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Changed salt appetite and central angiotensin II-induced cellular activation in rat offspring following hypoxia during fetal stages

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Abstract

Hypoxia in pregnancy may induce fetal growth restriction and cause functional abnormalities during development. The present study determined the long-term influence of hypoxia in fetal life on dipsogenic behavior linked to central angiotensin (Ang) network in the offspring rats. Fetal blood pO_2 and body weight were decreased by hypoxia during pregnancy, followed by a postnatal “catch-up” growth. Subcutaneous hypertonic saline or intracerebroventricular Ang II significantly increased salt intake in the offspring prenatally exposed to hypoxia, while water intake was the same between the two groups. Ang II-induced c-fos expression was detected in the paraventricular nuclei, median preoptic nuclei, supraoptic nuclei, and subfornical organ in the brain, in association with reduced forebrain AT₂ receptor protein abundance in the offspring prenatally exposed to hypoxia. Levels of central AT₁ receptor protein were not changed between the two groups. Hypoxia during pregnancy could be linked to developmental problems related to behavioral dysfunctions in body fluid regulations in later life, in association with the change in central angiotensin II-mediated neural activation and expression of the Ang II receptor in the brain.

Keywords

Hypoxia; Appetite; Ang II receptor; Programming

1. Introduction

A number of factors, including chronic diseases (such as anaemia and hypertension), drugs, smoking, and placenta abnormality, may lead to hypoxia in pregnancy [4,12,28]. The hypothesis on fetal origins of adult diseases proposes that an altered *in utero* environment may impair fetal development and physiological functions, increasing susceptibility to diseases after birth [1,5,9,29]. A series of epidemiological and basic science studies have demonstrated that hypoxia during pregnancy has been associated with numerous adverse perinatal outcomes, including fetal growth retardation, cardiovascular diseases, eclampsia, placenta angioemphrax, and hemophthisis [5,16,30]. Recent extensive studies have demonstrated that the offspring with a history of hypoxia during pregnancy have a high incidence of cardiovascular malformations at adult stage [1,5,9]. The increased vascular

risks in the offspring were linked to changes of the renin-angiotensin system (RAS) that plays a critical role in cardiovascular and body fluid homeostasis [6].

Angiotensin (Ang) II, a major biologically active peptide of the RAS, acts through G-protein-coupled receptors of two pharmacological classes, AT₁ and AT₂ subtypes [8,11,12,31,32]. Those receptors are expressed in the brain, mediate Ang II-induced actions, including regulation of water intake and salt appetite. Recent studies have shown that environmental insults during pregnancy such as maternal hypoxia could affect the development of Ang II receptors in peripheral systems [6,16]. Nevertheless, chronic impact of hypoxia during fetal stages on dipsogenic regulations and central Ang II receptors in the offspring is unclear. Therefore, the present study sought to determine the long-term effect of hypoxia during the early developmental period on salt appetite and dipsogenic behavior associated with central Ang II-mediated cellular activation and Ang II receptors in the offspring. The information gained added new and interesting information on further understanding of mechanisms of fetal origin-adult health problems.

2. Materials and methods

2.1. Experimental animals

Time-dated pregnant Sprague-Dawley rats were randomly divided into two groups: control and hypoxia group ($n = 14$, each group). Animals in the hypoxia group were housed in a mionectic chamber with oxygen level maintained at 10.5% (normal oxygen concentration at sea level is 21%) from *day 4* to *day 21* of gestation. The chamber for hypoxia was filled with a mixture of nitrogen gas and air as previously described [5]. The normoxic control group was housed in the similar chamber filled with only room air. Food and water were provided. Maternal food intake and body weight were measured daily during pregnancy. All procedures and protocols used were approved by the Institutional Animal Care Committee and followed the guidelines by the National Institutes of Health.

