Developmental changes in renal renin mRNA half-life and responses to stimulation in fetal lambs

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Wang, J injuan, and J ames C. Rose. Developmental changes in renal renin mRNA half-life and responses to stimulation in fetal lambs. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1130–R1135, 1999.—In the perinatal period there is increased renin gene expression in the kidney compared with other stages of development. This may be related to changes in responsiveness of the renin gene to stimulation and/or differences in renin mRNA stability as development progresses. To ascertain if either responsiveness or stability changes in fetal life, we studied renin mRNA levels in primary cultures of renal cortical cells obtained from fetal lamb kidneys at two stages (0.7 and 0.9) of gestation after stimulation with isoproterenol, forskolin, or isobutyl methylxanthine and after inhibition of transcription with actinomycin D. Forskolin and isobutyl methylxanthine rapidly increased renin mRNA by at least twofold in the cultured cells from fetuses of both ages, with the sensitivity to stimulation higher in the cells from the mature fetal kidneys. Isoproterenol was effective only in mature fetal cells. In addition, the decay of renin mRNA after cessation of transcription was slower in mature cells compared with immature cells, the half-life being 11.6 ± 0.8 h in mature cells and 6.6 ± 0.6 h in immature cells (P < 0.05). The data suggest that increases in both renin mRNA sensitivity to stimulation and in stability can contribute to the enhanced renin expression in the perinatal period.

renin gene expression; renin gene stability; adenosine 3′,5′-cyclic monophosphate; actinomycin D; mature and immature kidney cortical cells

The renin-angiotensin system (RAS) plays a key role in the control of blood pressure, renal hemodynamics, and fluid and electrolyte homeostasis in adults (15). In the developing animal its role may be expanded to include modulation of renal growth and angiogenesis (13, 14). In adult animals, renin and synthesis secretion are regulated by a complex array of factors, including 1) vascular stretch (renal perfusion pressure); 2) sodium chloride concentration at the macula densa; and 3) renal sympathetic, especially b-adrenergic, tone (7, 15). The final common effector for all of these factors is probably intracellular cAMP, because both renin gene expression and renin secretion are increased when intracellular levels of this second messenger increase (7, 15, 18).

During fetal life, activity in the RAS changes markedly, with renin mRNA and protein levels in the kidney cortex increasing to peak in the perinatal period (3, 28, 35, 37). Although the full explanation for this maturational change in renin expression is unknown, adrenergic input may be involved. Studies in fetal animals have shown that the renal nerves are active during fetal development (21, 29, 33), and renin secretory responses to b-adrenergic stimulation are developmentally regulated, with greater responses observed in the perinatal period than earlier (22, 23, 26, 27). Moreover, denervation of the fetal kidneys abolishes the substantial increase in renin mRNA expression associated with birth (25). Thus it is possible that the increase in renin expression seen in the fetal kidney in late gestation may be related to differences in renin gene responses to b-adrenergic stimulation. Alternatively, considering that cellular mRNA levels are determined both by the rate of nuclear gene transcription and by the rate of mRNA degradation (30), developmental differences in renin mRNA stability may also factor into the increase in renin expression close to term. Therefore in the present studies, we compared renin gene responses to stimulation in dispersed renal cortical cells obtained from fetuses at two stages of development to determine if age-related differences exist. We also compared the disappearance of renin mRNA in immature and mature fetal kidney cells after blockade of transcription by actinomycin D to establish if there are developmental changes in renin gene stability in the ovine fetus.

MATERIALS AND METHODS

Preparation of fetal sheep kidney and culture of renin-containing cells. All the procedures used on the animals were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine. Two groups of ovine fetuses, 0.69–0.72 (immature fetuses) and 0.93–0.97 (mature fetuses) gestation, were used for this study. Term is ∼145 days of gestation in our flock. Pregnant ewes were anesthetized with ketamine (10 mg/kg) and pentobarbital sodium (0.6 mg/kg) and ventilated with halothane in oxygen. Fetuses were delivered through an abdominal hysterotomy and given a lethal dose of pentobarbital sodium (85 mg/kg) intravenously, and the kidneys were quickly removed. Cortical tissue was separated from medulla, and a fraction of renal cortical cells enriched with renin-containing cells was prepared as described previously (35). In brief, fetal kidney cortex was minced with two surgical blades, then incubated in dissociation buffer (in mM: 130 NaCl, 5 KCl, 2 CaCl2, 10 glucose, 20 sucrose, and 10 Tris, pH 7.4) with 0.1% collagenase for three 30-min periods at 37°C. The dispersed cells were harvested after each 30-min incubation, and fetal calf serum was added to the cell suspension to a final concentration of 20%. When all three incubations were finished, the dispersed cells from each incubation were pooled and filtered through 20-µm nylon mesh. Single cells were collected, washed, and reresuspended in

