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Effects of Hypothydroidism on the Gene Expression of Progesterone Receptors in the Neonatal Rat Brain

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The effects of neonatal hypothyroidism on the gene expression of progesterone receptors (PRs) in the 8-day-old female rat brain were examined. The levels of PR ('A' + 'B')-, PR ('B')-, estrogen receptor (ER)- and β-actin mRNAs in the cerebral cortex and hypothalamus-preoptic area of propylthiouracil-treated rats (PTU group) or untreated rats (control group) were analyzed using a quantitative reverse transcription-polymerase chain reaction-Southern blotting assay. When the levels of PR mRNAs were calibrated by respective levels of β-actin mRNA, the levels of PR ('A' + 'B')- and PR ('B') mRNAs in the cerebral cortex of the PTU group were markedly less than those in the control group with no significant changes in the levels of PR messages in the hypothalamus-preoptic area of both groups. No significant difference in the calibrated levels of ER mRNA between both groups was found in these tissues. These results on the PR-, and ER mRNAs were essentially similar to those on the levels of PR- and ER proteins previously reported suggesting that thyroid hormones affect the transcriptional machinery of PR in the developing brain, in a region-specific manner.

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INTRODUCTION

In previous experiments [1] we have demonstrated that neonatal hypothyroidism causes a drastic decrease in the levels of progesterone receptors (PRs) in the cerebral cortex (CC) with no changes in those in the hypothalamus-preoptic area (HPOA) of the neonatal rat brain, suggesting a regulatory role of thyroid hormones in the developing rat brain cortex. The molecular basis of those region-specific modulations have not been elucidated so far. Since rat PRs have been shown to exist in two forms A and B [2], a quantitative reverse transcription-polymerase chain reaction-Southern blotting (RT-PCR-SB) assay estrogen receptor (ER) mRNA '92 [3], PR mRNA '92 [4] has been further employed for measurement of the levels of PR ('A' + 'B')- and PR ('B') mRNAs [5]. Our data on the distribution and the ontogeny of the levels of brain PR mRNAs indicated that mRNAs for two isoforms were differentially transcribed in a region-specific and stage-related manner in the rat brain [5]. In the present study, we investigated the effects of propylthiouracil (PTU)-treatment on the levels of PR ('A' + 'B')- and PR ('B') mRNAs in the CC and the HPOA of the 8-day-old female rat using the assay to elucidate the effect of neonatal hypothyroidism on the gene expression of brain PRs.

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MATERIALS AND METHODS

Animals

PTU-treated female rats ("PTU group") were prepared according to the method of Kikuyama *et al.* [6]. Briefly, female rats of the Wistar strain (SLC, Shizuoka, Japan) were fed PTU (Tokyo Kasei Chemical, Tokyo, Japan) at a concentration of 2.0% (w/w) in tap water from day 18 of pregnancy to day 8 of lactation. Normal control female rats ("control group") were obtained from females treated without PTU. Each group of the 8-day-old female rats was subdivided into 3 subgroups ($n = 3$/subgroup).

Chemicals and laboratory equipment

All reagents used in these studies were the highest grade available. Water was either double distilled and autoclaved, or treated additionally with 0.1% diethyl-

pyrocarbonate and reautoclaved. All equipment was autoclaved or sterilized at 200°C for the elimination of RNase activity. Microtubes, pipette tips and centrifugation tubes were used only once to avoid contamination with amplified or cloned genes.

RNA extraction

The rats were killed by decapitation and the CC and the HPOA were dissected. Total RNA was extracted from each tissue by the guanidium–cesium chloride ultracentrifugation method [7] and the RNA concentration was determined by UV absorption.

Reverse transcription (RT)

Total RNA (20 ng) from each tissue was reverse transcribed to synthesize single stranded cDNA. Briefly, total RNA was incubated at 42°C for 60 min with 5 U of RAV-2 reverse transcriptase (Takara, Kyoto, Japan) in a 25 μl reaction volume containing 50 mM Tris–HCl (pH 8.3), 100 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreithol (DTT), 1 mM of each dNTP and 10 μM random hexadeoxynucleotide primer (Takara).