2.2. Fatal experiments

On gestational day 21, half of pregnant rats ($n = 7$, each group) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A small cut was made at the middle of abdomen of the maternal rat. Fetuses were removed immediately and their body weight was measured. Fetal blood samples were collected in chilled heparinized tube by intracardiac puncture. Blood gas index were determined with a Nova analyzer (Nova Biochemical, Model *pHOx* Plus L, Waltham, MA) at 39 °C base. The remained blood samples were centrifuged at 3000 round/min for 10 min, and 20 μ l plasma was used for measurement of osmolality (mean of duplicates/each sample).

2.3. Offspring experiments

Other half of pregnant rats (380–450 g) ($n = 7$, each group) were allowed to give birth. Newborn pups were kept with their mothers until weaning. At weaning, male and female offspring were separated randomly into control group and hypoxia group. Four months after birth, offsprings were weighted and used for experiments. All animals were adapted to tap water and 0.3 M NaCl solution *ad libitum* for at least 5 days before the beginning of behavioral experiments.

2.3.1. Determination of hypertonic saline-induced water intake and salt appetite—Following 5 days of adaptation to 0.3 M NaCl salt solution, offspring were subcutaneously injected with hypertonic saline (2 M NaCl, 0.2 ml/100 g) and were tested for their drinking. Intake of water and 0.3 M NaCl solution were recorded at 5, 15, 30, 60, and 120 min following injections.

2.3.2. Determination of intracerebroventricular (i.c.v.) Ang II-induced water intake and salt appetite—Under the condition of anesthesia (ketamine and xylazine) (Hengrui Medicine, JiangSu, China), all rats were placed in a stereo-taxic apparatus, a stainless steel guide cannula was implanted just above the roof of the right lateral ventricle (coordinates with respect to bregma: 1-mm caudal and 1.5-mm lateral) [15] and was lowered 2.4 mm below the surface of the skull. The guide cannula was anchored to the skull by using acrylic dental cement. Following surgical recovery and 5-day adaptation to 0.3 M NaCl salt solution, Ang II (100 ng/rat, 1 μ l) (Sigma, St Louis, MO) was injected by inserting a stainless steel internal cannula into the guide cannula in the lateral ventricle of the offspring, and then intake of water and 0.3 M NaCl solution were measured at 5, 15, 30, 60, and 120 min after i.c.v. injections.

2.3.3. Determination of morphology index and blood values—Male and female offsprings in two groups ($n = 7$, each group) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Their body weights were measured. The brains were used for western-blot analysis. Blood samples were collected from the abdominal aorta in chilled heparinized for the measurement of blood gas index with a Nova analyzer.

2.3.4. Immunohistochemistry experiments—Male offspring brains in two groups ($n = 5$ /each group) were used for c-fos studies. Sixty minutes after i.c.v. injection of Ang II (100 ng/rat, 1 μ l), rats were perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Post-fixation was performed in the same PFA solution for 12 h, after which the brains were placed in 20% sucrose in 0.01 M phosphate buffer overnight. Thirty-micrometer coronal sections were cut through the brain on a cryostat. Every other section of the brain was used for c-fos immunoreactivity (FOS-ir) staining using the avidin–biotinperoxidase technique. Tissue sections were incubated on a gentle shaker overnight at 4 °C in the primary antibody (1:10,000, Santa Cruz Biotech, CA). The sections were further incubated in a goat anti-rabbit serum (1:200) for 1 h and then processed using the Vectastain ABC kit for 1 h (Vector Labs, Burlingame, CA) at room temperature. The sections were treated with 1 mg/ml diaminobenzidine tetrahydrochloride (0.02% hydrogen peroxide) (Sigma). All sections were mounted on slides, dehydrated in alcohol, and then coverslipped. All sliced sections with the interested areas were used for FOS-ir counting.