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dissociation buffer and then further separated using Percoll density gradient. Usually, cells from one fetal kidney cortex were mixed with 120 ml 40% isosmotic Percoll, and then divided into four tubes each containing 30 ml 40% Percoll-cell suspension. After 25 min of centrifugation at 4°C and 27,000 g, three cell layers were obtained. The second cell layer was collected, washed and resuspended in culture medium (RPMI 1640) containing 0.66 U/ml insulin, 100 U/ml penicillin, 100 U/ml streptomycin, and 2% fetal calf serum. Then cells were plated onto standard plastic tissue culture plates or flasks at an average density of 1 \times 10^5 cells/cm^2 and cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Experimental protocols. All experiments were conducted after the renal cortical cells were cultured for 20 h. At that point, medium was removed and replaced with serum-free culture medium containing vehicle or drug. Studies examining the effects of isoproterenol and forskolin included time course and dose response experiments, whereas those with 3-isobutyl-1-methylxanthine (IBMX) only included time course studies. In the time course studies (n = 6, each age group), renal cortical cells were exposed to vehicle, isoproterenol (10^{-7}, 10^{-8}, or 10^{-9} M), forskolin (5 \times 10^{-5}, or 10^{-4} M), or IBMX (10^{-4} M) for 2, 4, 8, and 24 h, respectively. In the dose response studies (n = 6, each age group), renal cortical cells were incubated in media with vehicle, isoproterenol, or forskolin (10^{-7}–10^{-4} M) for 2 h. The experiments examining the disappearance of renin mRNA (n = 9, mature fetuses; n = 6, immature fetuses) were done by adding media with 5 µg/ml actinomycin D to culture plates and incubating them for 0, 2, 6, and 11 h. At the end of each experiment, the cells were harvested and total RNA was extracted. Renin mRNA was measured using RNase protection assay. Total RNA isolation. Cells were harvested and total RNA was extracted by adding Trizol reagent (GIBCO, Grand Island, NY) to the culture dishes (3 ml/75 cm^2). The purified total RNA was dissolved in RNase-free water, quantitated, and then stored at -70°C for renin mRNA detection.

Preparation of ovine renin probe and renin mRNA detection. To generate ovine renin sense and antisense RNA by transcription in vitro, we constructed a plasmid (pgEM-RW1) by inserting an ovine renin cDNA fragment into the vector pGEM-7Zf(+) according to the published ovine renin cDNA sequence. Then, an 870-bp ovine renin cDNA fragment was prepared by RT-PCR amplification using these primers and total RNA from mature ovine fetal kidney cortex. After checking the sequence, this cDNA fragment was subcloned into the pGEM-7Zf(+)- vector (Promega, Madison, WI). Renin sense RNA (870 bp) was made using T7 RNA polymerase and Cla I linearized plasmid. Renin antisense RNA probe (262 bp) was made using SP6 RNA polymerase and EcoRI linearized plasmid according to protocols described in literature provided with the kit from Promega.

Renin mRNA was quantitated using the RNase Protection Assay Kit II (Ambion, Austin, TX). Total RNA extracted from samples (20 µg) or standard renin sense RNA (40, 20, 10, 5, or 1 pg) was hybridized to 32P-labeled ovine renin antisense probe (1 \times 10^6 cpm, specific activity -5 \times 10^8 to 1 \times 10^9 cpm/µg) at 45°C overnight and then digested with RNase A/T1 cocktail at 37°C for 30 min. The protected fragments were precipitated with RNase inactivation/precipitation solution and separated on a 5% urea denaturing gel. Signals in the gel were visualized by autoradiography and quantitated by a densitometer. For quantitation of renin mRNA levels, a standard curve was made by plotting the optical density values of the signals versus the concentration of renin sense RNA (Fig. 1), and the renin mRNA in the samples was estimated using the regression expression from the standard curve. An aliquot of a pool of fetal kidney RNA, which was stored at -80°C, was analyzed in each assay to provide an internal control for the RNase protection assay. The coefficient of variation for the RNase protection assay was 8.6 ± 0.6%.

Data analysis. The data are presented as the means ± SE. To assess the overall effects of treatments within and between groups, the ratio of the hybridization signals in the treated versus control samples were compared by ANOVA. Subsequently, specific differences were established by simple main effects or Newman-Keuls test where appropriate. To ensure homogeneity of variance, the data were transformed (log) before analysis.

The first-order decay kinetics of renin mRNA were used to determine the renin mRNA half-life. The half-life for each animal in both groups was calculated, and the groups were compared by t-test. Differences between groups were considered significant when P < 0.05.

RESULTS

Basal levels of renin message. There was an age-related increase in steady-state levels of renin mRNA in renal cortical cells obtained from immature and mature fetuses. The renin mRNA was greater in mature than in immature cells (3.3 ± 0.4 vs. 1.6 ± 0.5 pg/20 µg total RNA, P < 0.01, t-test).

