Kidney angiotensin and angiotensin receptor expression in prenatally programmed hypertension

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Vehaskari, V. Matti, Tyrus Stewart, Derek Lafont, Christopher Soyez, Dale Seth, and Jennifer Manning. Kidney angiotensin and angiotensin receptor expression in prenatally programmed hypertension. Am J Physiol Renal Physiol 287: F262–F267, 2004.—Adult hypertension may be programmed by the prenatal environment in humans and in experimental animals. The potential role of the intrarenal renin-angiotensin system (RAS) in prenatally programmed hypertension was investigated. Hypertension in rat offspring was induced by maternal protein restriction during pregnancy. The offspring were studied on day 1 of life and immediately preceding the development of hypertension on day 28. ANG I and II contents were determined by radioimmunoassay. Angiotensin receptor protein and mRNA levels were quantified by immunoblotting and real-time RT-PCR, respectively. Plasma and kidney ANG I and II were unchanged in the offspring from low-protein pregnancies (LP). ANG II type 1 receptor (AT1R) protein abundance was low in the newborn LP kidney (P < 0.05) but rose above control values by 28 days of age (P < 0.05); the rise was associated with an increase in AT1R subtype A (P < 0.01), but not subtype B, mRNA level. ANG II type 2 receptor protein expression was decreased on day 1 (P < 0.05) and increased on day 28 (P < 0.05) in LP kidneys. The results show that prenatal programming of hypertension is associated with an abnormal pattern of intrarenal RAS ontogeny that may play a pathogenetic role, for instance, by constitutively altering renal hemodynamics or Na reabsorption.

fetal origins of adult disease; kidney ontology; low-protein diet

The concept of “prenatal programming of adult disease” refers to modulation of future adult phenotype by nongenetic environmental factors in utero. Programming of angiotensin receptor protein and mRNA levels was quantified by immunoblotting and real-time RT-PCR, respectively. Plasma and kidney ANG I and II were unchanged in the offspring from low-protein pregnancies (LP). ANG II type 1 receptor (AT1R) protein abundance was low in the newborn LP kidney (P < 0.05) but rose above control values by 28 days of age (P < 0.05); the rise was associated with an increase in AT1R subtype A (P < 0.01), but not subtype B, mRNA level. ANG II type 2 receptor protein expression was decreased on day 1 (P < 0.05) and increased on day 28 (P < 0.05) in LP kidneys. The results show that prenatal programming of hypertension is associated with an abnormal pattern of intrarenal RAS ontogeny that may play a pathogenetic role, for instance, by constitutively altering renal hemodynamics or Na reabsorption.

THE CONCEPT OF “PRENATAL PROGRAMMING OF ADULT DISEASE” REFERS TO MODULATION OF FUTURE ADULT PHENOTYPE BY NONGENETIC ENVIRONMENTAL FACTORS IN UTERO. PROGRAMMING OF ANGIOTENSIN RECEPTOR PROTEIN AND mRNA LEVELS WAS QUANTIFIED BY IMMUNOBLOTTING AND REAL-TIME RT-PCR, RESPECTIVELY. PLASMA AND KIDNEY ANG I AND II WERE UNCHANGED IN THE OFFSPRING FROM LOW-PROTEIN PREGNANCIES (LP). ANG II TYPE 1 RECEPTOR (AT1R) PROTEIN ABUNDANCE WAS LOW IN THE NEWBORN LP KIDNEY (P < 0.05) BUT ROSE ABOVE CONTROL VALUES BY 28 DAYS OF AGE (P < 0.05); THE RISE WAS ASSOCIATED WITH AN INCREASE IN AT1R SUBTYPE A (P < 0.01), BUT NOT SUBTYPE B, mRNA LEVEL. ANG II TYPE 2 RECEPTOR PROTEIN EXPRESSION WAS DECREASED ON DAY 1 (P < 0.05) AND INCREASED ON DAY 28 (P < 0.05) IN LP KIDNEYS. THE RESULTS SHOW THAT PRENATAL PROGRAMMING OF HYPERTENSION IS ASSOCIATED WITH AN ABNORMAL PATTERN OF INTRARENAL RAS ONTOGENY THAT MAY PLAY A PATHOGENETIC ROLE, FOR INSTANCE, BY CONSTITUTIVELY ALTERING RENAL HEMODYNAMICS OR Na REABSORPTION.

