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The effect of fetal and neonatal nicotine exposure on renal development of AT₁ and AT₂ receptors

Caiping Mao^{a,1}, Jiawei Wu^{a,1}, Daliao Xiao^b, Juanxiu Lv^a, Yang Ding^a, Zhice Xu^{a,b,*}, and Lubo Zhang^{a,b}

^aPerinatal Biology Center, Soochow University School of Medicine, Suzhou, PR China

^bCenter for Perinatal Biology, Department of Physiology & Pharmacology, Loma Linda University School of Medicine, Loma Linda, CA 92350, USA

Abstract

Aims—Maternal cigarette smoking accompanied with fetal and neonatal growth restriction causes abnormalities in organ development in the postnatal life. The present study determined the effect of maternal administration of nicotine on the development of the kidney in rats by examining the expression of renal angiotensin II receptors at mRNA and protein levels as well as kidney weight during postnatal development.

Methods—Nicotine was administered to pregnant rats *via* subcutaneous osmotic minipumps throughout gestation and up to 10 days after delivery. Kidneys were removed and collected from both male and female offspring at ages of 14-day-old, 30-day-old, and 5-month-old. Maternal nicotine administration significantly reduced renal AT₂ receptor (AT₂R) mRNA and protein abundance in both males and females at all three developmental ages examined.

Results—Although AT₁ receptor (AT₁R) mRNA and protein levels were not significantly changed between the control offspring and the offspring exposed to maternal nicotine during the early developmental period, the renal AT₁R/AT₂R ratio was significantly increased. This was associated with a significant decrease of kidney weight in both male and female offspring.

Conclusions—The results demonstrated that the development of renal angiotensin II receptor could be changed following exposure to perinatal nicotine, and such change in the kidney could be long-term in postnatal life.

Keywords

Nicotine; Kidney; Angiotensin receptor; Programming

1. Introduction

Maternal cigarette smoking with fetal nicotine exposure is one of the most widespread prenatal insults that cause fetal growth restriction in humans as well as in experimental animals [1-3]. Prenatal and postnatal growth restriction is associated with poor organ development in the postnatal life. Given that the kidney plays a vital role in the regulation of vascular volume and body fluid homeostasis, several reports have indicated that multiple factors during pregnancy,

including nutrition restriction, cause poor renal development in the fetus and in the offspring [4,5]. A previous study showed that infusion of nicotine in mothers during pregnancy resulted in a decreased kidney weight in male offspring in spontaneously hypertensive rats [6]. However, the potential gender differences and postnatal development of kidney in response to nicotine exposure during the early developmental stage (fetal and neonate period) have not been determined.

The renin-angiotensin system (RAS) plays a key role in physiological function of the kidney in the regulation of body fluid balance and cardiovascular homeostasis [7,8]. Previous studies have shown that adverse intrauterine environment, including those of drug exposure and poor nutrients, can affect expression of angiotensin receptor subtypes AT₁R and AT₂R, as well as angiotensinogen, in the kidney of ovine fetuses in late gestation [9,10]. These changes induced by prenatal factors are likely to influence the renal development and function in postnatal life. It is unknown whether and to what extent maternal nicotine administration alters AT₁R and AT₂R expression pattern in the kidney during postnatal development. Herein, we determined the effect of early developmental exposure of nicotine in fetus and neonate through maternal nicotine administration on AT₁R and AT₂R mRNA and protein abundance in the kidney at three postnatal ages, 14-day-old, 30-day-old, and 5-month-old. The corresponding kidney and body weights were measured. To determine the potential gender effects of prenatal nicotine exposure on the kidney, both male and female offspring were included in the studies.

