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Original Article

Developmentally-induced Hypothyroidism Alters mRNA Expression of Cerebral Angiotensin II Type 1 and Type 2 Receptors of Offspring in a Mouse Model

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Abstract The impact of hypothyroidism on cerebral renin-angiotensin system (RAS) remains poorly understood. This study was aimed to examine the impact of methimazole (MMI)-induced hypothyroidism on mRNA expression of angiotensin II type 1 receptor-a (AT1a) and type 2 (AT2) receptors in the mouse brain. Pregnant C57BL/6J mice in hypothyroid (H) group received an administration of 0.03% MMI in the drinking water from gestational day 10, and at postnatal day (P) 7, these dams were divided into two subgroups: perinatal hypothyroid (H1) and permanent hypothyroid (H2) groups. Dams in the H1 group stopped receiving MMI, and their litters received pure water after weaning (P21). Dams in the H2 group continued to receive MMI treatment until weaning, and their litters received MMI treatment until sacrifice. The relative expressions of cerebral AT1a and AT2 mRNA were determined by quantitative real-time polymerase chain reaction (PCR). In the H1 group, the cerebral AT1a mRNA expression of offspring was significantly decreased during their first two postnatal weeks, and recovered to normal level thereafter. However, cerebral AT1a mRNA expression of H2 group was reduced persistently during postnatal development. Conversely, cerebral AT2 mRNA expression in the H2 group was significantly increased compared with the controls at P14 and P21. In conclusion, we found that developmentally-induced hypothyroidism may alter mRNA expression of cerebral AT1a and AT2 in the mouse offspring.

Key words Angiotensin II receptor; Hypothyroidism; Renin-angiotensin system; Thyroid hormone

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Introduction

The classic renin-angiotensin system (RAS) was initially described as a circulating endocrine system that mediating cardiovascular and body fluid regulation. A major advance in this field is the discovery of a complete local RAS in the brain, independent from the peripheral system.[1] The brain RAS is involved in blood pressure control, drinking behavior, sodium intake and cognitive performance.[2] The functional components of brain RAS include angiotensinogen, peptidases, angiotensin, and specific receptors.[3,4]

Among them, angiotensin II is the most powerful effector that exerts its action mainly through two principal types of angiotensin II receptors, AT1 and AT2. They are both widely expressed in the brain of the mammals and rodents, and they can be antagonised by losartan and PD123319, respectively.[5,6] In rodents, there are two homologous AT1

subtypes, AT1a and AT1b. AT1a is predominantly expressed in the central nervous system.[7,8] Most biological effects of the brain RAS including vasoconstriction, cellular growth, and proliferation are ascribed to the activation of AT1.[2] On the contrary, AT2 may counterbalance the effects of AT1, promoting vasodilatation, apoptosis, and antigrowth effects.[9,10]

Classic RAS as well as local RAS can be modulated by various kinds of hormones, especially thyroid hormone (TH).[11,12] There are growing evidences that deficiency of TH during early or adult life has direct effects on RAS functions. One clinical study revealed that the circulating angiotensin converting enzyme level was significantly reduced in children with congenital hypothyroidism.[13] With respect to local RAS, changes in its components induced by TH deficiency seem to be tissue specific. Adult hypothyroidism can cause AT1 and AT2 gene overexpression in the rat heart.[14] Chen et al[15,16] performed thyroidectomy surgery on fetal sheep and they found AT1 mRNA expression was down regulated but AT2 mRNA expression was up regulated in the kidney. In the rat brain, both perinatal and adult hypothyroidism resulted in a marked decrease of angiotensinogen mRNA.[17-19] However, little is known about the impact of TH deficiency on cerebral AT1 and AT2 expressions.

It's now well established that brain AT1 and AT2 expressions are developmentally regulated.[20-22] Therefore, in the present study, we aimed to compare the expression patterns of cerebral AT1 and AT2 mRNA between the normal mice and those with developmentally-induced hypothyroidism.

Materials and Methods

Animals and Induction of Experimental Hypothyroidism

Pregnant C57BL/6J mice were purchased from the Center of Animal Experiments at Zhejiang University. Dams were randomly assigned to the hypothyroid (H) and control (CN) groups. Dams in the H group were exposed to an anti-thyroid drug methimazole (MMI) (Sigma, USA), at a concentration of 0.03% in drinking water. MMI treatment was started at gestational day 10. And at postnatal day (P) 7, the hypothyroid dams were divided into two subgroups: perinatal hypothyroid (H1) and permanent hypothyroid (H2) groups. Dams in the H1 group stopped receiving MMI, and their litters received pure water and normal diets after weaning at P21. Dams in the H2 group continued to receive MMI treatment until weaning, after which their litters received MMI treatment until sacrifice. Both dams and their litters in the CN group received pure water and normal diet throughout the experimental period. Animals were housed in a temperature-controlled animal facility with a reversed light/dark cycle. The experimental protocol was approved by the Animal Ethics Committee of Zhejiang University.