2.3.5. Determination of AT1R and AT2R in the offspring forebrain—The forebrains 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 μ g/ml leupeptin, and 5 μ g/ml aprotinin, pH 7.4. Homogenates were ultrasonicated for 15 s and then centrifuged at 4 °C for 10 min. Supernatants were collected and protein concentrations were determined. Samples with equal protein were loaded and separated on 10% PAGE-SDS. 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 and AT₂R (1:500, Santa Cruz Biotech; CA) overnight at 4 °C. After washing, membranes were incubated with a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3000). Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. β -Actin was blotted in the same membrane as an internal control for normalizing the relative density. Results were quantified with the Kodak electrophoresis documentation and analysis system with Kodak ID image analysis software.

2.4. Statistical analysis

Two-way ANOVA was used for analysis of food and fluids intake as well as for maternal body weight. Comparison between the treatments was determined with one-way ANOVA

followed by a Tukey's test and by *t*-test, the data were expressed as mean $\pm$ SEM, and the differences were evaluated for statistical significance.

3. Results

3.1. Fetus

All pregnancies reached their full term. Hypoxia during pregnancy did not affect the litter size, and the fetal number was the same between the control and hypoxia group. As shown in Fig. 1, maternal hypoxia during pregnancy starting at day 4 of pregnancy caused a moderate and transient increase in food intake (Fig. 1A) and a moderate decrease in maternal body weight gain (Fig. 1B) during the period of treatment ($F = 2.89$ and 2.24 , respectively, both $p > 0.05$).

At gestational day (GD) 21, fetal body weight in the hypoxia group was significantly lower than that of the control group (4.02 ± 0.12 versus 2.80 ± 0.13 , $n = 5-6$, $p < 0.05$) (Fig. 2A). Exposure to hypoxia during pregnancy significantly decreased fetal pO_2 (50.30 ± 2.20 versus 42.28 ± 2.02 , $n = 5-6$, $p < 0.05$) (Fig. 3A) and oxygen saturation ($SO_2\%$) (59.80 ± 2.35 versus 49.21 ± 1.87 , $n = 5-6$, $p < 0.05$) (Fig. 3B). It had no effect on fetal blood pH, pCO_2 , Na^+ and K^+ concentrations, osmotic pressure, and other blood values ($p > 0.05$) (Table 1).

3.2. Offspring

3.2.1. Morphology index—In both male and female offspring at 4 months old, there was no significant difference in body weight between the normoxic control and hypoxia group (male, 426.60 ± 13.34 versus 424.32 ± 7.03 ; female, 245.67 ± 5.70 versus 242.25 ± 7.31 , $n = 6-8$, $p > 0.05$) (Fig. 2B).

3.2.2. Water intake and salt appetite following subcutaneous hypertonic saline

—Following subcutaneous injection of hypertonic saline (2 M NaCl, 0.2 ml/100 g), water intake (100 g b.w.) was significantly increased in the two groups male and female offspring. However, water intake (100 g b.w.) was not significantly changed in the male and female ($F = 2.48$ and 2.17 , respectively, both $p > 0.05$) offspring between the two groups. Salt appetite (100 g b.w.) was also significantly increased in the male and female offspring exposed to prenatal hypoxia (male: $F = 9.09$, and female: $F = 9.13$, both $p < 0.05$) (Fig. 4A and B). Salt appetite in the offspring increased significantly within 60 min (control: male, 0.95 ± 0.09 ml; female, 0.97 ± 0.09 ml; hypoxia: male, 1.60 ± 0.21 ml, and female, 1.65 ± 0.22 ml) after subcutaneous injection of hypertonic saline.

3.2.3. Water intake and salt appetite following i.c.v. Ang II

—Following i.c.v. Ang II (100 ng/rat, 1 μ l), water intake (100 g b.w.) was significantly increased in both male and female offspring, however, water intake (100 g b.w.) was not significantly changed in the male and female ($F = 1.28$ and 1.19 , respectively, both $p > 0.05$) offspring between the two groups. Salt appetite (100 g b.w.) was also significantly increased in the male and female offspring exposed to prenatal hypoxia (male: $F = 12.39$, $p < 0.01$, and female: $F = 13.62$, $p < 0.01$). Salt appetite (100 g b.w.) of in the offsprings increased significantly within 30 min (control: male, 1.43 ± 0.22 ml; female, 1.31 ± 0.48 ml; hypoxia: male, 2.62 ± 0.32 ml, female, 3.02 ± 0.26 ml) after i.c.v. Ang II (Fig. 5A and B).

