Acta histochemica 110 (2008) 497—504

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acta histochemica
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Effects of maternal undernutrition on renal angiotensin II and chymase in hypertensive offspring

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Received 23 December 2007; received in revised form 28 January 2008; accepted 29 January 2008

KEYWORDS
Angiotensin;
Chymase;
Glomerulus;
Intrauterine growth restriction;
Rats

Summary

Intrauterine growth restriction (IUGR) can program the future development of hypertension in adulthood. The renin–angiotensin system has been reported to play a role in IUGR-induced hypertension. The aims of this study were to investigate the effects of IUGR on renal angiotensin-converting enzyme (ACE), angiotensin II (Ang II) and chymase in IUGR-induced hypertension. Timed pregnant Sprague–Dawley rats received 50% rations of control food intakes from days 15 to 21 of gestation. Control rats received regular food throughout the pregnancies. Arterial blood pressure and glomerular number were measured and immunohistochemical studies were performed on kidney tissues in adult male offspring at 16 weeks of age. IUGR rats exhibited significantly lower body and kidney weights and reduced number of glomeruli when compared with control rats. IUGR rats had significantly higher systolic blood pressure than control rats. Immunoreactivity of ACE was comparable between control and IUGR rats whereas immunoreactivities of chymase and Ang II were significantly higher in IUGR rats than in control rats. In conclusion, immunohistochemical studies document up-regulation of ACE-independent Ang II and chymase in IUGR kidney and indicate that overactivity of chymase may result in increased intrarenal Ang II production, which could contribute to the development of hypertension in intrauterine undernourished rats.

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doi:10.1016/j.acthis.2008.01.002

Introduction

Epidemiological studies suggest that factors operating in prenatal life are important determinants of the risk of cardiovascular disorders in adult life. Data from several human studies have shown that intrauterine growth restriction (IUGR) is a risk factor for the later development of hypertension, *Diabetes mellitus* and ischemic heart disease in adults (Curhan et al., 1993; Barker, 1996; Moore et al., 1996; Rich-Edwards et al., 1997). Animal studies have demonstrated that some prenatal factors, probably related to maternal nutrition, can program the future development of cardiovascular and renal dysfunction in later life (Marchand and Langley-Evans, 2001). Mechanisms implicated have included alteration of the renin–angiotensin system (RAS) and disturbance of the hypothalamic–pituitary–adrenal axis (Clark, 1998; Langley-Evans, 2001; Woods et al., 2001). Rats with IUGR caused by either maternal undernutrition or vascular placental insufficiency during pregnancy had a significant reduction in glomerular number (Vehaskari et al., 2001; Woods et al., 2001). These findings were supported by the observation that human neonates with IUGR have a significant reduction in glomerular number and are prone to develop hypertension in adulthood (Manalich et al., 2000).

Hypertension is a common complication in offspring with IUGR. Little information is available regarding the mechanism of hypertension in this condition. The RAS is a key regulator of blood pressure and fluid homeostasis (Peach, 1977). Angiotensin II (Ang II) is the main effector of the RAS and is produced from the substrate angiotensinogen through sequential enzymatic cleavages by renin and angiotensin-converting enzyme (ACE). RAS blockade using ACE inhibitor and Ang type 1 receptor antagonist abolish hypertension in adult growth-restricted offspring from intrauterine undernourished dams (Ceravolo et al., 2007). These results suggest that RAS may play a role in IUGR-induced hypertension. It is clear that such a simplified view of the RAS cannot completely explain the physiological complexities of the system in health and disease. Various components of the RAS are synthesized in tissues throughout the body where their expression may be subject to local control (Dzau et al., 1987). Consequently, it has been suggested that compartmentalized RAS may work within individual organ systems with some degree of autonomy to influence regional response. Chymase is a major chymotrypsin-like serine protease that is expressed in the secretory granules of mast cells in many mammalian species (Takai et al., 1996). Chymase can convert Ang I to Ang II more efficiently than ACE (Ihara et al., 1999). Chymase has been reported to be involved in the Ang II-generating activity in human diabetic nephropathy and in rat ischemic kidney with renovascular hypertension (Huang et al., 2003; Sadjadi et al., 2005). A recent report has documented increased renal RAS activity in adult growth-restricted offspring from reduced uterine perfusion dams (Grigore et al., 2007). Despite the relationship between local RAS and adult hypertension, little is known about the role of chymase in hypertensive adult rats exposed to intrauterine undernutrition. The aims of this study were to investigate the effects of IUGR on glomerular number and renal ACE, Ang II and chymase immunolocalisation in IUGR-induced hypertension.