Sample Collection

Mouse offspring were sacrificed at P7, P14, P21 and P60. To evaluate MMI-induced changes of cerebral AT1 and AT2 mRNA levels, their cerebrum were immediately removed on ice for RNA extraction. Total cerebral RNA was extracted using an AxyPrep TM Multisource Total RNA Miniprep kit (Axygen Biosciences, USA).

Serum TH Analyses

For each litter, trunk blood was collected upon decapitation and kept on ice until centrifuged to collect serum. Serum total triiodothyronine (TT3) and total thyroxine (TT4) concentrations were analysed by an IMMULITE 1000 immunoassay system (Siemens Medical Solutions, USA).

Quantitative Real-time PCR Analysis

Total RNA was reverse transcribed using a PrimeScript® RT reagent kit (TaKaRa, Japan). The target genes were AT1a (Agtr1a, 11607) and AT2 (Agtr2, 11609). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous reference gene. Their specific primer sequences are listed in Table 1. The quantitative real-time PCR analyses were performed in Sequence Detection System (SDS 7500; Version 1.4.0; Applied Biosystems, USA) using SYBR Green detection. They were performed according to the following thermo-cycling parameters: 94°C for 180 seconds, followed by 40 two-step cycles at 94°C for 20 seconds and at 60°C for 45 seconds. We used $2^{-\Delta\Delta Ct}$ method[23] to analysed the data: ΔCt=Ct(target gene)-Ct(GAPDH) and $\Delta\Delta Ct$=ΔCt(tested sample)-ΔCt(calibrator). The mean ΔCt(CN group on P7) was used as a calibrator (fold change equals one) for each gene. By using the formula that relative expression= $2^{-(\Delta Ct(\text{tested sample})-\text{mean }\Delta Ct(\text{CN group on P7}))}$, the fold change in target gene expression, normalised to GAPDH and relative to mean ΔCt (CN group on P7), was calculated for each tested sample.

Statistical Analyses

Data were analysed using SPSS 22.0 software. All biological data were examined for normal distributions. Normally distributed data were expressed as mean ±

standard deviation (SD). Differences between the normal and hypothyroid groups at P7 were compared by independent-samples *t* test. Differences between three groups were compared by one-way ANOVA and LSD multiple comparison test. *P* values <0.05 was considered significant.

Results

Changes in Serum TT3 and TT4 Levels of the Offspring (Table 2)

Serum TT3 and TT4 levels of mice offspring were evaluated at P7, P14, P21 and P60. In the H1 group, serum TT3 and TT4 levels were significantly lower than those in the CN group from birth to P14 and P21, respectively. Afterwards they reversed to normal levels. In the H2 group, both of serum TT3 and TT4 concentrations were persistently decreased by approximately 30% to 40% as compared with their controls, and the differences had statistical significances (P<0.05) at all investigated ages.

Changes in Cerebral AT1a mRNA Expressions of the Offspring (Figure 1)

AT1a mRNA expression in the normal mouse brain followed a developmental pattern with a general increase with advancing age. There was a four-fold increase in AT1a mRNA expression at P21 compared with that at P7, and the increased expression lasted into adulthood (P60). In the H1 group, the cerebral AT1a mRNA expression level was obviously decreased during the first two postnatal weeks, but it returned to normal at P21, and then significantly exceeded the control level at P60 (P<0.05). In the H2 group, there was a persistent significant reduction in cerebral AT1a mRNA expression compared with the controls during postnatal development (P<0.05).

Changes in Cerebral AT2 mRNA Expressions of the Offspring (Figure 2)

The AT2 mRNA expression in the normal mouse brain displayed an opposite developmental pattern of AT1 mRNA. In the CN group, cerebral AT2 mRNA expression

Table 1 Sequence of specific primers used for quantitative real-time PCR

	Forward	Reverse
Agtr1a	GGACACTGCCATGCCCATAAC	TGAGTGCGACTTGGCCTTTG
Agtr2	GTGCATGCGGGAGCTGAGTA	ATTGGTGCCAGTTGCGTTGA
GAPDH	CAATGTGTCCGTCGTGGATCT	TCACCACCTTCTTGATGTCATCAT

Agtr1a: Angiotensin II type 1a receptor; Agtr2: Angiotensin II type 2 receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Table 2 Effects of MMI treatment on serum TT3 and TT4 levels (nmol/L) of offspring

