). This ~45-kDa band was identified as renin in immunoblots of fetal and adult kidney homogenates using a polyclonal antibody specific for rat renin (Figure 1B). The intensities of the immunoreactive band were similar in fetal and adult kidney homogenates. This may be a characteristic of the immunoblotting conditions, which clearly detected renin but did not quantify it. Alternatively, incubation of kidney homogenates with pepstatin agarose may have provided an enrichment of renin that was greater in fetal samples.

Two-Dimensional Gel Separation of Fetal and Adult Renin Isoforms Characterization of isoelectric point (pI) and relative molecular size (MW) of fetal and adult rat kidney renin isoforms was done using 2D gel electrophoresis. In a pH gradient of 4 to 6.5, fetal samples had an ~45-kDa acidic band, pI 5.3 ± 0.1 (Figure 2A). In corresponding adult samples, this ~45-kDa band had a pI of 6.0 ± 0.05 (Figure 2B). When fetal samples were focused in a 2–5-pH gradient, the fetal isoforms were identified again with a MW of ~45 kDa and pI of 5.2 ± 0.1 (Figure 3A). In adult samples focused in a 2–5-pH gradient, no ~45-kDa isoforms were identified (Figure 3B). When fetal and adult samples were focused in a basic pH gradient of 7 to 9, no renin isoforms were identified (data not shown).

Renin Enzymatic Activity Renin enzymatic activity measured in fetal and adult rat kidney homogenates revealed some significant differences (Figure 4). There was significantly less active renin in fetal kidney homogenates compared with adult kidney homogenates. However, fetal kidney had significantly more trypsin-activated renin than adult rat kidney. Total renin activity in fetal kidney homogenates was significantly greater than in adult rat kidney homogenates.

DISCUSSION

Our results show for the first time a significant developmental difference in the expression of renin isoforms in the rat kidney. Fetal renin isoforms are more acidic than adult renin isoforms. The fetal kidney also has more total renin and inactive renin, but less active renin, than adult rat kidney.

Renin mRNA levels and immunohistochemical staining patterns have been shown to change during development.¹⁻⁴ Specifically, fetal kidney contains more renin mRNA than adult kidney.³⁻⁴ The immunohistochemical staining and in situ hybridization for renin in fetal and newborn kidney show that a greater portion of the protein and message are localized in the renal vasculature, in addition to juxtaglomerular cells, than is seen in the adult.¹⁻⁴ This pattern changes during maturation, so that in the adult rat kidney renin message and protein are localized primarily in juxtaglomerular cells.¹⁻⁴ The physiologic significance of these differences is hypothesized to relate to changes in renal blood flow and renal function in the maturing rat kidney.⁵⁻⁶
Developmental changes in stored and circulating renin have been reported by several investigators. There is a consensus of reports showing increased plasma renin activity and secreted renin in late-fetal and newborn mammals, including humans, compared to adults. Fetal kidney renin content varies with fetal age. Near-term fetal kidneys contain more renin protein than early fetal and adult kidney. The enzymatic activity of stored fetal renal renin has not been consistently evaluated and compared to adult stored renal renin. Our data suggest that there are significant differences in the enzymatic activity of fetal and adult stored renal renin. The fetal kidney has significantly more stored total renin activity compared to the adult kidney. However, a significantly greater fraction of stored fetal renal renin is enzymatically inactive and the fraction of active stored fetal renal renin is significantly lower compared to adult renal renin.

FIGURE 2. Representative silver stain of a 2D gel for pepstatin-treated fetal (A) and adult kidney homogenate (B). Isoelectric focusing was in pH gradient 4 to 6.5. Samples were normalized to contain equal amounts of total protein for incubation with pepstatin. Equal volumes of pepstatin-eluted protein were applied to gels.

FIGURE 3. This figure is a representative silver stain of a 2D gel for pepstatin-treated fetal (A) and adult kidney homogenate (B). Isoelectric focusing was in pH gradient 2 to 5.
adult kidney. This finding is supported in part by renin secretion studies from fetal ovine kidney slices, which showed that a greater amount of inactive renin was released from fetal ovine kidney compared to adult.

Renin is recognized to be an isoenzyme. Three glycoforms of rat renin have been described by differences in lectin affinity. Up to six enzymatically active isolectric forms of stored and secreted rat kidney renin have been found when native samples were focused in shallow isolectric gradients. Under denaturing conditions, our 2D isolectric focusing techniques reveal two different renin isoform bands. The bands have a similar estimated MW of ~45 kDa, but there is a difference in pI when comparing fetal and adult samples. The bands focus diffusely and, because of the small amount of renin in the samples being separated, it has not been possible to resolve individual renin isoforms within a given band.

Although we have described developmental biochemical differences in renal renin isoforms, this may have physiologic significance for the developing cardiovascular and renovascular systems. Reported examples of possible physiologic actions of renin isoforms include the observation that there is preferential release and clearance of the glycosylated renin isoforms found in rat kidney. This unique release and clearance of the glycosylated renin isoforms include the observation that there is preferential clearance of the glycosylated forms of renin in the developing rat kidney to perform physiologic studies has not been done and should be pursued in future developmental studies.

In conclusion, fetal rat kidney renin isoforms in the vasculature may vary from renin isoforms found in other cellular locations within the adult kidney. This concept is supported by our present findings showing that the isolectric points of fetal and adult renin are different. In addition, there is a difference in renin enzymatic activity in fetal rat kidney, which contains significantly more total renin and inactive renin, but less active renin, than the adult rat kidney. We speculate that differences in posttranslational modifications of fetal renin may be responsible for such differences.

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