Material and methods

Animals

This study was approved by the Institutional Committee for Animal Use at Taipei Medical University and was performed using timed pregnant Sprague–Dawley rats (vaginal smear positive, day 0; term, day 22). Because IUGR produced by placental insufficiency results in the development of hypertension only in male offspring (Ojeda et al., 2007), the present study was performed only on male offspring. All animals were individually caged and maintained at 22 °C with a 12-h light–dark cycle in an isolated room. Three pregnant control rats received food (regular rat chow containing 23.5% protein, 4.5% fat, and 53% carbohydrate) and water *ad libitum* from hanging containers throughout their pregnancies. Their food consumption was measured daily by weighing the container after carefully collecting spilled chow. The three experimental pregnant animals (maternal undernutrition) received 50% rations of the control food intakes during their last trimester from days 15 to 21 of gestation. The dams delivered spontaneously at term and were then immediately switched back to standard rat chow diets available *ad libitum*. The offspring were nursed by their mothers until being weaned at 4 weeks of age, and then they were switched to standard chow diet. At 16 weeks of age, male offspring were randomly selected from control and undernourished litters.

Blood pressure measurements

Arterial blood pressures were measured by direct femoral artery catheterization. Rats were

anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg, Abbott Laboratories, North Chicago, IL, USA). PE-50 catheters were placed in the abdominal aorta from the femoral artery for measurements of blood pressure. These catheters were filled with heparinized saline (100 U/ml), plugged with stainless-steel pins, tunneled under the skin to exteriorize and secured at the back of the neck. On recovery from anesthesia, each rat was placed in an individual cage for a 24-h recovery and habituation period. The arterial catheter was then attached to a pressure transducer (Gould P23ID, Cleveland, OH, USA) and blood pressure was measured using a polygraph system (Gould Inc., Cleveland, OH, USA).

Morphological studies and measurements of glomerular number

After blood pressure was measured, each rat was sacrificed with an intraperitoneal injection of pentobarbital (100 mg/kg) and kidneys were removed and weighed. The kidneys were immersed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The number of glomeruli was estimated by the dissector technique (Woods et al., 2001), briefly as follows. The fixed kidney was cut into 2 mm-thick horizontal slices and three slices were systematically, uniformly randomly sampled. The slices were sampled with a periodicity of two (e.g. 1, 3, 5 or 2, 4, 6). The sampled sections were serially dehydrated in increasing concentrations of ethanol before being processed for embedding in paraffin wax. Three 15 μm-thick sections were cut from the approximate center of each tissue slice then dewaxed in xylene and rehydrated through an ascending ethanol series. Sections were stained with hematoxylin and eosin. The first and third sections were used for glomerular number estimation. The glomeruli present on the first section, but not on the third section and *vice versa*, were counted as ΣQ^{-}. The total number of glomeruli was calculated according to the formula: $n = [\Sigma Q^{-}/(2h\Sigma a)]KW$, where h is the height of the dissector (30 μm), Σa is the total area of the kidney (2 mm × 2 mm) on sampled sections, and KW is the kidney weight converted to a kidney volume (1 g~1 cm^3).

Immunohistochemistry

Immunohistochemical labeling was performed on paraffin wax sections of kidney prepared as described previously. After deparaffinization in xylene and rehydration in an ethanol series, sections were first preincubated for 1 h at room temperature in 0.1 M phosphate-buffered saline containing 10% normal goat serum (Chemicon, Temecula, CA, USA) and 3% H_2O_2 (for ACE) or 0.3% H_2O_2 (for chymase) to block endogenous peroxidase activity and non-specific binding of antibody before being incubated for 20 h at 4 °C with rabbit polyclonal antibody against Ang II (1:350 dilution, Peninsula Laboratories, San Carlos, CA, USA) or mouse monoclonal antibodies against ACE (CD143, 1:500 dilution, Chemicon, Temecula, CA, USA) or chymase (1:100 dilution, Abcam, Cambridge, UK). Sections were then treated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (for rabbit anti-Ang II, 1:200, Vector, CA, USA) or anti-mouse IgG (for mouse anti-ACE and mouse anti-chymase, 1:200, Vector, CA, USA). This was followed by reaction with ABC complex (Avidin–Biotin Complex, prepared according to the manufacturer's recommendations, Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Reaction products were visualized by incubation with 0.5 mg/ml of 3,3-diaminobenzidine (Chemical, Louis, MO, USA) and 0.003% H_2O_2 in 0.5 M Tris buffer (pH 7.6) for 3–5 min. The slides were rinsed in distilled water, counterstained with hematoxylin (Koch-Light Laboratory, Colnbrook-Bucks-England, UK) for 60 s, washed extensively in running water before being mounted using Permount (Fisher Scientific, Pittsburgh, PA, USA). For controls, in place of primary antiserum, sections were incubated in buffer containing preabsorbed primary antiserum or buffer alone.