Oligonucleotide primers

The sequences of the primers for PR ('A' + 'B') mRNAs were P7: 5′-CCATGTGGCAGATCCCA-CAGGCGTT-3′, and P8: 5′-TGGAAATTCAACA-CTCAGTGCCCGG-3′, flanking the human PR (hPR) cDNA sequence from base 3142 to 3461 as numbered by Kastner *et al.* [8] which was considered to contain 2 splicing sites judging from the chicken PR gene structure [9]. The sequences of the primers for PR ('B') mRNA were P10: 5′-TTCTCCTCCCTCTGC-CCCTA-3′, and P11: 5′-ACGTCGGACAGCGACT-GCTG-3′, flanking base 646 to 991 as numbered by Kastner *et al.* [8] and corresponding to the first exon. The sequences of the primers for ER mRNA were rE1221s: 5′-TCCTTCTAGACCCTTCAGTGAA-GCC-3′ and rE1507 as: 5′-ACATGTCAAAGA-TCTCCACCATGCC-3′, flanking the rat ER cDNA sequence from base 1221 to 1507 as numbered by Koike *et al.* [10] which was considered to contain 2 splicing sites judging from the human ER gene structure [11]. The sequences of the primers for β-actin mRNA were as follows; βAs: 5′-ATCGTGGGC-CGCCCTAGGC-3′, and βAs: 5′-TGGCCTTAGG-GTTCAGAGG-3′. The primer set flanked the rat β-actin cDNA sequence from base 100 to 343, as numbered by Nudel *et al.* [12], which consisted of the first and second exons.

Polymerase chain reaction (PCR)

The single stranded cDNA was subjected to PCR [13]. Briefly, 1 μl of cDNA (1/25 of the obtained cDNA) was amplified in a 25 μl reaction volume containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 10 mM Tris–HCl (pH 8.3), 200 μM of each dNTP, 20 μM of each primer and 1.25 U of *Taq* DNA polymerase (Perkin–Elmer Cetus, Norwalk, CT, U.S.A.). The reaction was performed for 24 [to analyze PR ('A' + 'B') mRNAs, 22 [to analyze PR ('B')- and ER mRNA] or 17 cycles (to analyze β-actin mRNA) of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min with extension of the final 72°C incubation for an additional 9 min.

Nucleotide sequencing

In order to confirm the authenticity of the reaction, the RT-PCR product was subjected to direct nucleotide sequencing using *Taq* DNA polymerase according to the dideoxy method [14]. To determine the nucleotide sequence nearby a primer, the products were subcloned into pBS M13 vector (Stratagene, La Jolla, CA, U.S.A.) and sequenced using a Sequenase DNA sequencing kit (USB, Cleveland, OH, U.S.A.).

Southern blotting (SB)

One microliter of the RT-PCR product (1/25 of the obtained product) from each tissue was electrophoresed in a 2.0% agarose gel. After the electrophoresis, the product was transferred onto a nylon membrane (Hybond N+, Amersham, Bucks, England). The membrane was prehybridized in the buffer containing

