Reproductive Toxicology 28 (2009) 297–307

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Assessment of developmental effects of hypothyroidism in rats from in utero and lactation exposure to anti-thyroid agents

Makoto Shibutani[a,b,*], Gye-Hyeong Woo[a], Hitoshi Fujimoto[a], Yukie Saegusa[b], Miwa Takahashi[a], Kaoru Inoue[a], Masao Hirose[a,c], Akiyoshi Nishikawa[a]

[a] Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan
[c] Food Safety Commission, 2-13-10 Prudential Tower 6th Floor, Nagata-cho, Chiyoda-ku, Tokyo 100-8989, Japan

ARTICLE INFO

Article history:
Received 18 March 2009
Received in revised form 10 April 2009
Accepted 17 April 2009
Available online 3 May 2009

Keywords:
Developmental hypothyroidism
6-Propyl-2-thiouracil
Methimazole
Growth retardation
Neuronal migration
Oligodendroglial development

ABSTRACT

To clarify the developmental effects of hypothyroidism and to establish a detection system of resultant brain retardation, pregnant rats were administered 3 or 12 ppm of 6-propyl-2-thiouracil (PTU) or 200 ppm of methimazole (MMI) in the drinking water from gestation day 10 to postnatal day 20 and maintained after weaning until 11 weeks of age (adult stage). Offspring displayed evidence of growth retardation lasting into the adult stage, which was particularly prominent in males. Except for hypothyroidism-related thyroid follicular cell hypertrophy, most histopathological changes that appeared at the end of chemical exposure were related to growth retardation and reversed by the adult stage. A delayed onset of puberty and an adult stage gonadal enlargement occurred by exposure to anti-thyroid agents, both being especially evident in males, and this effect might be related to gonadal growth suppression during exposure. At the adult stage, the distribution variability of hippocampal CA1 pyramidal neurons reflecting mismigration could be detected in animals receiving both thyrotoxins, with a dose-dependent effect by PTU. Similarly, a reduction in the area of the corpus callosum and oligodendroglial cell numbers in the cerebral deep cortex, both reflecting impaired oligodendroglial development, were detected in rats administered both chemicals. Thus, all effects, except for impaired brain development, might be linked to systemic growth retardation, and the brain morphometric methods employed in this study may be useful to evaluate the potency of chemicals to induce hypothyroidism-related brain retardation.

1. Introduction

Groups of persistent organic pollutants (POPs), such as organochlorine pesticides and polychlorinated biphenyls (PCBs), have been shown to be ubiquitous environmental pollutants because of their great chemical stability and lipid solubility [1]. POPs have been reported to cause a variety of effects including immunologic, teratogenic, reproductive, carcinogenic, and neurological effects [2]. Also, many of these compounds are known to induce hypothyroidism [3].

Developmental hypothyroidism leads to growth retardation, neurological defects and impaired performance on a variety of behavioral learning ability [4–6]. Experimentally, rat offspring exposed maternally to anti-thyroid agents such as 6-propyl-2-thiouracil (PTU) and methimazole (MMI) show brain retardation, resulting in an impairment of neuronal migration as well as white matter hypoplasia involving limited axonal myelination and oligodendrocytic accumulation [7–9]. In humans, subclinical or mild hypothyroidism is common in women and in the elderly and has been associated with an increased incidence of depression by lowering the threshold for the development of major depressive disorders [10] and other mood disorders [11,12]. In addition, mild hypothyroidism has been linked with a diminished response to standard psychiatric treatment and with cognitive dysfunction [11]. These findings indicating that even small changes in the mother's thyroid hormone status early in pregnancy may cause adverse effects on her child and may lead to an increased concern for thyroid hormone disrupting chemicals in the environment. In addition to the effects on brain development, developmental hypothyroidism affects hearing function and the immune system [13,14].

Crosstalk between the estrogen receptors (ERs) and thyroid hormone receptors (TR) by the estrogen response element (ERE) has been reported in previous studies [15,16]. Because of the similarities in the DNA binding domain of ERE and thyroid hormone response element, TR can compete with ER on the ERE and influence transcription from ER target genes [15,16]. Therefore, there

* Corresponding author at: Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan. Tel.: +81 42 367 5874; fax: +81 42 367 5771.
E-mail address: mshibuta@cc.tuat.ac.jp (M. Shibutani).

0890-6238/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2009.04.011

is a possibility that the sexual differentiation of offspring can be affected by developmental hypothyroidism.

The present study was performed to clarify the systemic effect, including sexual differentiation, of developmental hypothyroidism as well as to establish a detection system for resultant brain retardation using rats to screen chemicals that may potentially induce developmental hypothyroidism. To distinguish chemical-specific toxicity from hypothyroidism-linked effects, two different anti-thyroid agents, PTU and MMI, were used, and dose-related responses were also examined with PTU. Both agents are known to exert inhibitory effect on thyroid hormone synthesis by interfering with thyroid peroxidase-mediated iodination of tyrosine residues in thyroglobulin [17].

