Identification of hepatic thyroid hormone-responsive genes in neonatal rats: Potential targets for thyroid hormone-disrupting chemicals

Nariaki Fujimoto[a,*], Shigeyuki Kitamura[b], Naoto Uramaru[b], Shinichi Miyagawa[c], Taisen Iguchi[d]

[a] Institute for Radiation Biology and Medicine (RIRBM), Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan
[b] Faculty of Pharmacology, Nihon Pharmaceutical University, 10281 Komuro, Ina, Kitaadachi-gun, Saitama 362-0806, Japan
[c] Institute for Advanced Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, Japan
[d] Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, Japan

ARTICLE INFO

ABSTRACT

There have been many concerns about the possible adverse effects of thyroid hormone-disrupting chemicals in the environment. Because thyroid hormones are essential for regulating the growth and differentiation of many tissues, disruption of thyroid hormones during the neonatal period of an organism might lead to permanent effects on that organism. We postulated that there are target genes that are sensitive to thyroid hormones particularly during the neonatal period and that would thus be susceptible to thyroid hormone-disrupting chemicals. Global gene expression analysis was used to identify these genes in the liver of rat neonates. The changes in hepatic gene expression were examined 24 h after administering 1.0, 10, and 100 ng/g body weight (bw) triiodothyronine (T3) to male rats on postnatal day 3. Thirteen upregulated and four downregulated genes were identified in the neonatal liver. Among these, *Pdp2* and *Slc25a25* were found to be upregulated and more sensitive to T3 than the others, whereas *Cyp7b1* and *Hdc* were found to be downregulated even at the lowest dose of 1.0 ng/g bw T3. Interestingly, when the responses of gene expression to T3 were examined in adult rats (8-week old), one-third of them did not respond to T3. The environmental chemicals with thyroid hormone-like activity, hydroxylated polybrominated diphenyl ethers, were then administered to neonatal rats to examine the effects on expression of the identified genes. The results showed that these chemicals were indeed capable of changing the expression of *Slc25a25* and *Hdc*. Our results demonstrated a series of hepatic T3-responsive genes that are more sensitive to hormones during the neonatal period than during adulthood. These genes might be the potential targets of thyroid hormone-disrupting chemicals in newborns.

1. Introduction

Several types of environmental chemicals with the ability to disrupt the function of thyroid hormones have been a concern for their possible adverse effects on thyroid hormone-related physiological functions (Boas et al., 2006; Zoeller, 2005). These chemicals include polychlorinated biphenyls (PCBs), perchlorates, polybrominated biphenyls (PBBs), polybrominated diphenyl ethers (BDEs), and phthalates and their metabolites (Jugan et al., 2010; Miller et al., 2009). Because thyroid hormones are critical to the regulation of the development and differentiation of organisms in the early stages of life, the consequences of thyroid hormone disruption are more serious in pre- and postnatal organisms than in organisms at other stages of life (Bernal, 2005; Costa et al., 2014; Smith et al., 2002). These chemicals could affect the thyroid hormone system in at least two ways. First, they could change serum thyroid hormone concentrations through mechanisms not fully understood (Ellis-Hutchings et al., 2006; Lee et al., 2010; Richardson et al., 2008). Second, they could directly bind to thyroid hormone receptors and induce or suppress the transcription of thyroid hormone-responsive gene expressions (Kojima et al., 2009; Nakamura et al., 2013). In a study that examined the effects of PCB exposure during the pre- and postneonatal periods, the expression of a thyroid hormone-responsive gene, *malic enzyme 1* (*Me1*), had increased despite the reduction in serum thyroid hormone levels (Giralt et al., 1991). A more recent study that investigated the expressions of thyroid hormone-responsive genes, including *Sopt 14*, *Me1*, and *iodothyronine deiodinase 1* (*Dio1*), showed similar inconsistent changes in gene expression owing to PCB exposure (Giera et al., 2011). These genes might not be suitable representatives of hepatic thyroid-hormone responses or useful markers for thyroid hormone disruption during the neonatal period because they are mainly expressed and hormonally regulated in adult tissues. We then postulated that the manner in which thyroid hormone is

regulated during the neonatal period would differ from that during the adult stages and that there would be a set of thyroid hormone-responsive genes regulated particularly during the neonatal period. Some of these genes might respond to lower levels of thyroid hormones or chemicals by manifesting in weak thyroid hormone-like activity.

In the present study, to identify these neonatally regulated hepatic genes, triiodothyronine (T3) was administered to F344 rats on postnatal day (PND) 3. After 24 h, the hepatic tissues were subjected to a cDNA microarray analysis. The results of our study identified a series of hepatic genes whose expressions were altered by thyroid hormones during the neonatal period. The responses were compared with those in the adult liver. The effects of the administration of hydroxyl-BDEs (OH-BDEs) to neonatal rats on the expression of the identified genes were also examined.

2. Materials and methods

2.1. Chemicals

T3 was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). T3 was dissolved in 0.01N NaOH and diluted in sterilized saline (0.9%(w/v) NaCl). The synthesis of 4-OH-BDE-42, 4′-OH-BDE-49, and 4-OH-PBDE has been previously described (Nakamura et al., 2013). Gas chromatography–mass spectrometry confirmed the purity of each chemical to be > 98%. They were dissolved in dimethyl sulfoxide (DMSO) and diluted in sterilized saline containing DMSO.