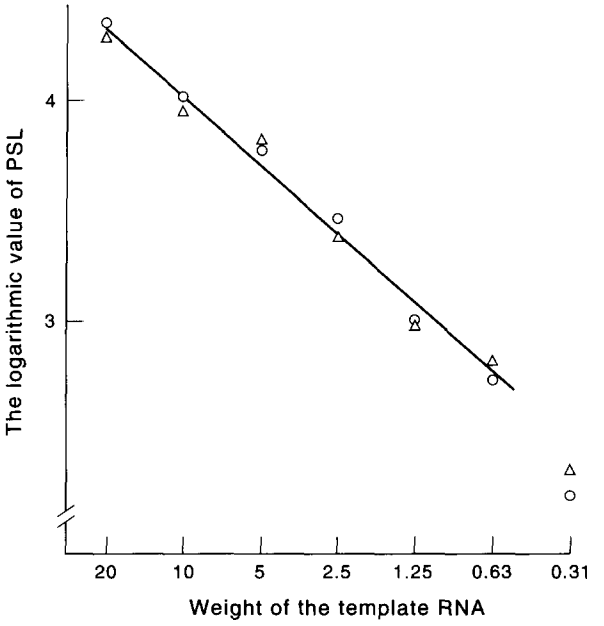

Fig. 1. A standard curve for analyzing PR ('B') mRNA. Graded diluted cortical total RNAs from the control group; 20, 10, 5, 2.5, 1.25, 0.63, and 0.31 ng; were subjected to RT-PCR-SB using primers for PR ('B') mRNA which flanked the human PR (hPR) cDNA sequence from base 646 to 991, as numbered by Kastner *et al.* [8]. The hybridization signal was analyzed by a bioimage analyzer, BAS2000 (Fuji Film). The ordinate is expressed as the logarithmic value of the radioactivity (PSL; photo stimulated luminescence, Fuji Film) of the signal and the abscissa as the logarithmic value of amount of total RNA.