Quantification of ACE, chymase and Ang II immunoreactivities

A minimum of four randomly selected kidney fields of immunohistochemically labeled kidney sections per animal, at 400 × magnification, were captured using a digital camera and imported into a computerized image analysis system (Image-Pro Plus 5.1 for Windows, Media Cybernetics, Silver Spring, MD, USA). The automatic object counting and measuring process was used to quantify the immunoreactivity in the sections. We used the "count/size" function command to perform a cell number counting operation. This generated a percentage of positively labeled cells and the values were expressed as labeling index (%).

Statistical analysis

Data are expressed as the means ± SD. Between-group comparisons at each age group were made

using Student's *t*-test. Differences were considered statistically significant when *P*-value was <0.05.

Results

Effects of undernutrition on maternal body weight during late gestation

Mean body weights before treatment of the control and undernourished dams were 317 ± 28 and 352 ± 15 g, respectively (Figure 1). Body weights of control dams increased gradually up to 420 g, while body weights of undernourished dams remained at around 350 g during the last 7 gestational days, and the values were lower than those of control rats from gestational days 17 to 21. Total body weight gain was significantly higher in control than that in undernourished dams (103 ± 26 and 1 ± 6 g, respectively).

Effects of maternal undernutrition on body weight, kidney weight and blood pressure in offspring

Effects of maternal undernutrition on postnatal body weight, kidney weight, kidney/body weight ratio and blood pressure are presented in Table 1. IUGR rats exhibited significantly lower body weights. Mean kidney weight was significantly lower in the IUGR rats; when adjusted for body weight, the difference was not statistically significant. IUGR rats had significantly higher systolic blood pressure than control rats. Diastolic blood pressures were comparable between control and IUGR rats.

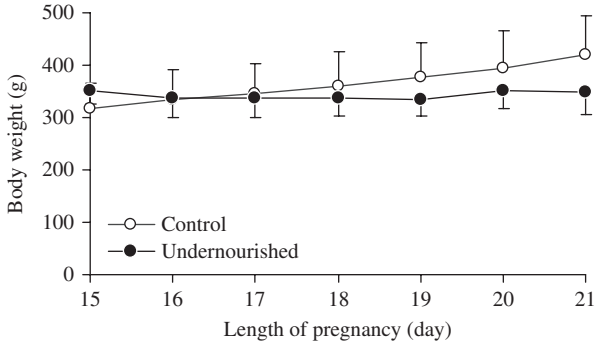

Figure 1. Mean body weights of control (open symbols) and undernourished (filled symbols) pregnant rats during the last 7 days of gestation. Body weights of control rats increased gradually up to 420 g, while body weights of undernourished rats remained about 350 g during the last 7 gestational days.

Effects of maternal undernutrition on glomerular number in offspring

The number of glomeruli was significantly reduced in IUGR rats at 16 weeks of age (Figure 2); the IUGR kidney had 40% fewer glomeruli compared with controls.

Immunohistochemistry of ACE, chymase and Ang II

Immunoreactivity of ACE was mainly localized to the distal tubules and rarely in the proximal tubules and glomeruli, and the immunoreactivity was similar between control and IUGR rats (Figure 3). Chymase immunolocalisation was detected in the distal tubules and glomerular mesangial cells, with weaker immunolabeling present in the proximal tubules. Chymase immunoreactivity was significantly higher in IUGR rats than in control rats (Figure 4). Ang II was mostly immunolocalized to the proximal and distal tubules and seldom in the glomeruli. Ang II immunoreactivity was significantly higher in IUGR rats than in control rats (Figure 5).