2. Materials and methods

2.1. Chemicals and animals

The two chemicals, 6-propyl-2-thiouracil (PTU; CAS No. 51-52-5) and methimazole (2-mercapto-1-methylimidazole: MMI; CAS No. 60-56-0), were obtained from Sigma Chemical Co. (St. Louis, MO). Pregnant Crj:CD®(SD)IGS rats were purchased from Charles River Japan Inc. (Yokohama, Japan) at gestation day (GD) three (the day when vaginal plugs were observed was designated as GD 0). Dams were housed individually in polycarbonate cages (SK-Clean, 41.5 cm × 26 cm × 17.5 cm in size; CLEA Japan, Inc., Tokyo, Japan) with sterilized softwood chips (Sankyo Lab Service Corp., Tokyo, Japan) as bedding in a barrier-sustained animal room conditioned at 24 ± 1 °C and 55 ± 5% humidity, with a 12 h light/dark cycle. A soy-free diet (Oriental Yeast Co. Ltd., Tokyo, Japan) was chosen as the basal diet for dams to eliminate possible effects of phytoestrogens on the evaluation of this study, and water was given *ad libitum* throughout experimental period including the one-week acclimation. On the other hand, all offspring consumed a regular CRF-1 basal diet (Oriental Yeast Co. Ltd.) and water *ad libitum* from postnatal day (PND) 20 onwards (PND 0: the day of delivery). Although the formula is not open, CRF-1 contains soybean/alfalfa-derived proteins and oil including daidzin and genistin at concentrations of 87 and 102 ppm in diet according to the supplier's analysis, and coumestrol of less than 3 ppm based on the content of lucerne meal in the diet (supplier's comment). Soy-free diet was prepared based on the formulation of the NIH-07 open formula rodent diet, in which soybean meal and soy oil were replaced with ground corn, ground wheat, wheat middlings and corn oil. Values for phytoestrogens in this diet were below the detection limit (0.5 ppm), except for coumestrol with 3 ppm. Estrogen equivalents of phytoestrogens included in each CRF-1 and soy-free diet were roughly calculated as 0.91 and 0.06 ppm of β-estradiol, respectively, based on the relative binding affinities in a rat endometrial-derived experimental model [18]. Nutritional standards did not differ between SF diet and CRF-1 (supplier's analysis).

2.2. Experimental design

Dams were randomly divided into four groups including untreated controls. Eight dams per group were treated with PTU at 3 or 12 ppm or MMI at 200 ppm in the drinking water from GD 10 to PND 20. Dose finding study on PTU and MMI was preliminarily performed based on the dose range to show changes in neuronal or oligodendroglial parameters in previous reports [8,19–21]. With the dose setting at the level of 9 or 12 ppm for PTU and 200 or 250 ppm for MMI in the drinking water, dams (n = 2/dose) were treated from GD 10 to PND 20, apart from the untreated control dams (n = 2). As a result, PTU at 12 ppm and MMI at 200 ppm exhibited clear hypothyroidism-linked effects to dams, i.e., increased relative thyroid weights and thyroid follicular cell hypertrophy, but did not affect pregnancy, implantation, delivery, or nursing until PND 20 (data not shown).

In the main study, food consumption and body weight gains of dams were measured throughout the experimental period. On PND 1, the number, weights and anogenital distance (AGD) of neonates were recorded, and on PND 2, litters were culled randomly to adjust to four male and four female offspring. The offspring were weaned on PND 20. Twenty male and twenty female offspring (at least one male and one female per dam) per group were subjected to prepubertal necropsy for histopathological assessment (10 males and 10 females per group) and for other experimental purposes (10 males and 10 females per group) [22]. Other remaining males and females were allocated to four rats per cage and further maintained until they were 11 weeks old. The age and body weight at the onset of puberty as determined by vaginal opening for females and preputial separation for males were recorded for the offspring assigned for adult examination. Estrous cycles of females were examined by daily microscopic observation of vaginal smears from postnatal week (PNW) 8 to PNW 11. Classification was divided into proestrus, estrus, and diestrus, depending on whether specimens contained nucleated epithelial cells, cornified epithelial cells, or leukocytes, respectively. When estrus or diestrus continued for at least 3 or 4 days within cycles, 'extended estrus' or 'extended diestrus' was concluded [23,24]. At PNW 11, offspring were sacrificed and tissues were subjected to histopathological assessment and thyroid-related hormone measurement. Male offspring were killed on the first day of week 11. For female offspring, killing was delayed for up to 4 days after the first day of week 11 until the animal entered the diestrus stage of the estrus cycle.