2. Materials and methods

2.1. Experimental animals

Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI). Nicotine was administered through osmotic minipumps implanted subcutaneously as described previously [11,12]. In brief, on the fourth day of pregnancy, rats were anesthetized with ketamine and xylazine and an incision was made on the back to insert osmotic minipumps (Type 2ML4, Alza Corp., Palo Alto, CA). The incision was closed with four sutures. Half of pregnant rats were implanted with the minipumps containing nicotine at a concentration of 102 mg/ml, and the other half were implanted with the minipumps containing only saline, which served as the vehicle control. The flow rate of the minipumps was 60 µl/day, which delivered a dose of 2.1 mg nicotine free-base per day. In rats of an average of 350 g body weight, this corresponds to a dose rate of 6 mg/kg day, which closely resembles those occurring in moderate to heavy human smokers [2,13]. According to the manufacturer's specifications, the delivery period for the pumps is 28 days, and thus delivery continued after birth until postnatal day 10. As previously reported [14], the nicotine treatment did not affect the litter size and the length of gestation, and all of the pregnancies reached their full term. Rats were allowed to give birth naturally. Pups born to the dams were kept with their mothers until weaning. At weaning, male and female pups were separated and transferred to cages where they were housed in groups of two. Male and female offspring were sacrificed at three different ages of 14-day-old, 30-day-old, and 5-month-old, and the kidneys were isolated for studies. Kidney and body weights were obtained. Maternal body weight and food consumption was measured using the same protocols in our laboratory already, and the results were reported recently [28]. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory.

2.2. Western immunoblotting

The kidneys were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, 0.1% Tween-20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, and 5 µg/ml aprotinin, pH 7.4. Homogenates were ultrasonicated

for 15 s and then centrifuged at 4°C for 10 min at $10,000 \times g$. Supernatants were collected and protein determined using a protein assay kit from Bio-Rad. Samples with equal protein were loaded and separated on 10% SDS-PAGE. The membranes were treated with a Tris-buffered saline solution containing 5% dry-milk on a gentle shaker for 1 h, followed by incubation with rabbit polyclonal antibodies against AT₁R or AT₂R (Santa Cruze Biotechnology, Santa Cruz, CA) overnight at 4°C. After washing, membranes were incubated with a secondary horseradish peroxidaseconjugated goat anti-rabbit antibody. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. GAPDH was blotted in the same membrane as an internal control for normalizing the relative density. Results were analyzed and quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software.

2.3. Real-time RT-PCR

RNA was extracted from the kidneys using TRIzol reagents (Invitrogen, Carlsbad, USA). mRNA abundance of AT₁R and AT₂R was determined by real-time RT-PCR using Icyler Thermal cycler (Bio-Rad, Hercules, CA). The primer sequences for rat AT₁R and AT₂R [15] are shown in Table 1. Real-time RT-PCR was performed in a final volume of 25 µl. Each PCR reaction mixture consisted of 600 nM of primers, 33 units of MMLV reverse transcriptase (Promega, Madison, WI), and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) containing 0.625 unit Taq polymerase, 400 µM each of dATP, dCTP, dGTP, and dTTP, 100 mM KCl, 16.6 mM ammonium sulfate, 40 mM Tris-HCl, 6 mM MgSO₄, SYBR Green I, 20 nM fluorescein and stabilizers. RT-PCR was performed under the following conditions: 42°C for 30 min, 95°C for 15 min, followed by 45 cycles of 95°C for 20 s, 52°C for 1 min. GAPDH was used as an internal reference and serial dilutions of the positive control were performed on each plate to create a standard curve. PCR was performed in triplicate, and threshold cycle numbers were averaged.

2.4. Statistics

Data analysis was expressed as means $\pm$ SEM. Statistical significance ($P < 0.05$) was determined using repeated measures of ANOVA and *t*-test.

3. Results

3.1. Kidney and body weight

Litter size was the same between the control and experimental groups. Maternal nicotine administration during pregnancy resulted in a significant decrease in body weight in offspring at ages of 14-day-old and 30-day-old, regardless of sex (Fig. 1a). At 5-month-old, there was no significant difference in body weight in either male or female offspring between the saline control and nicotine-treated animals (Fig. 1a). Unlike body weight, the kidney weight was significantly decreased in the nicotine-treated offspring at all three ages in both males and females (Fig. 1b). The kidney/body weight ratio showed a postnatal development-dependent decrease in both males and females (Fig. 2). There was a significant decrease in the kidney/body weight ratio in both male and female offspring of 14-day-old, 30-day-old, and 5-month-old after prenatal nicotine treatment (Fig. 2). However, there was no significant difference in the kidney/body weight ratio between males and females in either control or nicotine-treated animals (Fig. 2).