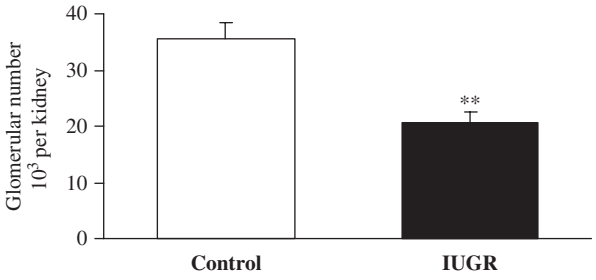

Figure 2. Effects of maternal undernutrition on the glomerular number in control and IUGR rats. Glomerular number per kidney was significantly reduced by ~40% in IUGR rats compared with control rats at 16 weeks of age (**$P<0.01$).

Table 1. Body weight, kidney weight and blood pressure in control and IUGR rats at 16 weeks of age

	n	BW (g)	Kidney (g)	Kidney/BW (%)	Systolic BP (mmHg)	Diastolic BP (mmHg)
Control	8	475 ± 41	3.17 ± 0.44	0.69 ± 0.11	167.8 ± 12.9	135.0 ± 22.7
IUGR	8	408 ± 46**	2.58 ± 0.19*	0.64 ± 0.07	190.6 ± 5.2*	145.0 ± 15.8

Values are means$\pm$SD. *$P<0.05$, **$P<0.01$ vs. control. BW, body weight; BP, blood pressure.

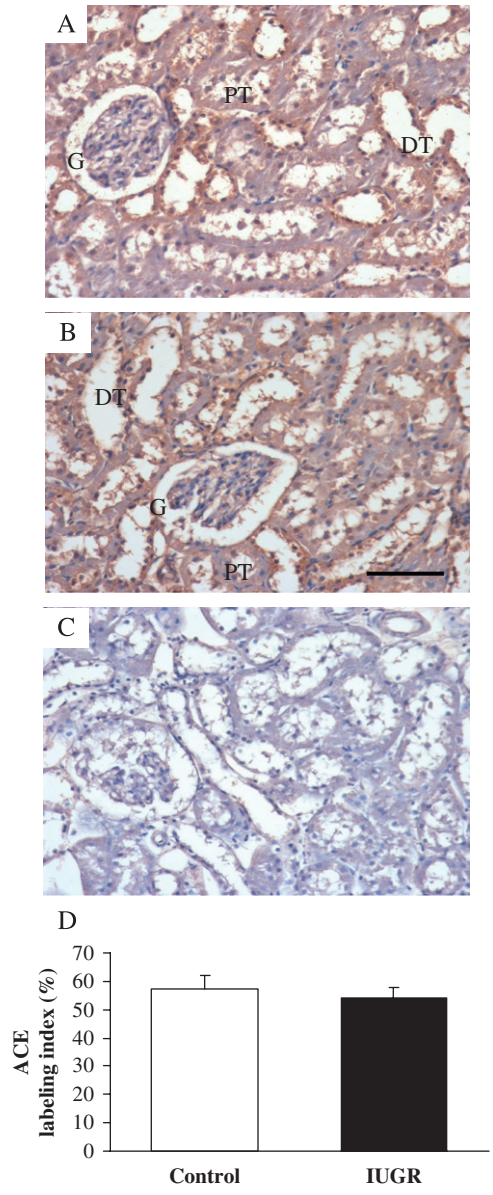

Figure 3. Immunolabeling of ACE in kidney of (A) control and (B) IUGR rats. Bar = 100 μm. ACE was mainly immunolocalized to the distal tubules (DT) and rarely in the proximal tubules (PT) and glomerulus (G), and the immunoreactivity was comparable between control and IUGR rats. (C) Negative control without primary antibody shows no labeling. (D) Quantitative analysis of ACE immunoreactivity in control and IUGR rats.