	Groups	Postnatal day (P)			
		P7	P14	P21	P60
TT3 (nmol/L)	*CN (n)*	*2.24±0.32 (7)*	*2.56±0.31 (8)*	*2.0±0.28 (7)*	*1.63±0.28 (7)*
	H1 (n)	*1.5±0.35 (9)**	*1.94±0.17 (7)**	*1.94±0.1 (7)*	*1.5±0.28 (7)*
	H2 (n)		*1.53±0.42 (9)*#*	*1.27±0.26 (7)*#*	*1.21±0.22 (7)**
TT4 (nmol/L)	*CN (n)*	*92.53±10.63 (7)*	*103.26±5.66 (8)*	*95.24±8.35 (7)*	*88±6.72 (7)*
	H1 (n)	*63.06±10.03 (9)**	*73±7.38 (7)**	*84.2±7.81 (7)**	*80.5±6.4 (7)*
	H2 (n)		*73.29±6.6 (9)**	*64.09±4.56 (7)*#*	*64.06±4.07 (7)*#*

Serum total triiodothyronine (TT3) and total thyroxine (TT4) concentrations were measured in the normal control (CN), perinatal hypothyroid (H1) and permanent hypothyroid (H2) groups at P 7, 14, 21 and 60.

Data are expressed as mean±SD. *indicates significant differences compared with the CN group, P<0.05. #indicates significant differences compared with the H1 group, P<0.05.

was progressively decreased during postnatal development, with a fold change of 0.29 at P60 relative to P7. At P14 and P21, we detected a significant increase in cerebral AT2 mRNA expression of the H2 group comparing with other groups (P<0.05). However, there was no significant difference in cerebral AT2 mRNA expression levels between the H1 and CN groups.

Discussion

In the normal mouse brain, AT1a mRNA expression increased gradually during lactation and then maintained a high level, whereas AT2 mRNA expression declined steadily during postnatal development. The pattern of reciprocal changes in cerebral AT1a and AT2 mRNA expression during normal maturation is consistent with several previous protein studies.[20-22] This phenomenon may be due to the negative crosstalk between AT1 and AT2 signaling. One previous *in-vivo* study reported that a sustained peripheral administration of AT2 antagonist PD123319 into adult male rats increased AT1 mRNA expression in the brain.[24] *In vitro*, AT1 blockade increased AT2 mRNA expressions in endothelial cells while overexpression of AT2 significantly decreased AT1 gene expression in vascular smooth muscle cells.[25-27]

TH is essential for normal brain development and deficiency of TH in early development leads to a series of neurological disorders.[28] The anti-thyroid drug MMI can cross the placenta freely and be excreted into milk,[29,30] so we duplicated both perinatal and persistent hypothyroid models by orally giving MMI to dams and/or offspring during different developmental time frames.[31] We observed a persistent reduction of serum TT3 and TT4 levels in the H2 group and a recovery of reduced serum TT3 and TT4 levels in the H1 group. Serum T4 crosses the blood-brain

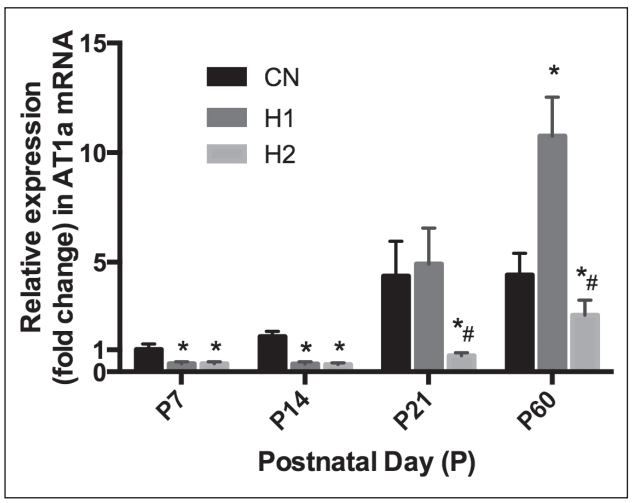

Figure 1 Effect of hypothyroidism on cerebral AT1a mRNA expression of offspring.

Total RNA was purified from mice cerebrum in normal control (CN) group, perinatal hypothyroid (H1) group and permanent hypothyroid (H2) group at postnatal day (P) 7, 14, 21 and 60. Total RNA was reverse transcribed and then analysed by quantitative real-time PCR analysis. Data of relative AT1a mRNA expression were obtained by using the $2^{-\Delta\Delta Ct}$ method. The fold change in AT1a mRNA expression, normalised to endogenous reference gene (GAPDH) and relative to mean ΔCt (CN group on P7), was calculated for each sample.

Data are expressed as mean±SD. *indicates significant differences compared with the CN group, P<0.05. #indicates significant differences compared with the H1 group, P<0.05.

