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Neuronal expression of inducible nitric oxide synthase in hypothyroid rat

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Key words: inducible nitric oxide synthase; hypothyroidism; neuronal development; real-time polymerase chain reaction; Western blot

Abstract

The expression of iNOS mRNA and protein levels was studied in hypothyroid rat cerebral cortex during early days after postnatal life.

The homogenate of the cerebral cortex was examined using Real-time reverse transcription polymerase chain reaction (real-time RT PCR) and Western blot analysis.

ΔCt in hypothyroid group on day 7, 14, 21 was 18.89±0.92, 18.83±0.99, 16.48±0.29, separately. While in control group, ΔCt was 20.32±0.92, 20.07±0.86, 17.96±0.50, separately. Meanwhile, the protein level was detected only in hypothyroid group of 59±5% on day 14. On day 21, iNOS protein expression showed a decrease ($p<0.05$) in hypothyroid group (37±3%), compared with control group (58±4%).

Our findings show the successive changes of the iNOS mRNA and protein levels in early postnatal days of hypothyroid rat brain, and confirm the cross-talk between the TH and NO signaling pathway in developing cerebral cortex of rats.

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Abbreviations:

CNS	- central nervous system
ECL	- enhanced chemiluminescence method
eNOS	- endothelial nitric oxide synthase
iNOS	- inducible nitric oxide synthase
MMI	- 2-mercapto-1-methylimid-azole
nNOS	- neuronal nitric oxide synthase
NO	- nitric oxide
NOS	- nitric oxide synthase
PCR	- polymerase chain reaction
PTU	- 6-n-propyl-2-thyouracil
ROS	- reactive oxygen species
TH	- thyroid hormone

INTRODUCTION

TH is a major physiological regulator of mammalian brain development. Thyroid status affects the maturation of the CNS and causes irreversible dysfunction of the brain in both rodents and humans (Wong & Leung 2001). A reduction or absence of TH during brain maturation yields molecular, morphological and functional alterations in the cerebral cortex, hippocampus and cerebellum (Lee *et al.* 2003). TH controls various proteins for cell differentiation, migration and gene expression (Bernal 2005). NO, a diffusible messenger molecule, is produced from the amino acid L-arginine by the members of the NOS family of proteins and has both autocrine and paracrine activities. Studies showed that NO was associated with cognitive function, its role spanning from the induction and maintenance of synaptic plasticity to the control of sleep, appetite, body temperature and neurosecretion in the CNS (Rivier 2001). The NOS family consists of three isoforms: nNOS (type I); iNOS (type II) and eNOS (type III) (Guix *et al.* 2005). nNOS and eNOS are constitutively expressed and require the formation of Ca^{2+}–calmodulin complexes for their activation, whereas iNOS exerts its activity in a Ca^{2+}-independent manner (Calabrese *et al.* 2007).

It has been confirmed that the expression of nNOS, which is abundant in brain areas, is regulated by the physiological state of the thyroid gland in developing brain (Serfozo *et al.* 2008). Though iNOS level is low in brain, NO induced by iNOS is involved in the control of neuronal and glial activation, proliferation, differentiation and survival, thus influencing the development and regeneration of the CNS (Muñoz-Fernández & Fresno 1998). There is no evidence dealing with the possible interaction between TH and iNOS expression in the nervous system. The aim of this study is to investigate the role of TH in the regulation of iNOS in the rat brain during postnatal development.

METHODS AND MATERIAL

Animals

Healthy adult C57BL/6J mice were used for the experiments. In order to stimulate a hypothyroid state, 0.03% (w:v) of MMI (Sigma, St. Louis) contained water was fed from the 10th day after mate to 7th day after the offsprings' birth. Control (sham treated) mice were fed with clean drinking water. The offsprings were sacrificed on day 7, 14 and 21 (p7, p14 and p21) under anesthesia with chloral hydrate. The cerebral cortex was collected after saline perfusion.

The study was proved by Ethic Committee of the Children's Hospital of Zhejiang University School of Medicine.

Real-time polymerase chain reaction

Real-time PCR reactions were carried out by using standard conditions in the ABI Prism 7300 thermal cycler (Applied Biosystems, Foster City, CA). GAPDH was used as the endogenous control gene for normalization. Primers of iNOS were GGCAAGACAGACTACACGAC (forward) and ATCGCCGCAGACAAACAT (reverse). GAPDH forward and reverse primers were CAATGTGTCCGTCGTGGATCT and TCACCACCTTCTTGATGTCATCAT, respectively. The PCR thermocycling conditions started with an initial denaturation step of 95 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 15 s, extension at 58 °C for 15 s and annealing at 72 °C for 35 s. ΔCt, calculated as Ct of target gene minus Ct of GAPDH, was presented for target gene mRNA levels. Hence, higher ΔCt means lower mRNA levels. ΔCt = average iNOS Ct – average GAPDH Ct, was used for calculation.