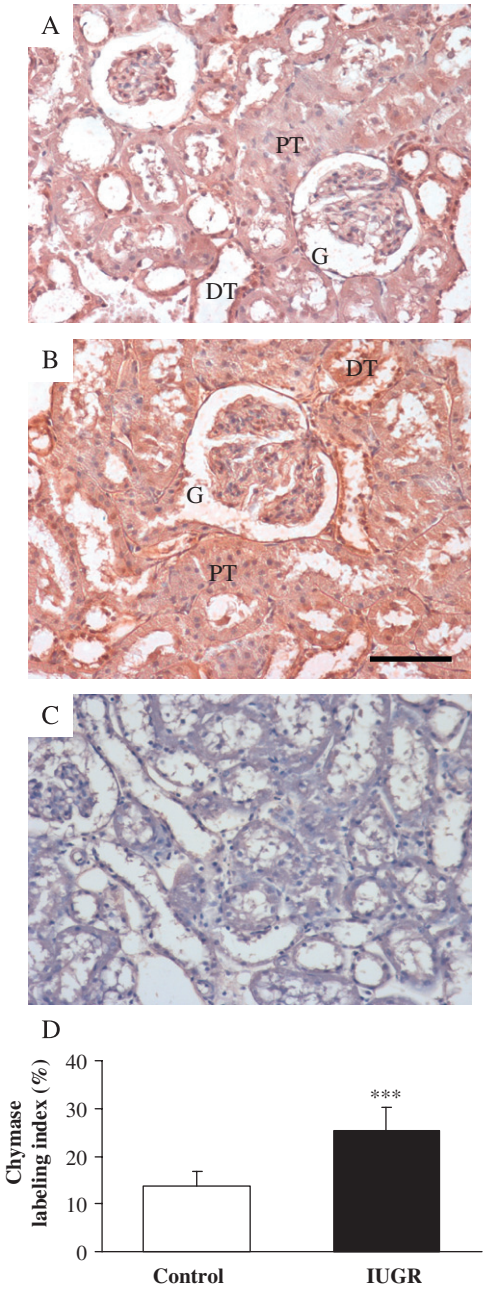

Figure 4. Immunolabeling of chymase in kidney of (A) control and (B) IUGR rats. Bar = 100 μm. Chymase was immunolocalized in the distal tubules (DT) and glomerular mesangial cells (G), with weaker labeling present in the proximal tubules (PT) and the immunoreactivity was significantly higher in IUGR rats than in control rats (***$P<0.001$). (C) Negative control without primary antibody shows no labeling. (D) Quantitative analysis of chymase immunoreactivity in control and IUGR rats.

Discussion

In the present study, late gestational exposure of rat pups to maternal undernutrition was found to result in hypertension and lower glomerular number in animals at 16 weeks of age. Greater ACE-independent Ang II and chymase immunopositivity was detectable in IUGR kidneys. These results agree with the findings of Rivière et al. (2005) that adult (4-month old) offspring from rat dams who experienced 70% food-restriction throughout gestation present with hypertension, reduced number of nephrons, and comparable renal ACE mRNA expression and ACE activity. These results suggest that

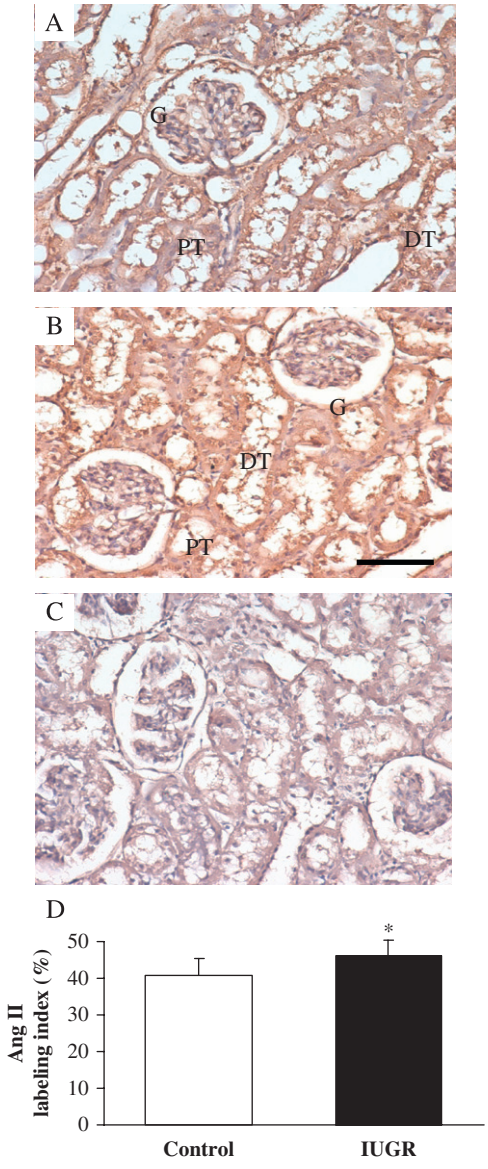

Figure 5. Immunolabeling of Ang II in kidney of (A) control and (B) IUGR rats. Bar = 100 μm. Ang II was mostly immunolocalized to the proximal (PT) and distal tubules (DT) and seldom in the glomerulus (G) and the immunoreactivity was significantly higher in IUGR rats than in control rats (*$P<0.05$). (C) Negative control without primary antibody shows no labeling. (D) Quantitative analysis of Ang II immunoreactivity in control and IUGR rats.

overactivity of chymase might result in increased intrarenal Ang II production, which may contribute to the development of hypertension in intrauterine undernourished rats.