Time course studies. Isoproterenol (10^{-7} M) increased the hybridization signal ratio in the cultured renal cortical cells (Fig. 2), and there was a significant interaction between age and incubation time (F = 3.2, P < 0.04). There was no effect of time after treatment in the immature cells, but there was a decline in the ratio in the mature cells (F = 3.6, P < 0.03). At 2 h after treatment with isoproterenol, the hybridization signal ratio was greater in the mature than in the immature cells (F = 9.6, P < 0.004), which indicates that the renin mRNA response was greater at this time in the mature cells.
The changes in hybridization signal ratio with time after IBMX treatment are shown in Fig. 3. There were significant effects of age ($F = 10.5, P < 0.02$) and time ($F = 5.5, P < 0.01$). At both ages there was a marked increase in signal at the earliest time point (2 h) examined. The ratio increased further in the mature cells at 4 and 8 h, but after 24 h had returned to levels observed at 2 h. In the immature cells, the ratio peaked at 8 h and then declined to levels similar to those found after 2 h. Thus a phosphodiesterase inhibitor increased renin mRNA levels in cells obtained from mature and immature fetal kidneys.

Forskolin (Fig. 4) also increased the hybridization signal ratio quickly in renal cortical cells from both groups ($F = 16.8, P < 0.01$), i.e., there was an effect of time after treatment in both groups. There was also a significant interaction between gestational age and time after treatment ($F = 3.4, P < 0.04$). At the 4-h time point, the ratio was higher in mature cells compared with immature cells ($F = 5.1, P < 0.03$). The results indicate that direct stimulation of adenylyl cyclase can increase renin mRNA in cells from immature and mature fetuses. They also suggest a potential increase with gestation in the ability of the renin gene to respond to direct stimulation of cyclase.

Dose-response studies. Figure 5 shows a representative autoradiogram of a ribonuclease protection assay of renin mRNA in forskolin-treated cells from mature and immature kidneys. There was an increase in the hybridization signal with treatment. The response to forskolin was dose related ($F = 3.32, P < 0.04$) and was greater in the mature cells at 1 and 10 µM concentrations of the drug ($F = 6.7, P < 0.02$, Fig. 6). Isoproterenol produced a dose-related increase in the hybridization signal ratio in the mature cells only ($F = 4.62, P < 0.01$), and the ratio was greater at the two highest concentrations of isoproterenol in the mature compared with the immature cells (Fig. 7).

mRNA stability. After treatment of cells with actinomycin D there was a decline in the hybridization signal ratio (Fig. 8) at both ages ($F = 56.4, P < 0.01$). There was a greater decline in the signal from RNA extracted from immature than mature cells at all time points ($F = 11.5, P < 0.01$). The calculated half-life of renin message was shorter in immature than mature cells ($6.0 \pm 0.6$ vs. $11.6 \pm 0.8$ h; $P < 0.01$).
DISCUSSION

In the present work we examined potential mechanisms by which fetal renal renin mRNA levels are regulated and compared responses in primary cultures of renal cortical cells obtained at two gestational stages to identify possible explanations for the enhanced renin expression observed in the perinatal period. We employed a modification of methods previously used for dispersing (5, 11, 19) and preparing primary cultures (19) of adult mouse and rat juxtaglomerular cells and obtained a relatively pure population of renin-containing cells from the fetal renal cortex for the studies. Our results suggest there are at least two ways that the steady-state levels of renin mRNA are increased in the fetus as gestation proceeds: first, gene responsiveness to cAMP stimulation can increase and, second, renin gene stability can increase. Thus the increase in renal renin mRNA levels observed in the perinatal period may result from both increased transcription and decreased message degradation.

The stimulatory effect of β-adrenergic agonists on renin secretion is widely accepted (15) and there are data from adult animals indicating that stimulation in vivo with large doses of isoproterenol increases the level of renin mRNA found in the kidney (10). In vitro there is also evidence indicating that stimulation of β-receptors on juxtaglomerular cells from adult kidneys increases renin mRNA levels (8). The present data show that the ability of a β-adrenergic stimulus to increase renin mRNA levels is developmentally regulated. Thus, although no response to isoproterenol was observed in immature fetal kidney cells, in kidney cells from mature fetuses there was a dose-related increase in renin mRNA levels occurring within 2 h of adding the drug to the cells in culture. This lack of responsiveness in the immature cells may represent some deficit at the level of the β-receptor, inadequate activation of the appropriate signal transduction cascade, or the presence or absence of various transcriptional control factors. Both positive and negative regulatory elements have been identified on the renin gene (2, 24), and, although it seems unlikely that they change during development, the factors interacting with them could.