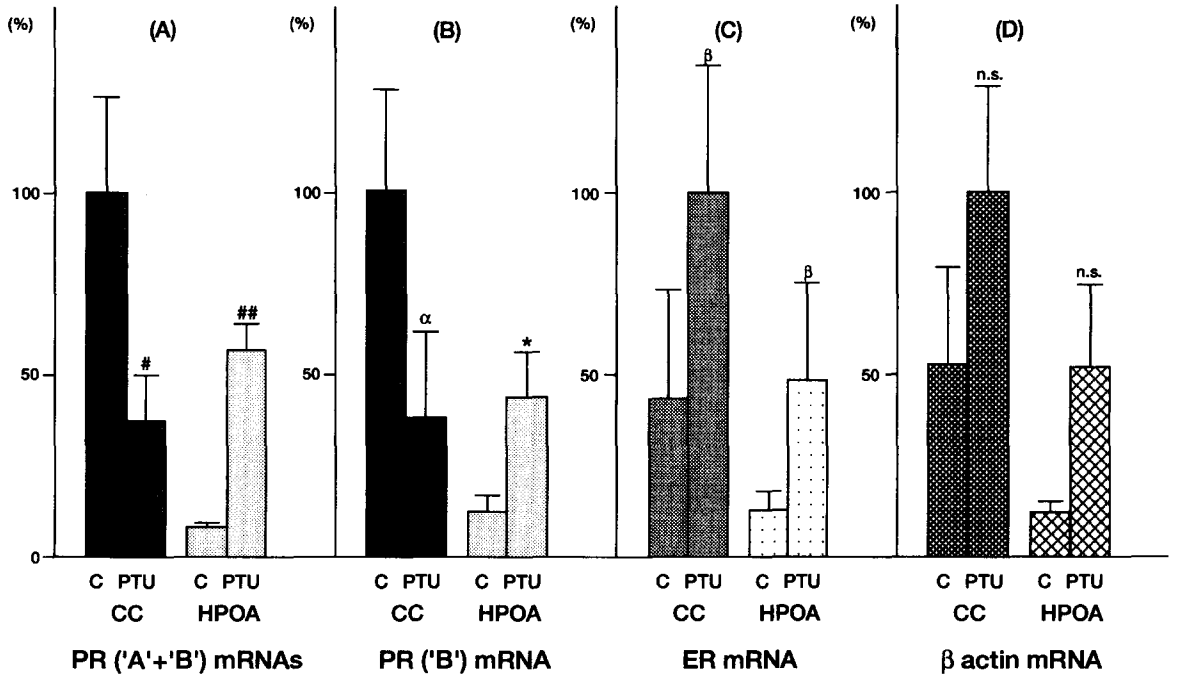

Fig. 2. Effect PTU-induced hypothyroidism on the level of PR ('A' + 'B') mRNAs (A), PR ('B') mRNA (B) ER mRNA (C) and β-actin mRNA (D). 20 ng of total RNA from the CC and the HPOA were subjected to a quantitative RT-PCR assay [3, 4] using primers specific for PR ('A' + 'B') mRNAs (A), PR ('B') mRNA (B) [5], ER mRNA (C) [3] and β-actin mRNA (D) [12]. The mean level of each mRNA obtained from the CC of the control group (PR mRNAs) or PTU group (ER- and β-actin mRNAs) was assigned a value of 100%. All other values were expressed as a percentage of this value. Each value, based on 3 subgroups ($n = 3$ in each subgroup), represents the mean and SD (vertical bar). #Significantly lower than the level of PR ('A' + 'B') mRNAs in its control group ($P < 0.01$). ##Significantly higher than the level of PR ('A' + 'B') mRNAs in its control group ($P < 0.01$). αTended to be lower than the level of PR ('B') mRNA in its control group ($P < 0.10$). *Significantly higher than the level of PR ('B') mRNAs in its control group ($P < 0.05$). βTended to be higher than the level of ER mRNA in the respective control groups ($P < 0.10$). n.s., no significant difference with respective controls.

6 × SSC (1 × SSC; 15 mM sodium chloride, 15 mM sodium citrate), 50 mg/ml denatured salmon sperm DNA, 1% sodiumdodecilsulfate (SDS) at 42°C for 3 h, followed by hybridization with ^{32}P-labeled rat PR ('A' + 'B')-, rat PR ('B')-, rat ER- or rat β-actin cDNA probe in the same buffer at 65°C for 12 h. The probes were synthesized by the random priming method using the respective RT-PCR product as a template. The membrane was rinsed twice in 2 × SSC, 1% SDS at room temperature for 10 min followed by washing twice in 0.1 × SSC, 1% SDS at 65°C for 20 min. The hybridization signal was analyzed by a bioimage analyzer, BAS2000 (Fuji Film, Tokyo, Japan).

Quantitative comparison of the levels of PR-, ER- and β-actin mRNAs

In order to compare the levels of PR-, ER- and β-actin mRNAs in a quantitative fashion, graded dilution cortical total RNAs from the control group; 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.63, 0.31, and 0.16 ng; were simultaneously subjected to RT-PCR-SB to generate a standard curve [a standard curve for analyzing PR ('B') mRNA is shown in Fig. 1]. The level of PR- and β-actin mRNAs were calculated using the standard curve. The mean level of each mRNA obtained from the CC of the control group (PR mRNAs) or PTU group (ER- and β-actin mRNAs) was assigned a value of 100% (Fig. 2). Moreover, individual data on PR ('A' + 'B')-, PR ('B')- and ER mRNAs were calibrated by respective data on β-actin mRNA. The mean calibrated level of each mRNA obtained in the CC of the control group was assigned a value of 100% (Fig. 3). All other values were expressed as a percentage of this value. Each value, based on 3 subgroups ($n = 3$ in each subgroup), represents the mean and SD.

"RT"-PCR without reverse transcriptase

As for the primer set for PR ('B') mRNA, the gene amplication of genomic DNA could occur because the set flanked the region within the first exon of PR cDNA. To rule out this possibility, the same reaction was carried out on the same samples without reverse transcriptase ("RT"-PCR).

RT-PCR blank

When distilled water as an RT-PCR blank was simultaneously subjected to RT-PCR-SB with the same reagents, no specific signal could be obtained,

indicating that no contamination of any reagents occurred in these experiments.