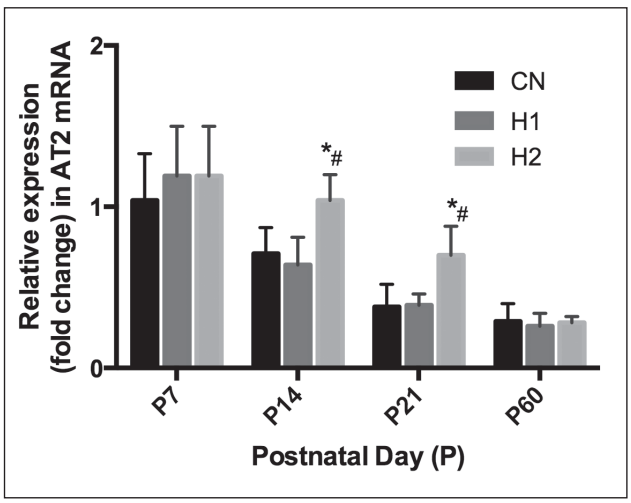

Figure 2 Effect of hypothyroidism on cerebral AT2 mRNA expression of offspring.

Total RNA was purified from mice cerebrum in normal control (CN) group, perinatal hypothyroid (H1) group and permanent hypothyroid (H2) group at postnatal day (P) 7, 14, 21 and 60. Total RNA was reverse transcribed and then analysed by quantitative real-time PCR analysis. Data of relative AT2 mRNA expression were obtained by using the $2^{-\Delta\Delta Ct}$ method. The fold change in AT2 mRNA expression, normalised to endogenous reference gene (GAPDH) and relative to mean ΔCt (CN group on P7), was calculated for each sample.

Data are expressed as mean±SD. *indicates significant differences compared with the CN group, P<0.05. #indicates significant differences compared with the H1 group, P<0.05.

barrier or blood-cerebrospinal fluid barrier more easily than T3.[32] In the brain, T4 undergoes local deiodination into active T3, which is transferred to neurons or oligodendrocytes and then binds to thyroid receptors. As a result, the declined serum TT4 levels in the hypothyroid offspring may affect brain TH levels.

TH functions by regulating target genes, the transcriptional products of which are the essential proteins underlying neurobiological events.[18,28] To our knowledge, present research findings suggest for the first time that permanent hypothyroidism starting from fetal period significantly down regulates AT1a mRNA expression but up regulates AT2 mRNA expression in the mouse brain. In the H1 group, cerebral AT1a mRNA expression was decreased during first two weeks, but later it was restored to normal and was significantly increased compared with the controls at P60. This interesting phenomenon is indicative of a compensatory of AT1a gene expression in perinatal hypothyroid offspring once anti-thyroid treatment ceases at P7.

The precise mechanism underlying the effect of hypothyroidism on brain AT1a and AT2 gene expression remains unknown. Several previous studies have demonstrated that hypothyroidism in early development results in partial arrest of astrocyte differentiation[33] and down regulates angiotensinogen gene expression in the brain.[18,19] Astrocyte is the principal cellular source of brain angiotensin II as it produces angiotensinogen; angiotensin II in turn acts on AT1 in astrocytes via different kinase signaling pathways to stimulate astrocyte proliferation.[3,4] In addition, chronic intraventricular infusion of angiotensin II has been reported to increase AT1 gene expression but decrease AT2 gene expression in the rat brain.[34,35] Accordingly, we speculate that the delayed astrocyte development and reduced angiotensinogen expression may contribute to changes of AT1a and AT2 gene expression in the hypothyroid brain.

AT2 is highly expressed in the fetal brain and declines rapidly after birth, coinciding with cessation of developmental apoptosis, suggesting a role in cell apoptosis.[20-22,36] AT2 was considered to be essential to mediate the effect of angiotensin II on enhancing apoptosis of cultured neurons from newborn rat brain.[37] In addition, the overexpression of AT2 itself is a ligand-independent signal for cell apoptosis.[38] We previously reported that the number of apoptotic neurons was increased in the hippocampus of hypothyroid rat offspring.[39] Thus, hypothyroidism-induced gene overexpression of cerebral AT2 may be implicated in the enhanced neuronal apoptosis observed in the hypothyroid brain.

There was a limitation in our study that we used mRNA expression as the indicator of AT1a and AT2 changes. However, in most studies, there is a rather good agreement in the direction of changes between their mRNA and protein expression.[14-16,35]

In conclusion, our findings indicate an important role of TH in regulating cerebral AT1a and AT2 mRNA expression. Further research work is needed to investigate the impacts of hypothyroidism on the related protein expression.

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Declaration of Interest

We declare no conflict of interest.

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