Western blot

Proteins were extracted by RIPA and the concentration was measured by BCA Protein Assay Kit. A 40 μg total protein was used for 6% SDS-PAGE electrophoresis. The proteins from the gels were blotted onto nitrocellulose membrane at 100 V for 60 min. The membranes were blocked in for 2 h, then incubated for overnight at 4 °C with diluted (1:600) iNOS anti-body (zhongshan, China). After washing three times with TBST, horseradish peroxidase-conjugated affinipure goat anti-rabbit secondary antibody (diluted in 1:3,000) was applied for 1h. The immunocoplexes were visualized by the ECL. Finally, scan the radiation autoradiography strip and analysis the protein bands with

gray-scale. The iNOS/β-actin ratio was calculated as the relative levels of iNOS. β-actin was used as the endogenous control gene for normalization.

Statistic analysis

Data reprsent mean±SEM Statistic analysis was carried out using the two-tailed Stuedent's t-test or one way ANOV with LSD multiple comparison where appropriate. $p<0.05$ was considered significant difference.

RESULTS

Effect of hypothyroid status on level of iNOS mRNA

ΔCt in hypothyroid group on day 7, 14, 21 were 18.89±0.92, 18.83±0.99, and 16.48±0.29, respectively. There was a decrease trendency (compared p14 with p7, $p>0.5$; compared p21 with p7 and p14, $p<0.001$) of Δct with aging, which was coincident with the result in control group (Δct on p7, p14 and p21 was 20.32±0.92, 20.07±0.86, 17.96±0.50, respectively). It represented the tendency of the increase of iNOS mRNA in rat developing brains. Compared hypothyroid group (18.89±0.92), iNOS mRNA expression of control group (20.32±0.92) on p7 was raised ($p<0.05$). Moreover, this difference didn't appear until on p21. Since p14, there was no difference of iNOS between hypothyroid group (18.83±0.99) and control group (20.07±0.86, $p>0.05$). On p21, hypothyroid group again (16.48±0.29) showed a significant decrease ($p<0.001$), as showed in Table 1.

Tab. 1. The mRNA levels of iNOS in 2 group at different time points (ΔCt).

	Hypothyroid group	Control group	t	*p*-value
D7	18.89	20.32	2.82	0.02
D14	18.83	20.07	2.12	0.07
D21	16.48	17.96	5.71	0.00
F-value	19.04	13.72		
p-value	0.00	0.001		
LSD	D7 vs D14 - 0.89 D7 vs D21 - 0.00 D14 vs D21 - 0.00	D7 vs D14 - 0.63 D7 vs D21 - 0.00 D14 vs D21 - 0.001		

Effect of hypothyroid status on level of iNOS protein

The iNOS antibody detected a single protein band at a molecular mass of about 130 kDa (Figure 1). Similar intensity of the β-actin bands indicated that the comparison of the iNOS levels was carried out at about the same amount of tissue proteins. iNOS protein didn't express on p7 in both hypothyroid and control rat. We only detected iNOS protein (59±5%) on p14 in hypothyroid rat, but not in control rat. On p21, protein was detected in both groups. Compared hypothyroid group (37±3%) with control group (58±4%), iNOS protein expression shows a significant decrease ($p<0.05$), as showed in Table 2.

Tab. 2. The protein levels of iNOS in 2 group at different time points.

	Hypothyroid group	Control group	t	*p*-value
D7	0.00	0.00		
D14	0.59	0.00	–21.75	0.00
D21	0.37	0.58	7.46	0.002
F-value	233.57	803.31		
p-value	0.00	0.00		
LSD	D7 vs D14 - 0.00 D7 vs D21 - 0.00 D14 vs D21 - 0.00	D7 vs D14 - 1.00 D7 vs D21 - 0.00 D14 vs D21 - 0.00		

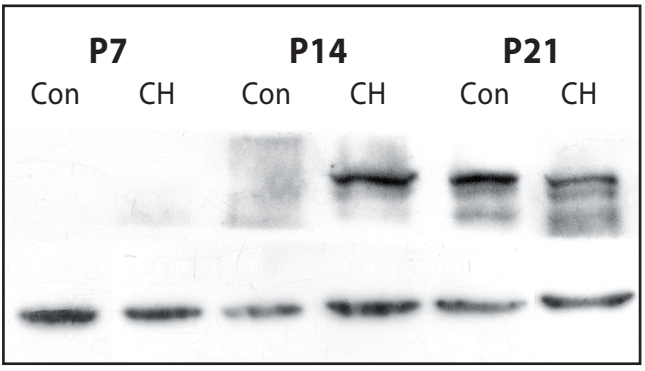

Fig. 1. Western blot analysis of the iNOS protein level in the cerebral cortex of p7, p14 and p21 hypothyroid and control rats. Animals were feeded with 0.03% MMI containing water for hypothyroid state and clean drinking water for control from the 10th day after mate to 7th day after the offsprings' birth. Data ploted for densitometric tracing are mean±SEM for three animals with the same treatment. The two-tailed Stuedent's t-test was used for stastical analysis. *$p<0.05$ hypothyroid group versus control group on p21.