3.2.4. Blood values—There was no significant difference in blood Na^+ and K^+ concentrations, and osmotic pressure in the offspring between the control and the prenatal hypoxia group in both male and female. In addition, there were no significant differences in

blood pH, pO_2 , pCO_2 , $SO_2\%$, and other blood values in the offspring between the two groups ($p > 0.05$) (Table 1).

3.2.5. FOS-immunostaining—After i.c.v. Ang II (100 ng/rat, 1 μ l), a significant increase of FOS-ir was observed in the brain of the offspring prenatally exposed to hypoxia. The areas of increased FOS-ir in the brain included the paraventricular nuclei (PVN) (Fig. 6A and B, Fig. 7), median pre-optic nuclei (MnPO) (Fig. 7), supraoptic nuclei (SON) (Fig. 6C and D, Fig. 7), and subfornical organ (SFO) (Fig. 6E and F, Fig. 7) (all $p < 0.05$). However, there was no significant difference of FOS-ir in the organum vasculosum lamina terminalis (OVLT) between the two groups ($p > 0.05$).

3.2.6. Forebrain AT₁R and AT₂R protein expression—Hypoxia during pregnancy had no significant effect on AT₁R protein abundance in the forebrain of offspring at age of 4-month old, regardless of sex (Fig. 8A). In contrast, AT₂R protein abundance was significantly decreased in the forebrain of the male and female offspring with a history of prenatal hypoxia (Fig. 8B).

4. Discussion

In the present study, we found an alteration of salt appetite in response to subcutaneous hypertonic saline and i.c.v. Ang II in the offspring rats with a history of prenatal exposure to hypoxia. This behavioral change was accompanied with an increased neural activation marked by c-fos expression and altered Ang II receptor levels in the forebrain. The results provided new information on development of fatal origin-adult health problems.

A number of studies have shown that environmental insults such as undernutrition can cause fetal growth restriction and low birth weight, followed by a “catch-up” growth during postnatal development [10,23]. The present study demonstrated that exposure to hypoxia during pregnancy also caused a significant decrease of fetal body weight in rats. This change, however, disappeared at adult age in the offspring in both male and female. These observations are in agreement with the previous results obtained in the same animal model [5,9] and other studies [16], suggesting a “catch-up” growth during postnatal development in the prenatally hypoxia-treated animals. This also raises a question that the decreased fetal weight was a result from hypoxia itself or indirectly caused by possible changes in maternal feeding behavior because undernutrition is a well known cause of in utero growth restriction [16]. The measurement during pregnancy showed that food intake and body weight of the pregnant rats in the hypoxia environment were not significantly changed if compared to the control, indicating that fetal growth restriction was due to hypoxia directly, not indirectly via limitation of maternal food intake. In addition, fetal pO_2 and $SO_2\%$ levels at GD 21 were significantly decreased in the hypoxia environment. Previous studies demonstrated that “catch-up” growth in the offspring with fetal growth problems due to malnutrition during pregnancy [23]. Notably, the present study provided new information that “catch-up” growth also can occur after birth in those “hypoxia”-induced fetal growth restriction.

In the present study, we determined dipsogenic responses and salt appetite in response to hypertonic challenge following prenatal exposure to hypoxia associated with fetal developmental restriction. Subcutaneous hypertonic saline-induced water intake (thirst responses) was the same between the control and the hypoxia offsprings, while salt appetite was significantly increased in the offspring with history of prenatal exposure to hypoxia. Under normal physiological conditions, an increase of salt appetite usually is accompanied with an enhanced drinking of water [27]. However, in the offspring prenatally exposed to hypoxia, water intake was not increased like salt appetite in face of hypertonic stimulation,

indicating that adjusted mechanisms for a balance of salt and water intake may be disturbed. This is worth further studies.