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as previously described (36). The dams were allowed to deliver. No premature deliveries or immediate postnatal abnormalities in the pups were observed. The birth weights of the pups from protein-restricted pregnancies (LP) were ~15% lower than those from control pregnancies, consistent with our previous observations (22, 36). At birth, all litters were reduced to 8–10 pups; the extra pups were killed and their kidneys were harvested as described below. After birth, all dams were on a standard 20% protein diet and were allowed to nurse their own offspring until weaning at 21 days of age. After being weaned, the offspring were given the standard 20% protein diet and water ad libitum.

Specimens were harvested from the offspring killed at birth and at 28 days of age. The animals used for renal RNA and protein isolation were killed with a lethal dose (100 mg/kg) of intraperitoneal pentobarbital sodium. The 28-day-old rats used for ANG I and II determination were killed by guillotine decapitation. Each experimental group included pups from at least three different litters. Additional pups from each litter were allowed to reach the age of 8 wk and the LP offspring were shown to develop hypertension as previously described by us (22, 36).

The study protocol was approved by the Animal Care and Use Committees of Louisiana State University Health Sciences Center and the Research Institute for Children. The American Physiological Society’s “Guiding Principles in the Care and Use of Animals” were followed.

Protein quantification. The abundances of ANG II type 1 receptor (AT1R) and ANG II type 2 receptor (AT2R) were determined by semiquantitative immunoblotting as previously described (21). Briefly, for total protein isolation, one kidney was cut into small pieces, homogenized in ice-cold isolation solution of 250 mM sucrose, 10 mM triethanolamine, 1 μg/ml leupeptin, and 0.1 mg/ml phenylmethylsulfonyl fluoride, centrifuged, and the pellet was discarded. Final protein concentration was adjusted to 2 μg/ml and the samples were solubilized in Laemmli sample buffer. Importantly, an initial preloading SDS-PAGE gel, stained with Coomassie blue, was done for each set of samples to confirm equal loading among samples (21). After subsequent SDS-PAGE on 10% polyacrylamide gels, the bands were transferred electrophoretically to polyvinylidene difluoride membranes, blocked with 5% milk, probed overnight at 4°C with the desired primary antibody, and then incubated with secondary antibody. The bands were visualized by chemiluminescence (SuperSignal West Dura Extended Duration Substrate, Pierce Biotechnology), exposed with the Versa Doc Imaging System (Bio-Rad Life Sciences), and analyzed by Quantity One software (Bio-Rad Life Sciences). The readings of LP samples were normalized to those of the control samples.

The rabbit anti-AT1R and goat anti-AT2R antibodies were purchased from Santa Cruz Biotechnology. Anti-AT1R is known to recognize both of the rodent AT1R subtypes, subtype A (AT1RA) and subtype B (AT1RB). The specificity of each antibody was determined by showing elimination of the band by a blocking peptide. The secondary antibody for anti-AT1R was goat anti-rabbit IgG (Pierce Biotechnology) and that for anti-AT2R was donkey anti-goat IgG (Santa Cruz Biotechnology).

mRNA quantification by real-time RT-PCR. Small pieces from freshly harvested kidneys were immediately placed in RNA-preserving solution (RNAlater, Ambion). Total RNA was then isolated using TRIzol Reagent (Life Technologies) according to the manufacturer’s instructions. The RNA was treated with DNase I (Ambion) to eliminate contamination by genomic DNA, and the final RNA concentration was standardized to 0.75 μg/μl. The integrity of the RNA was assessed by agarose gel electrophoresis.

One-step real-time RT-PCR was carried out on a real-time thermal cycler (iCycler, Bio-Rad Life Sciences) using a QuantiTect SYBR Green RT-PCR kit (Qiagen). The method allows the reverse transcription and PCR to be carried out in a single step in the same reaction tube. The fluorescent dye SYBR Green I is included in the PCR master mix; in addition, the reaction was spiked with 0.5 μl of 1 μM fluorescein for background reference. The threshold cycle number (Ct) for real-time PCR was set by the cycler software.

PCR primers (18 to 22 bp) for AT1RA, AT1RB, AT2R, and GAPDH were designed using commercial software (Beacon Designer, Bio-Rad Life Sciences) to produce an amplicon length of 75–150 bp. Optimal primer concentration for PCR was determined separately for each primer pair. Each reaction was run in triplicate, and reaction tubes with target primers and those with GAPDH primers were always included in the same PCR run. To test primer efficiencies, the one-step RT-PCR was run with each target primer/GAPDH primer combination on an mRNA template dilution series up to a dilution factor of 1:100. The ΔCt [Ct[target] – Ct[GAPDH]] over the dilution range was constant for each primer pair, indicating equal primer efficiencies of the target and reference (GAPDH) primers, as required for the comparative Ct method (20).