The experimental animals were weighed and sacrificed by exsanguination from the abdominal aorta under deep anesthesia with ether. The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

2.3. Thyroid-related hormone measurement

At the necropsies of animals sacrificed on PND 20 and PNW 11, blood samples of male offspring were collected from the abdominal aorta under anesthesia. Serum was prepared and stored at −30 °C to measure thyroid-stimulating hormone (TSH), triiodothyronine (T_3) and thyroxine (T_4) concentrations at SRL, Inc. (Tokyo, Japan).

2.4. Histopathological assessment

At prepubertal necropsies of animals sacrificed on PND 20, the brain, liver, kidneys, thyroid, pituitary, adrenals, mammary glands, testes, epididymides, other male accessory sex glands (ventral prostate + seminal vesicle + coagulating gland + dorsolateral prostate), ovaries, uterus, and vagina were removed, and weights of the brain, liver, kidneys, adrenals, testes, epididymides, ovaries, and uterus were measured. Removed organs were fixed in 10% buffered formalin (pH 7.4) for three days at room temperature, except for brains and testes, which were fixed in Bouin's solution at room temperature overnight. For PNW 11 necropsies, the brain, liver, kidneys, thyroid, pituitary, adrenals, mammary glands, testes, epididymides, ventral prostate, other male accessory sex glands (seminal vesicle + coagulating gland + dorsolateral prostate), ovaries, uterus and vagina were removed and fixed in 10% buffered formalin for three days at room temperature, except for testes, which were fixed in Bouin's solution at room temperature overnight. Weights of all organs excluding the vagina and mammary glands were recorded before fixation except for those of the pituitary and ventral prostate, and other male accessory sex glands after fixation. Removed organs were routinely processed for paraffin embedding, sectioned at 3 μm, and stained with hematoxylin and eosin (HE) for light microscopy.

2.5. Immunohistochemistry

Brains of male offspring obtained at PNW 11 were subjected to immunohistochemistry for 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) and neuron-specific nuclear protein (NeuN) to stain oligodendrocytes and neurons, respectively. Deparaffinized coronal brain slices at the position of −3.5 mm from the bregma were serially sectioned at 3 μm. For detection of CNPase signals, microwave treatment was carried out with the deparaffinized brain sections for 10 min at 90 °C in 1×10^{-2} M citrate buffer (pH 6.0) using a microwave oven H2850 (EBSciences, East Granby, CT, USA). Nonspecific endogenous peroxidase activity was blocked by treatment with 0.3% H_2O_2 in absolute methanol for 30 min. After masking with 1.0% normal horse serum/0.01 M phosphate-buffered saline (PBS; pH 7.4), sections were exposed to mouse anti-human CNPase antibodies (1:300 in 0.5% casein/0.01 M PBS; Chemicon, Billerica, MA, USA) or mouse anti-mouse NeuN (1:1000 in 0.5% casein/0.01 M PBS; Chemicon) overnight at 4 °C and then subsequently to biotinylated secondary antibody for 60 min at room temperature. Immunodetection was carried out with the horseradish peroxidase–avidin–biotin complex method and a VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA), with 3,3′-diaminobenzidine/H_2O_2 as the chromogen. Sections were then counterstained with hematoxylin and coverslipped for microscopic examination.

2.6. Morphometric assessment

For the evaluation of the irreversible effects on neuronal migration, quantitative measurement of the variability in the distribution of neurons located within and lateral to the pyramidal cell layer of the hippocampal CA1 region was performed at PNW 11 using brain sections stained with NeuN (Fig. 1A). The mean distance of the location of neurons positive for NeuN from the innermost margin of the pyramidal cell layer adjacent to the lucid layer was bilaterally measured at 0.9 mm lateral to the boundary with the subiculum under 200× magnification (Fig. 1B). Numbers of NeuN-positive nuclei within the pyramidal cell layer and outside of this layer (polymorphic layer) were also counted in the same view area (Fig. 1C).

To evaluate the effect on oligodendroglial development, areas of the white matter tract immunoreactive for CNPase and the number of CNPase-positive oligodendrocytes surrounding myelinated axons distributed in the cerebral cortical area were measured (Fig. 2). In detail, the area of the corpus callosum medial to the cerebral white matter at the uppermost position of the cingulum was measured (Fig. 2A). Also, numbers of CNPase-positive oligodendrocytes were counted at layer V of the parietal isocortex dorsolateral to the cingulum under 200× magnification (Fig. 2B).

For the quantitative measurement of each tissue component, digital photomicrographs at each magnification were taken using a Vanox-S microscope (Olympus Optical Co., Ltd., Tokyo, Japan) attached to a Fujix Digital Camera System (Fujifilm, Tokyo, Japan), and quantitative measurements were carried out with the aid of the MacSCOPE image analysis software package (version 3.61, Mitani Corp., Fukui, Japan).