Dipsogenic responses and salt appetite can be induced via multiple pathways, including osmoregulatory and central Ang II mechanisms [14]. In the present study, we found that i.c.v. Ang II produced the same responses in water and salt intake as that observed in the hypertonic saline-treated offspring. Osmoreceptor activation usually prevents hypertonic sodium intake by inhibitory mechanisms. If these mechanisms are deactivated, then osmolality may activate sodium intake and ANG II-induced sodium intake also increases [3,7]. Perhaps some inhibitory mechanisms might be impaired in the hypoxia group, which would explain the increase in hyperosmolality-induced sodium intake.

C-fos is an immediate early gene that has been widely used as a marker for cellular activation in the central nervous system [13,19,21,33–35]. Previous studies have shown that i.c.v. Ang II induced FOS-ir in the rat brain [2]. Intracerebroventricular administration of Ang II can activate the nuclei in the forebrain, including SFO, MnPO, and OVLT [13,19], those central regions are closely linked to dipsogenic regulations via Ang II-pathways [2,10,25,27]. In the present study, FOS-ir labeled cellular activation in the SFO, MnPO, SON, and PVN, not of the OVLT, was higher in the brain of the offspring exposed to hypoxia during fetal stages than that of the control, while physiological status remained stable as arterial values (pH, $p\text{CO}_2$, $p\text{O}_2$, plasma osmolality, and electrolyte levels) were not changed. Central Ang II can stimulate both water intake and salt intake via the SFO [2,10,27,31]. Blackburn et al. [3] reported that oxytocin released from the PVN could inhibit Ang II-induced sodium intake. The increased cellular activation in the SFO may be a possible reason for Ang II-enhanced salt intake in the offspring prenatally exposed to hypoxia.

Previous studies have demonstrated that the development of the RAS could be affected by environmental factors [6,16]. Changes of Ang II receptor expression by prenatal factors could influence postnatal life. In determination of hypoxia on the brain RAS, the present study was the first to examine the impact of prenatal hypoxia on the expression of Ang II receptors in the brain. We found that AT_2R , not AT_1R , was significantly decreased in the forebrain of the adult offspring exposed to hypoxia in fetal stages. This interesting and new evidence suggests that maternal hypoxia could affect the development of central angiotensin receptors and produce a persistent alteration on the protein of AT_2 into adult life.

It is established that AT_1R is the main mediator of the effect of Ang II, but AT_2R may reinforce its action [18]. A balance between AT_1R and AT_2R expression contributes to central functions in both health and diseases [10,27]. Although there was no significant increase of AT_1R proteins, AT_1R relatively increased as amount of AT_2R was decreased in the present study. This could affect actions of Ang II in the forebrain and influence salt intake or dipsogenic responses in the offspring. This finding also supports the hypothesis that developmental changes following environmental insults during fetal periods could be a trigger for influence of adult health and diseases, as well as longevity [15,26].

Finally, previous studies on fetal origin-programmed diseases have shown that there might be differences between male and female [20,24]. However, in the present study, we did not observe any significant difference in “catch-up” growth, behavioral responses, and central expression of Ang II receptors between the male and female offspring. Notably, those observations were mainly related to the central nervous system. Therefore, the results also suggest prenatal exposure to hypoxia related changes in the brain could be the same to both male and female.

In conclusion, the present study demonstrated that prenatally exposure to hypoxia could cause growth restriction in association with low oxygen levels in the fetus, and followed by a “catch-up” growth during postnatal development. In the offspring prenatally exposed to hypoxia, dipsogenic responses to osmotic challenge were changed in salt appetite, and such changes were the same as that caused by i.c.v. Ang II, indicating the behavioral change could be related to central Ang II signal pathways. Cellular activation labeled with FOS-ir in the central network in the control of dipsogenic responses was higher in the brain of the offspring exposed to hypoxia during fetal stages, in association with alterations in expression of the Ang II receptor in the forebrain. Together, the results suggest that hypoxia during pregnancy could be linked to developmental problems related to behavioral dysfunctions in body fluid regulations in later life, in association with the change in central angiotensin II-mediated neural activation and expression of the Ang II receptor in the brain. Importantly, the data also provided new information on development of fetal origin-adult health problems.