Several animal IUGR models exist which rely on dietary restriction, protein restriction and placental insufficiency (Creasy and Resnik, 1994). Maternal dietary deprivation is unlikely to be limited to protein restriction alone. Therefore, we investigated the responses to restriction of all dietary constituents in this study. Maternal undernutrition during late gestation has been associated with poor maternal weight gain and reduced offspring body weight in rats (Lesage et al., 2002). In this study we found that maternal undernutrition during the last week of pregnancy reduced maternal body weight and offspring body weight at 16 weeks of age. Kidney weight was significantly lower in IUGR rats than in control rats; when adjusted for body weight the difference was not statistically significant. These results are consistent with the findings of Woods et al. (2004). Although the experimental dams were immediately transferred back to unrestricted chow diet after delivery, during the time that they nourished their offspring, until 4 weeks of age, they would still themselves be undernourished. As a consequence, newborn rats received insufficient nutrients in the postnatal period and this may contribute to the differences of body weight between control and IUGR rats.

The pathogenesis of hypertension induced by intrauterine dietary restriction is not clear. Three theories have been proposed to explain how the fetus is programmed to develop hypertension: alteration of the RAS, disturbance of the hypothalamic–pituitary–adrenal axis, and reduced glomerular number (Clark, 1998; Langley-Evans, 2001; Woods et al., 2001). The mechanism by which hypertension is associated with a reduction of nephron number appears to be caused by impaired renal sodium excretion, thus preventing pressure-induced natriuresis from restoring blood pressure toward normal levels (Brenner et al., 1988). The circulating RAS has been recognized as an important regulator of blood pressure and fluid homeostasis (Peach, 1977). Ang II is the main effector of the RAS and is synthesized in tissues throughout the body where expression may be subject to local control (Dzau et al., 1987). Ang II produced locally in the kidney exerts an important regulatory influence on renal functions as a paracrine factor (Paul et al., 2006). Inappropriate activation of intrarenal Ang II causes reductions in renal function and sodium excretion that contributes to progressive hypertension and leads to renal injury (Navar et al., 2003). Previous studies suggest that intrarenally derived Ang II is a potent regulator of systemic blood pressure, despite the absence of an increase in peripheral RAS (Navar et al., 1997; Davisson et al., 1999). Because circulating renin, ACE or Ang II concentrations are not constant, identification of local RAS activity is essential for understanding the mechanisms mediating hypertension in IUGR offspring (Langley-Evans and Jackson, 1995; Ceravolo et al., 2007). ACE and Ang

II are abundant in the rat kidney and are predominantly expressed in renal proximal and distal tubules (Tikellis et al., 2003; Fukada et al., 2005). In the study reported here, we performed immunohistochemical studies on kidney tissues from control and IUGR rats and found comparable ACE and increased Ang II immunolabeling in IUGR compared to control kidney. In contrast to our results, other investigators have found no difference in RAS gene expression in IUGR kidneys of fetal sheep exposed to placental insufficiency (Grigore et al., 2007). The discrepancy might be related to differences in the IUGR procedures and age of animals used.

Chymase is capable of acting on angiotensinogen to form Ang II from Ang I and has been reported to be up-regulated in human diabetic nephropathy and in rat ischemic kidney with renovascular hypertension (Huang et al., 2003; Sadjadi et al., 2005). These data suggest that chymase might play a significant role in renal Ang II formation. However, there is little information regarding chymase expression in the IUGR kidney. In this study, we found increased chymase immunolabeling in adult growth-restricted kidney from intrauterine undernourished dams. This result suggests that chymase might be involved in the Ang II-generating activity in IUGR kidney.

Our results suggest that chymase might participate in increased intrarenal Ang II production, which may contribute to the development of hypertension in intrauterine undernourished rats. These results imply that use of a chymase inhibitor might be a potentially useful strategy to control hypertension induced by intrauterine undernutrition.

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