Relative quantification was achieved by the comparative 2−ΔΔCt method (20). The relative increase/decrease (fold-induction/repression) of mRNA of target x in the experimental group (LP) was calculated using the control group as the calibrator: 2−ΔΔCt, where ΔΔCt is: [Ct[x][LP] – Ct[GAPDH][LP]] − [Ct[x][control] – Ct[GAPDH][control]].

Fig. 1. Angiotensin type 1 receptor (AT1R) expression in kidneys of 1-day-old offspring from control (C) and low-protein (LP) pregnancies; n = 8 in each group. A: relative AT1R protein abundance determined by immunoblotting, total of both AT1R subtypes. B: relative AT1RA mRNA content measured by real-time RT-PCR. C: relative AT1RB mRNA content.
**ANG I and II determination.** Plasma and kidney ANG I and ANG II contents were measured using solid-phase extraction and radioimmunoassay as previously described (41). Briefly, kidney samples were weighed, homogenized in ice-cold methanol, and centrifuged. The soluble fractions were dried and reconstituted in phosphate buffer. For plasma samples, trunk blood was collected into an inhibitor cocktail and centrifuged. Plasma and kidney samples were extracted on phenyl C18 reverse-phase bond elution columns (Varian). RIA was performed using rabbit anti-ANG I or anti-ANG II antiserum (Phoenix Pharmaceuticals) and 125I-labeled ANG I or ANG II (New England Nuclear Life Sciences) with commercially available standards (Phoenix Pharmaceuticals). Results were expressed as femtomoles per milliliter or femtomoles per gram of tissue.

**Statistical methods.** All results are expressed as means ± SE. Statistical comparisons were done with an unpaired t-test, and a P value of < 0.05 is considered significant.

**RESULTS**

**One-day-old offspring.** AT1R protein abundance was determined by immunoblotting using an antibody that recognizes both the A and B rodent subtypes of the receptor. As shown in Fig. 1A, the level was decreased in the LP kidneys when compared with control kidneys. AT1RA and AT1RB mRNA levels were measured separately using appropriate primers. AT1RA mRNA abundance was numerically but not statistically significantly lower in the LP kidneys (P = 0.058; Fig. 1B). The mRNA level of AT1RB was significantly lower in the LP group (Fig. 1C). AT1R protein abundance in the LP kidneys was significantly lower than that in the control kidneys (Fig. 2A). In contrast, the AT2R mRNA level in LP kidneys was significantly higher than that in control kidneys (Fig. 2B).

**Twenty-eight-day-old offspring.** The age of 28 days was chosen because it immediately precedes the development of hypertension in our model (22, 36). In contrast to the newborn period, AT1R protein abundance at this age was significantly increased in the LP group compared with that of the control group (Fig. 3A). The upregulation was corroborated by the PCR results showing a markedly increased mRNA level of the subtype A receptor in the LP pups (Fig. 3B). AT1RB mRNA content was not significantly different between the two groups (Fig. 3C). AT2R protein abundance in the kidney at this age was slightly higher in the LP group than that in the control group (Fig. 4A). However, the difference in mRNA level was not significant (Fig. 4B).

Systemic angiotensin levels at 28 days of age are illustrated in Fig. 5. Both ANG I and II concentrations in the plasma in the LP group were similar to those in the control group. Figure 6 shows kidney ANG I and II contents. The values of the LP group did not differ significantly from those of the control group.

**DISCUSSION**

The present study explored the role of intrarenal ANG II and its receptors in a rat model of prenatally programmed hypertension. The most important finding was that the expression of AT1R in the kidney is upregulated at 4 wk of age. Because we...
previously showed that this is the period preceding the development of hypertension (22, 36), the finding may be important for the pathogenesis. Interestingly, AT1R expression at birth was suppressed and there were changes, albeit less consistent, in AT2R expression, suggesting that the ontogeny of the intrarenal RAS is altered throughout the perinatal and early postnatal period. Kidney ANG I and ANG II contents at 4 wk did not differ from those of the controls, indicating that there was no feedback downregulation of the upstream components of the intrarenal RAS in response to the increased AT1R expression.