3.2. Renal AT₁R and AT₂R protein abundance

Maternal nicotine administration during pregnancy had no significant effect on AT₁R protein abundance in the kidney of offspring at ages of 14-day-old, 30-day-old, and 5-month-old, regardless of sex (Fig. 3A). In contrast, AT₂R protein abundance was significantly decreased

in the kidney of both male and female offspring at 14-day-old, 30-day-old, and 5-month-old after prenatal nicotine treatment (Fig. 3B). Additionally, there was no significant difference in the nicotine-induced decrease in renal AT₂R protein abundance between male and female offspring. Nonetheless, the extent of decrease in renal AT₂R protein abundance in the nicotine-treated animals was less in 5-month-old offspring than that in 30-day-old offspring (Fig. 3B), suggesting a recovery in AT₂R during the postnatal development.

3.3. Renal AT₁R and AT₂R mRNA abundance

Consistent with the finding of protein abundance, fetal and neonatal nicotine exposure caused a significant decrease in AT₂R, but not AT₁R, mRNA abundance in the kidney of both male and female offspring at postnatal ages of 14-day-old, 30-day-old and 5-month-old (Fig. 4). Although there was no significant difference in the nicotine-induced decrease in renal AT₂R mRNA abundance between male and female offspring at 30-day-old and 5-month-old, there was a tendency of greater decrease in male offspring. Unlike the protein data, the extent of decrease in renal AT₂R mRNA abundance in the nicotine-treated animals was not significantly different between 30-day-old and 5-month-old offspring (Fig. 4), suggesting that the recovery of AT₂R during the postnatal development occurred at translational level. Fig. 5 shows the renal AT₁R/AT₂R mRNA ratio of 30-day-old and 5-month-old offspring. Both male and female offspring showed a significant increase in the AT₁R/AT₂R mRNA ratio in the kidney after prenatal nicotine exposure (Fig. 5). Whereas the nicotine-induced increase in renal AT₁R/AT₂R mRNA ratio was not significantly different between males and females at 30-day-old offspring, females showed significantly greater increase in the AT₁R/AT₂R mRNA ratio than males in 5-month-old offspring after prenatal nicotine treatment (Fig. 5).

4. Discussion

The present study demonstrated that fetal and neonatal nicotine exposure caused a significant decrease in body weight in rats at early postnatal ages up to 30-day-old. No significant difference was observed in the adult offspring between control and nicotine-treated animals. Additionally, no sex difference in body weight was observed. These findings are in agreement with the previous results obtained in the same animal model [16] and other studies [1,2,17, 18], and suggest a “catch-up” growth during postnatal development in the nicotine-treated animals. The previous study showed that maternal nicotine administration starting at day 4 of pregnancy in rats caused a rapid and transient reduction in food intake and a moderate decrease in maternal body weight gain during the period of treatment [16]. A number of studies have shown that *in utero* undernutrition causes fetal growth restriction and low birth weight, which is associated with a “catch-up” growth during postnatal development [3,19]. This raises the possibility that the decreased body weight in early developmental ages and the “catch-up” growth observed in the present study is caused by potential fetal malnutrition in the nicotine-treated animals. However, studies of maternal food restriction mimicking that observed in nicotine-treated animals with an equivalent decrease in maternal body weight gain showed no significant effect on body weight in offspring [16]. Taken together, these studies suggest a specific effect of fetal and neonatal nicotine exposure on early postnatal development in rats. It has been shown that low birth weight with accelerated postnatal growth is a trigger for the development of adult disease and ultimately can affect longevity [22,23].