RESULTS AND DISCUSSION

Authenticity of the RT-PCR

Using the primers for PR ('A' + 'B')- and PR ('B') mRNAs, the RT-PCR products of 320 and 350 bp were generated respectively from the total RNA of the CC and HPOA of the control and PTU groups. The nucleotide sequences of these products were identical with those of the RT-PCR products with the same primers generated from the rat uterine total RNA which was shown by Kato *et al.* [5]. The primers for PR ('B') mRNA could have originated from genomic DNA because those flanked the region within the first exon of PR cDNA, but no specific signal was obtained by the "RT"-PCR without reverse transcriptase (data not shown). From these results, RT-PCR products from these tissues have originated from the PR mRNAs in those tissues and not genomic DNA. The authenticity of the RT-PCR product generated with the primer set for ER- and β-actin mRNAs was confirmed in the same manner [3].

Standard curve for quantitative comparison of the levels of mRNAs

The standard curves for each mRNA were derived from the radioactivities of the signals of the RT-PCR products which were generated from graded diluted cortical total RNAs from the control group. The standard curves show a linear correlation, between logarithmic values of the radioactivities (PSL; photo stimulated luminescence, BAS2000, Fuji Film) of the product signals and the weights of RNAs [a standard curve for analyzing PR ('B') mRNA is shown in Fig. 1]. These results indicated that the levels of rat cortical PR-, ER- and β-actin mRNAs could be compared with each other in a highly sensitive and quantitative fashion by RT-PCR-SB.

The effects of PTU-induced hypothyrodism on the levels of PR mRNAs in the rat brain

The levels of the PR ('A' + 'B')-, PR ('B')-, ER- and β-actin mRNAs in the CC and HPOA of the control and PTU groups were determined using the respective standard curves (Fig. 2). In the CC, the levels of PR ('A' + 'B')- and PR ('B') mRNAs in the PTU group were lower than those in the control group [Fig. 2(A and B)]. This inhibition of PR mRNAs in the CC by PTU-treatment was more distinct when each level of the messages was calibrated by respective levels of β-actin mRNA [Fig. 3(A and B)] because the level of β-actin mRNA in the CC of the PTU group tended to be higher than that of the control group [Fig. 2(D)]. On the other hand, the levels of PR mRNAs in the HPOA