2.2. Animals

The animal experiment was approved by the Animal Experiment Committee of Hiroshima University (document # A11-17) and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals at Hiroshima University. Three-month-old pregnant F344 rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The rats were at 16–18 days of gestation, which allowed 3–5 days to acclimate to their new surroundings before delivery. They were maintained with free access to a basal diet (MF, Oriental Yeast Co., Tokyo, Japan) and tap water. Animal rooms were maintained at $23.0\,°C \pm 2.0\,°C$ with $50.0\% \pm 10.0\%$ relative humidity and a 12-h light cycle. After delivery, male pups were selected and divided into four groups. T3 was injected intraperitoneally (i.p.) into one of the three groups at 1.0, 10, or 100 ng/g body weight (bw); the fourth group was used as the control and received a saline (vehicle) solution. The groups receiving either saline, 1.0 or 100 ng/g bw T3 comprised 5 rats each; the group receiving 10 ng/g bw T3 comprised 3 animals. Each group was placed randomly with different mothers. At 24 h after administration (on PND 4), the animals were sacrificed using ether anesthesia. Whole blood was collected from the axillary vessel, left for 3 h at room temperature, and centrifuged at $1500 \times g$ for 15 min to obtain serum, which was stored at $-30\,°C$. Pieces of liver tissue were excised, quickly frozen in liquid nitrogen, and stored at $-80\,°C$. The necropsy was performed between 13:00 and 15:00 h to reduce any potential circadian-dependent variation in gene expression. For BDE administration, male pups were divided into four groups. Three of the groups received 0.5 μg/g bw i.p. 4-OH-BDE42, 4′-OH-BDE49, or 4-OH-BDE90 on PND 3; the fourth group was used as the control and received saline containing 20% (v/v) DMSO (vehicle). Each group comprised either 5 or 6 animals placed randomly with different mothers. At 24 h after administration (on PND 4), the animals were sacrificed using ether anesthesia and pieces of liver tissue were excised, quickly frozen in liquid nitrogen, and stored at $-80\,°C$. To examine the hepatic gene expression in adult animals, 7-week-old male F344 rats were purchased from Charles River Laboratories Japan and allowed to acclimate to their surroundings. At the age of 8 weeks, the animals were divided into three groups comprising 3 rats per group. T3 in saline was injected i.p. at 0, 10, or 100 ng/g bw. The rats were sacrificed using ether anesthesia

24 h after treatment. Whole blood was collected from the abdominal aorta, left for 3 h at room temperature, and centrifuged at $1500 \times g$ for 15 min to obtain serum, which was stored at $-30\,°C$. The liver tissue was excised, frozen in liquid nitrogen, and stored at $-80\,°C$.

2.3. cDNA microarray array

One animal was randomly selected from each neonatally T3 treated group (0, 1.0, 10, and 100 ng/g bw T3) and subjected to cDNA microarray array analysis. Total RNA was extracted from the liver tissue using NucleoSpin RNA II (Macherey-Nagel GmbH & Co. KG, Düren, Germany). RNA quality was verified with RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). Each 200 ng total RNA was labelled with Cy3 using a Quick Amp labeling kit 1-color (Agilent Technologies). The samples were hybridized with a Rat GE $4 \times 44\,K$ v3 Microarray Kit (Agilent Technologies) at 65 °C for 17 h. The hybridized array slide was washed according to the manufacturer's standard protocols and scanned with the G2565CA Microarray Scanner (Agilent Technologies). The scanned image was processed with Feature Expression software (Agilent Technologies) to extracts the signal data. All the gene expression data of the four different groups were normalized using 'Baseline' and 'AALoess' functions of the Agilent expression array processing package (agilp), an R/Bioconductor package (www.bioconductor.org) (Chain et al., 2010).

2.4. Determination of mRNA levels by quantitative reverse transcription polymerase chain reaction

Determination of mRNA levels by the quantitative reverse transcription–polymerase chain reaction (RT-PCR) method was described previously (Matsubara et al., 2012). Total RNA was prepared from the liver tissues using NucleoSpin RNA II, and reverse transcribed using MMLV-RT with an oligo-dT primer (3 μg of total RNA/sample). A Step One Plus RT-PCR system (Applied Biosystems/Life Technologies Co., Carlsbad, CA, USA) was used for quantitative measurement of the cDNA using a KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Inc., Wilmington, MA, USA). The mRNA levels were normalized with reference to β-actin mRNA levels that were confirmed to be similar among samples (Matsubara et al., 2012). Specific primer sets were designed for each mRNA (Table 1). Before performing the quantitative analysis, each PCR product was separately prepared and confirmed to be a single band by gel electrophoresis. The PCR fragment of each band was extracted with a Gel Extraction kit (Favorgen Biotech Corp, Ping-Tung, Taiwan), which was used as the standard for quantification. The DNA sequence was confirmed using the 310 Genetic Analyzer (Applied Biosystems/Life Technologies Co., Carlsbad, CA, USA). In addition, the specificity of PCR amplification was further confirmed by the melting curve analysis with Step One Plus RT-PCR system.

2.5. Serum total T3

Total T3 levels in serum were measured using an enzyme-linked immunosorbent assay kit from Alpco Diagnostics (Salem, NH, USA). Fifty microlitter of serum or diluted serum was used for each well of the assay. The detection range of the kit is between 0.2–10 ng/mL (the calibrator concentrations are 0, 0.2, 1, 3, and 10 ng/mL).

2.6. Statistical analyses

Student's t-test was applied to compare differences between the control group and each treated group, after the normality of distribution and the equality of variances were checked by Kolmogorov-Smirnov test and F-test, respectively. To decrease the false discovery rate in multiple comparison, the Benjamini-Hochberg procedure was applied to correct the p-values, using the R stats package (an R/Bioconductor package).