Unlike changes of the body weight that showed “catch-up” growth, reduction of kidney weight in the offspring treated with maternal nicotine was observed not only in the early postnatal developmental ages but also in the adult in the present study. Notably, the kidney/body weight ratio was also significantly decreased at all three developmental ages determined, i.e., 14-day-old, 30-day-old, and 5-month-old. Although other factors such as nutrition restriction during pregnancy have been demonstrated to cause poor renal development in the fetus and

the offspring [4,5], to our knowledge, the present finding is the first to show that fetal and neonatal nicotine exposure impairs kidney development in offspring rats. A previous study showed that maternal administration of nicotine during pregnancy resulted in decreased kidney weight in spontaneously hypertensive male rats, but not in normotensive male rats [6]. These different findings are likely due to the difference in the nicotine treatment. In the present study, nicotine was administered to pregnant rats *via* subcutaneous osmotic minipumps throughout gestation and up to 10 days after delivery, whereas the nicotine treatment was stopped at gestational day 21 before birth in the previous study [6]. The extended exposure of the pups to nicotine *via* maternal milk together with fetal exposure in the present study may impact critically on the development of the kidney in offspring. The present finding of no significant difference in the nicotine-induced decrease in the kidney/body weight ratio between male and female offspring suggests a lack of gender difference in prenatal nicotine-mediated programming of renal development. Previous studies demonstrated the gender dimorphism in fetal programming of adult disease with males often being more susceptible than females on a diversity of measures [5,20,21]. Prenatal low-protein and glucocorticoid exposure reduced nephron numbers in the kidney in male offspring in a gender-dependent manner [22,23]. Consistent with the present finding, fetal and neonatal nicotine exposure resulted in the increased heart susceptibility to ischemia and reperfusion injury in both male and female offspring. These findings suggest different gender mechanisms of *in utero* renal programming caused by fetal nicotine as compared with fetal malnutrition and glucocorticoid exposure. However, nicotine-induced fetal programming of vascular response showed a gender difference with males being more susceptible than females [12,14], suggesting an organ and/or tissue specificity of gender-dependent programming induced by prenatal nicotine exposure. ion injury in both male and female offspring. These findings suggest different gender mechanisms of *in utero* renal programming caused by fetal nicotine as compared with fetal malnutrition and glucocorticoid exposure. However, nicotine-induced fetal programming of vascular response showed a gender difference with males being more susceptible than females [12,14], suggesting an organ and/or tissue specificity of gender-dependent programming induced by prenatal nicotine exposure.

The RAS plays an important role in physiological regulation, including renal functions [7,8]. Recent studies have shown that the development of the RAS could be affected by multiple prenatal factors. For example, when dexamethasone was administered between 26 and 28 days of gestation in sheep, the levels of mRNA for AT₁ and AT₂ receptors, as well as angiotensinogen, were increased in the kidney of ovine fetuses in late gestation [9,10]. The changes of angiotensin receptors by prenatal factors could influence postnatal life. In determination of environmental effects on the development of the renal RAS, the present study was the first to examine the impact of prenatal nicotine on the expression of renal angiotensin receptors. We tested both AT₁R and AT₂R proteins. Under the condition of exposure to maternal nicotine during the early developmental periods, AT₂R was significantly decreased in the offspring at all three tested ages (0.5, 1, and 5 months of old). However, the extent of decrease in renal AT₂R protein in the prenatal nicotine-treated group was less in 5-month-old offspring than that in 30-day-old offspring, suggesting a recovery in AT₂R during the development after the neonate stage and before the adult stage tested. This new evidence suggests that maternal smoking could affect the development of renal angiotensin receptors, and that the influence from *in utero* exposure to maternal nicotine on the protein of AT₂R persisted into adult life, even though partially recovering of AT₂R was observed in the adult offspring.

Recent progress has been made to show that prenatal insults may affect development of receptors not only at protein level, but also at gene level [11]. To further explore the mechanism of maternal nicotine-reduced renal AT₂R protein in the offspring, we examined mRNA expression of both AT₁R and AT₂R in the kidney at different ages. The results showed that

changes of AT₁R and AT₂R mRNA were consistent with their proteins (AT₁R unchanged while AT₂R significantly lowered in both neonate and adult offspring). However, unlike the protein data, the extent of decrease in renal AT₂R mRNA abundance revealed by real-time PCR in the nicotine-treated animals was not significantly different between the neonate and adult offsprings. This suggests that maternal nicotine could cause developmental problems at mRNA levels in the renal RAS, and those problems may not be corrected even though the reduced body growth and the receptor protein during fetal and neonate periods could be fully or partially recovered later. Therefore, the effect of nicotine during fetal and neonate periods on renal AT₂R in the offspring was more persistent at its transcriptional level than that at translational level.