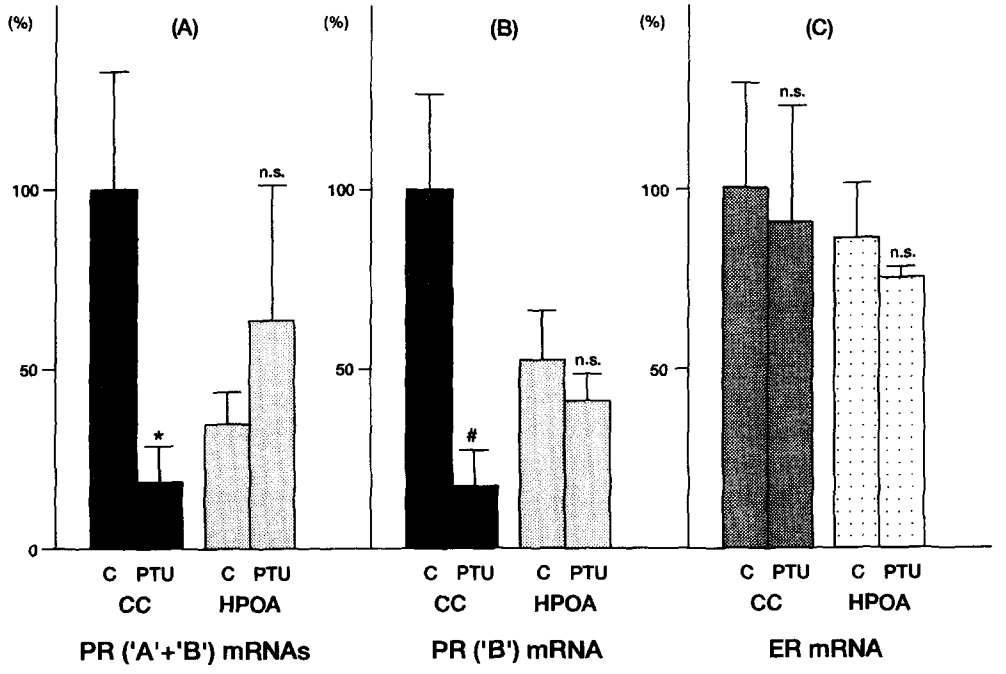

Fig. 3. Effect PTU-induced hypothyroidism on the calibrated level of PR ('A' + 'B') mRNAs (A), PR ('B') mRNA (B) and ER mRNA (C). Individual data on PR- and ER mRNAs shown in Fig. 2 were calibrated by respective data on β-actin mRNA. The mean calibrated level of each mRNA obtained in the CC of the control group was assigned a value of 100%. All other values were expressed as a percentage of this value. Each value, based on 3 subgroups ($n = 3$ in each subgroup), represents the mean and SD (vertical bar). *Significantly lower than the level of PR ('A' + 'B') mRNAs in its control group ($P < 0.05$). #Significantly lower than the level of PR ('B') mRNA in its control group ($P < 0.01$), n.s., no significant difference with respective controls.

tended to increase with PTU-treatment [Fig. 2(A, B)], however, this increase was neutralized after calibration by the levels of β-actin mRNA [Fig. 3(A and B)] because the level of β-actin mRNA in the HPOA also increased with PTU-treatment [Fig. 2(D)]. Furthermore, no significant changes in the calibrated levels of ER mRNA in both tissues were found between the control and PTU group [Fig. 3(C)], because the changes in the levels of ER mRNA in both tissues were almost similar to those of β-actin mRNA.

In the CC, a peak of PR protein around days 7–10 which was observed in postnatal development [15, 16] was markedly depressed by PTU-treatment [1]. Recently, Kato *et al.* [5] clearly demonstrated that the peak of cortical PR was caused mainly by changes in the level of PR ('B') mRNA and probably not by that in PR ('A') mRNA which remained relatively stable until days 8–12. In the present study, the level of cortical PR mRNAs significantly decreased with PTU-treatment indicating that a marked suppression of PR in the hypothyroid neonatal rat cortex was caused mainly by changes in the level of PR mRNAs. Because the level of PR ('A') mRNA still seemed to be low in the 8-day-old rat brain cortex [2], the decrease in the levels of cortical PR ('A' + 'B')- and ('B') mRNAs with PTU-treatment might be caused by the decrease in the level of the 'B' message. The calibrated levels of cortical ER mRNA show no significant change with PTU-treatment in agreement with the absence of changes in the ER protein previously reported [1]. Therefore, the inhibitory effect of PTU-induced hypothyroidism on cortical gene expression might be a non-specific event on the steroid hormone receptors but specific for PR mRNAs.

In the HPOA, the level of all PR- and ER mRNAs per total RNA increased with PTU-treatment [Fig. 2(A–C)]. However, the levels of these mRNAs per β-actin mRNA showed no significant change because of an increase in the level of β-actin mRNA with PTU-treatment. Since a similar increase in the level of β-actin mRNA was also observed in the CC [Fig. 2(D)], PTU-induced hypothyroidism might cause a decrease in ribosomal RNAs which results in an increase in the ratio of mRNAs per total RNA. Further study on the effects of hypothyroidism on β-actin mRNA and ribosomal RNAs is necessary to verify this hypothesis.

It is clearly demonstrated that PTU-induced hypothyroidism affects the gene expression of PR mRNAs in the developing rat brain in a region-specific manner, thus it was implicated that thyroid hormones may interact directly or indirectly with the upstream promoter region of the gene encoding PRs. Cloning of the upstream region of the rat PR gene is necessary to elucidate the mechanism by which thyroid hormones modulate the gene expression of PRs in the neonatal rat brain in a region-specific manner.

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