The intracellular mediator of the β-adrenergic-induced increase in renin mRNA is thought to be cAMP (1, 8, 11). Therefore, we used two agents that bypass the β-receptor to elevate intracellular cAMP in juxtaglomerular cell cultures to determine if the age-related differences in renin gene responsiveness are possibly related to some alteration in the cellular response downstream of the receptor. Forskolin, which directly activates adenylyl cyclase (31), and IBMX, which inhibits cAMP degradation, both rapidly increase the levels of renin mRNA found in the isolated renal cortical cells from both immature and mature fetal kidneys. Thus the renin gene appears capable of responding to elevations in cAMP even early in development. Although both of these agents increased renin mRNA levels in cells at both ages, the agonist isoproterenol was effective only in mature cells. This suggests that part of the reason renin mRNA sensitivity to adrenergic stimulation is attenuated in immature cells is related to a real or functional deficit in β-receptors. Although there was
about a doubling of renin mRNA within 2 h of treatment with either forskolin or IBMX, the dose-response study with forskolin revealed a significant effect of age with greater sensitivity found in the mature cells. This age effect is consistent with the dose-response data with isoproterenol and indicates an increase in sensitivity of the renin gene to stimulation in mature fetal kidney cells. Sensitivity in this instance is defined as the lowest dose that produces a detectable response.

Others have shown that forskolin can increase renin mRNA in adult mouse juxtaglomerular cells and rat kidney microvessels (8, 9, 11). Isoproterenol has also been reported to rapidly increase (8) renin mRNA in mouse juxtaglomerular cells. The fact that in the time course studies isoproterenol, forskolin, and IBMX all produced a prompt elevation in renin mRNA levels in mature fetal kidney cells suggests that this rapid response represents a cAMP-mediated increase in renin gene transcription. Several lines of evidence indicate elevations in cAMP can increase gene transcription. For example, dibutylryl cAMP or isoproterenol increased the rate of lactate dehydrogenase transcription in vitro, and the effect of isoproterenol was limited to concentrations that increased cAMP levels (17). Also, cAMP can produce a marked increase in promoter activity of the Ren-1<sup>1</sup> renin gene (24, 34). Moreover, forskolin stimulated the renin gene promoter in a pulmonary carcinoma cell line expressing human renin mRNA. Similarly, chorionic cells transfected with renin gene constructs have positive responses to cAMP (12, 20, 36). Thus it seems likely that in both mature and immature fetal kidney cells elevations in cAMP are capable of increasing renin gene transcription. The mechanisms responsible for the differences in sensitivity to stimulation require further investigation.

Our observations on the ability of both forskolin and IBMX to maintain elevations in renin message for 24 h in immature and mature fetal kidney cells are consistent with an increase in message stability occurring in response to an elevation in cAMP. Chen and colleagues (6) reported that cAMP increases the stability of renin mRNA in mouse juxtaglomerular cells. We found that the disappearance of renin message was more rapid in immature kidney cells compared with mature cells. This suggests that the stability of renin message is greater in fetal kidneys close to term. The actual mechanisms involved in changing stability with development are not clear but could be related to both protein kinase A and protein kinase C signal transduction pathways (16).

The calculated half-life for renin mRNA was 6.0 ± 0.6 and 11.6 ± 0.8 h in immature and mature fetal kidney cells, respectively. These values are different from each other and both are greater than the half-life reported for renin mRNA in adult mouse cells. This difference may represent a species effect or it could be that renin mRNA stability declines from fetal to adult life. Such a decline would be consistent with the overall increase in expression of renin observed in fetal kidneys compared with adult kidneys (3, 28, 37).

At this juncture, we do not know if the proportion of cells containing renin and its RNA at the two ages is identical after isolation and culture for at least 20 h. Clearly, differences in the proportion of cells expressing renin would influence the baseline levels of renin mRNA. However, such differences would not cause the dissimilarities in sensitivity to stimulation and in stability observed at the two ages because the data were referenced to the appropriate controls at each age. Indeed, any age-related differences in the number of cells expressing renin may be a consequence of developmental changes in renin gene responsiveness and/or renin mRNA stability.

**Perspectives**

To our knowledge these studies are the first to demonstrate a developmental difference in the ability of the renin gene to respond to stimulation and a maturational change in renin mRNA stability. The increase in sensitivity to stimulation as well as the enhanced message stability that occurs in late gestation are consistent with the significant elevation in renin expression observed in the perinatal period. It is interesting that the renin gene also shows increased responsiveness to negative regulation close to term such that cortisol infusions in the mature fetal lamb decrease renin mRNA levels, whereas in the immature fetus, elevations in cortisol have no detectable effect on renal renin mRNA (4, 32). This suggests that both positive and negative regulators of renin gene transcription and degradation are more active as gestation progresses. The factors mediating these changes are unidentified currently, but merit further investigation.

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