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Fig. 1.
The effect of prenatal hypoxia on maternal food intake (A) and body weight (B) during pregnancy. Control: the control rats; Hypoxia: the rats exposed to hypoxia ($p > 0.05$).

Fig. 2. The effect of prenatal hypoxia on body weight (BW) in the fetus at GD 21 (A) and the offspring at 4-month old (B). Control: the control animals; Hypoxia: exposed to hypoxia. * $p < 0.05$.

Fig. 3. The effect of prenatal hypoxia on pO_2 (A) and SO_2 % (B) in the fetus, and male/female offspring rats. Control: the control animals; hypoxia: exposed to hypoxia. GD21: gestation day 21. * $p < 0.05$.

Fig. 4.

The effect of prenatal hypoxia on salt (0.3 M NaCl salt solution) and water intake during 2 h period in the female (A) and male (B) offspring rats after subcutaneous injection of hypertonic saline (2 M NaCl, 0.2 ml/100 g). b.w.: body weight; C: the control animals, H: exposed to hypoxia. * $p < 0.05$.

Fig. 5.

The effect of prenatal hypoxia on salt (0.3 M NaCl salt solution) and water intake during 2 h period in the female (A) and male (B) offspring rats after i.c.v. Ang II (100 ng/rat, 1 μ l). b.w.: body weight; C: the control animals; H: exposed to hypoxia. * $p < 0.05$.

Fig. 6. FOS-ir induced by i.c.v. Ang II (100 ng/rat, 1 μ l) in the male offspring brain. A and B, paraventricular nuclei; C and D, supraoptic nuclei; E and F, subfornical organ. A, C, and E: the control offspring; B, D, and F: the offspring prenatally exposed to hypoxia. 100 $\times$.

Fig. 7.

The effect of i.c.v. Ang II (100 ng/rat, 1 μ l) on FOS-ir in the brain of the male offspring. Control: the control offspring; Hypoxia: the offspring prenatally exposed to hypoxia. PVN, paraventricular nuclei; MnPO, median preoptic nucleus; SON, supraoptic nuclei; SFO, subfornical organ; and OVLT, organum vasculosum of the lamina terminalis. * $p < 0.05$.

Fig. 8. The protein levels of AT₁ R (A) and AT₂ R (B) in the forebrain of the offspring. C: the control animals; H: exposed to hypoxia. * $p < 0.05$.

Table 1

The effect of maternal hypoxia during pregnancy on fetal and offspring blood values.

Index	Group			
	Fetal		Offspring	
	Control	Hypoxia	Female	Male
Ph	7.23 ± 0.01	7.29 ± 0.01	7.44 ± 0.02	7.45 ± 0.01
pCO ₂ (mm Hg)	51.84 ± 2.28	53.76 ± 1.15	33.31 ± 1.02	32.37 ± 1.51
Hemoglobin (g/dl)	10.53 ± 0.37	11.13 ± 0.25	10.02 ± 0.38	9.70 ± 0.84
Hematocrit (%)	31.27 ± 0.87	31.58 ± 0.81	35.60 ± 1.24	35.61 ± 1.68
Glucose (mmol/l)	2.94 ± 0.16	2.87 ± 0.11	8.18 ± 0.32	8.03 ± 0.49
Na ⁺ (mmol/l)	124.29 ± 0.76	124.45 ± 1.26	137.88 ± 1.73	138.23 ± 1.12
K ⁺ (mmol/l)	7.01 ± 0.25	7.18 ± 0.32	3.37 ± 0.13	3.45 ± 0.34
Osmolality (mOsm/kg)	288.86 ± 2.43	292.67 ± 2.79	302.61 ± 3.82	304.22 ± 2.50
				302.65 ± 4.31