Unlike humans, rodents have two AT1R subtypes, subtypes A and B (33). Although both subtypes are believed to be functional and to mediate similar effects of ANG II, AT1RA appears to be the predominant receptor as evidenced by mouse knockout models (6, 10, 14, 27, 35). In the present study, the upregulation of AT1R at 4 wk was seen only in AT1RA at the transcriptional level, with total AT1R (subtypes A and B combined) protein level paralleling that of AT1RA mRNA. The rise in AT1R receptor concurs with the findings of Sahajpal and Ashton (32), who reported an increase in AT1R protein at 4 wk of age in a slightly different model; their study did not distinguish between the receptor subtypes.

There is accumulating evidence that intrarenal RAS may regulate blood pressure independently of systemic RAS (25, 26, 31). Intrarenal RAS has been implicated in human low-renin hypertension, for instance, in diabetic subjects (13) and in hypertension in African-Americans (30). In experimental models, intrarenal ANG II concentration may greatly exceed systemic levels (12, 41), and the development of hypertension may be associated with activation of intrarenal RAS while the systemic RAS is suppressed (16). Our previous studies showed that prenatally programmed hypertension is responsive to angiotensin-converting enzyme inhibition despite low PRA (22), suggesting a role for intrarenal RAS, although an effect of angiotensin-converting enzyme inhibition on the bradykinin pathway is not ruled out. The present finding of upregulated intrarenal AT1R at 4 wk of age is consistent with the hypothesis that upregulation of AT1R in the kidney contributes to the development of hypertension in prenatally programmed hypertension.

The possible role of AT2R requires further study. Although hypertensive effects of RAS are believed to be mediated via AT1R, AT2R may function as a counterregulatory pathway (25). Our results show a slight increase in AT2R protein expression in LP kidneys at 4 wk without significant change in mRNA level, and in the newborn LP kidneys the protein and mRNA levels behaved in inverse fashion. The reason for the apparent discrepancy is not clear, but it may be related to the very low level of AT1R2 expression in the postnatal kidney (5).

Defining the precise mechanism by which AT1R upregulation may be involved in the programming of hypertension will require functional studies as well as localization of the intrarenal sites of upregulation. Our results show a quantitatively relatively modest upregulation of AT1R; however, in support of functional significance is the fact that the upregulation was seen at both the protein and the mRNA level. Moreover, determinations in whole kidney preparation may mask larger regional differences within the kidney. In the normal mature kidney, both AT1RA and AT1RB are expressed throughout the nephron and intrarenal vasculature (23). Constitutive activation of the receptor could lead to sustained hypertension by altering renal hemodynamics and/or tubular reabsorption of Na. Because luminal ANG II has been reported to stimulate distal tubule transport, probably via AT1 receptors (3, 37), the latter possibility is supported by our previous finding of upregulated
distal nephron Na transporters at the age of 4 wk (21), coinciding with the increase in \( \text{AT}_1 \text{R} \) in the present study.

The mechanisms leading to altered renal \( \text{AT}_1 \text{R} \) ontogeny in prenatally programmed hypertension are not clear. The kidney exhibits both spatial and temporal developmental regulation of angiotensin receptors (28). \( \text{RAS} \) is important for normal renal development; disruption of \( \text{RAS} \) by pharmacological means decreases renal blood flow (29), and the later upregulation of \( \text{AT}_1 \text{R} \) expression observed in our newborn rats does not reflect total absence of \( \text{RAS} \) activity during the period of kidney development. Decreased \( \text{RAS} \) activity of shorter duration or of lesser severity could, however, explain many of the features in our model. Woods and Rasch (40) showed that treatment of neonatal rats with an \( \text{AT}_1 \text{R} \) antagonist results in a decreased number of nephrons and later hypertension. Therefore, although the primary mechanism of abnormal \( \text{RAS} \) ontogeny is not known, the early suppression of \( \text{AT}_1 \text{R} \) could lead to reduced nephron number, and the later upregulation of \( \text{AT}_1 \text{R} \) may be a compensatory effort to increase glomerular filtration, with the possible consequence of inappropriate up-regulation of Na reabsorption.

In summary, the present study documents altered kidney \( \text{RAS} \) ontogeny in prenatally programmed hypertension. Of pathogenetic importance may be the upregulation of \( \text{AT}_1 \text{R} \) at 4 wk of age, coinciding with the previously documented onset of hypertension and upregulation of renal Na transporter expression in our model.

GRANTS

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REFERENCES