It is interesting to note that both renal AT₂R mRNA and protein were changed by maternal nicotine in the present study. A question raised immediately from this new finding is what kind of consequence in physiological functions or health problems would be induced by the change of AT₂R observed. A number of studies have shown that most physiological and pathophysiological actions of angiotensin II are mainly *via* AT₁R [7,8], while a few reports indicated, in the kidney, both the AT₁ and AT₂ receptors contribute to the regulation of renal hemodynamic and tubular functions. AT₂ receptor possesses functions that counteract the effects of the AT₁ receptor. The balance between the AT₁ and AT₂ receptors can determine the renal status in health and disease [24,25]. Although we did not perform functional experiments in the present study, a significant increase of AT₁R/AT₂R mRNA ratio in the kidney of both neonate and adult offspring whose mothers were infused with nicotine during pregnancy and early stage of lactation indicates that renal functions could be affected.

Finally, we paid attention to gender factor in our experimental design and analysis of data, because many studies have suggested that prenatal factors induced programming problems could be different between male and female [26,27]. For example, Vickers et al. showed the prenatal influence related to leptin in determining long-term energy homeostasis is modulated by gender [26]. However, in the present study, maternal nicotine-induced renal AT₂R changes associated with reduction of kidney weight not only in both neonate and adult offspring, but also in both male and female. In light of this, the data suggest that sex hormones may not be enough to protect the kidney from the insult due to maternal smoking at the early developmental stage.

In conclusion, the present study demonstrated that maternal exposure to nicotine could reduce renal AT₂R mRNA and protein in the neonate and adult offspring. Consequently, renal AT₁R/AT₂R ratio in mRNA was significantly increased associated with low weight kidney in both male and female offspring. These novel findings provide valuable information on subtle pathophysiological changes in the kidney due to exposure to perinatal nicotine or maternal smoking. Importantly, the results also indicate that renal health problems could be affected by nicotine in the fetal and neonate developmental period, and the affected gene of the angiotensin receptors may play a critical role in “programming” renal changes in the fetal origins.

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Fig. 1. Effect of maternal nicotine administration on body (a) and kidney (b) weight in offspring: M, male; F, female; 14d, 30d, and 5m: 14-day-old, 30-day-old, and 5-month-old. * $P < 0.05$, nicotine vs. control.

Fig. 2.

Effect of maternal nicotine administration on the kidney/body weight ratio in offspring: M, male; F, female; KW/BW, the kidney/body weight ratio; 14d, 30d, and 5m: 14-day-old, 30-day-old, and 5-month-old. * $P < 0.05$, nicotine vs. control.

Fig. 3. Effect of maternal nicotine administration on kidney AT₁R (A) and AT₂R (B) protein abundance in offspring: M, male; F, female; 14d, 30d, and 5m: 14-day-old, 30-day-old, and 5-month-old. * $P < 0.05$, nicotine vs. control.

Fig. 4.

Effect of maternal nicotine administration on kidney AT₁R and AT₂R mRNA abundance in offspring: M, male; F, female; 14d, 30d, and 5m: 14-day-old, 30-day-old, and 5-month-old.

* $P < 0.05$, nicotine vs. control.

Fig. 5. Effect of maternal nicotine administration on kidney AT₁R/AT₂R mRNA ratio in offspring: M, male; F, female; 30d and 5m: 30-day-old and 5-month-old. **P* < 0.05, nicotine vs. control.

Table 1Rat AT₁R and AT₂R primers for real-time PCR analysis.

Primer sequence	
Animal: rat	
AT ₁ receptor	Forward 5'-CGGCCTTCGGATAACATGA-3' Reverse 5'-CCTGTCACTCCACCTCAAAACA-3'
AT ₂ receptor	Forward 5'-CAATCTGGCTGTGGCTGACTT-3' Reverse 5'-TGCACATCACAGGTCCAAAGA-3'