DISCUSSION

In our study, we focus on the relationship between TH and iNOS on the early stage of the brain development. We found the responsiveness of the iNOS gene to TH in CNS. Though levels of iNOS in the CNS are low (Calabrese *et al.* 2007). We detected the mRNA level of iNOS showed up early on p7, and it had great increase on p21 both in normal and hypothyroid states. In hypothyroid rat, the mRNA level presented an increment at very early state of the brain on p7. The protein level of iNOS first raised in hypothyroid brains on p14, but the disparity of mRNA didn't show up on p14. The protein level of iNOS in hypothyroid brain was decreased on p21, made the protein level which is first appear in normal brain then take the advantage. On the other hand the mRNA level was still higher in hypothyroid rat on p21.

There were many evidences of the interaction of TH and iNOS in vivo and vitro. In some condition, the increased expression of iNOS displayed protective or toxic effect. Fernández et al study showed that TH triggered iNOS expression in rat liver, which could protect the liver from cytokine-mediated lethality and ROS toxicity (Fernández *et al.* 2005). On the other hand, MMI-induced hypothyroid status also could increase both the expression of mRNA and protein of iNOS at 8-wk, and the disparity enhanced at 16-wk in rat mesenteric arterie (Virdis *et al.* 2009). However, this increase would result in superoxide generation and induce endothelial dysfunction. Our findings suggested that both the mRNA and protein level of iNOS are changed in rat developing brains under hypothyroid status. These data imply that NO synthesis may depend on TH level in cerebral cortex.

Through the variation in both gene and protein levels in hypothyroid rats, we could infer that TH may regulate the process of iNOS mRNA transcription to iNOS protein expression. It caused the preferential appearance of the mRNA and the protein of iNOS under hypothyroid status at very early state. However, we see the advantage in hypothyroid rat on iNOS mRNA level, followed with the decrease on iNOS protein level on p21. So we infer that TH may control both iNOS pro-translation and translation level. Post-transcriptional regulation of iNOS gene expression is believed to occur predominantly via mechanisms that affect iNOS mRNA stability (Kiemer & Vollmar 1998).

MMI rats which characterizes as low-grade inflammation, is a well-demonstrated stimulus for iNOS induction (Kwak *et al.* 2005; Semmler *et al.* 2005). iNOS can be induced in astrocytes or microglial cells under inflammatory conditions in response to various proinflammatory stimuli (Bredt 1999;

Calabrese *et al.* 2006). Recent study shows a cross-talk between the TH and NO signaling pathway in the developing brain (Cano-Europa *et al.* 2008). T4 significantly increased nNOS protein level. In contrast, PTU decreased nNOS level both in cerebral cortex and cerebellum (Serfozo *et al.* 2008; 2009). Now we confirm that TH also regulates iNOS expression in cerebral cortex. NO induced by iNOS is involved in the control of neuronal and glial activation, proliferation, differentiation and survival, thus influencing the development and regeneration of the CNS (Muñoz-Fernández & Fresno 1999). But NO levels generated by iNOS appear to have both neuroprotective and neurotoxic effects in the nervous system (Pannu & Singh 2006). Once induction, iNOS synthesizes nanomolar concentrations of NO which is 100–1000 times those produced by nNOS. So its effects in the brain shouldn't been ignored. But the effect of iNOS is still confused. In vivo calture referred that inflammatory-activated glia would kill neurons mediated by high levels of iNOS expression in glia (Brown & Bal-Price 2003). Meanwhlie NO produced from iNOS may be protective by blocking brain cell death (Cho *et al.* 2005). And low levels of iNOS expression may also induce cell death (Borutaite & Brown 2005). We here still aren't sure whether the variance of the iNOS expression on mRNA and protein level represnets a protective or toxic role in hypothyroid status on different stages.

In conclusion, our paper first demonstrates the successive changes of the iNOS levels in early postnatal days of hypothyroid rat brain and suggests a role of iNOS in hypothyroid status. However, the exact mechanism should still be researched.

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