

Prolonged high iodine intake is associated with inhibition of type 2 deiodinase activity in pituitary and elevation of serum thyrotropin levels

Ningna Li^{1†}, Yaqiu Jiang^{1†}, Zhongyan Shan¹ and Weiping Teng^{2*}

¹Department of Endocrinology and Metabolism, The First Hospital of China Medical University, Shenyang, People's Republic of China

²The Endocrine Institute of China Medical University; The Liaoning Provincial Key Laboratory of Endocrine Diseases, Shenyang 110001, People's Republic of China

(Received 16 February 2011 – Revised 17 April 2011 – Accepted 31 May 2011 – First published online 28 July 2011)

Abstract

Our previous epidemiological study indicated that excessive intake of iodine could potentially lead to hypothyroidism. In the present study, we aimed to investigate the time and dose effect of iodine intake on serum thyrotropin (thyroid-stimulating hormone, TSH) levels and to explore the non-autoimmune regulation of serum TSH by pituitary type 2 deiodinase (D2). A total of 360 Wistar rats were randomly divided into five groups depending on administered iodine dosages (folds of physiological dose): normal iodine (NI), 3-fold iodine (3HI), 6-fold iodine (6HI), 10-fold iodine (10HI) and 50-fold iodine (50HI). At 4, 8, 12 and 24 weeks after administration of sodium iodide, blood was collected for serum TSH measurement by chemiluminescent immunoassay. Pituitaries were also excised for measurement of TSH β subunit expression, D2 expression and activity, monocarboxylate transporter 8 (MCT8) and thyroid hormone receptor β 2 isoform (TR β 2) levels. The results showed that iodine intake of 10HI and 50HI significantly increased pituitary and serum TSH levels from 8 to 24 weeks ($P < 0.05$ v. NI). Excess iodine had no effect on D2 mRNA or protein expression; however, 10HI and 50HI administration significantly inhibited pituitary D2 activities from 8 to 24 weeks ($P < 0.05$ v. NI). Iodine had no effect on MCT8 or TR β 2 protein levels. We conclude that prolonged high iodine intake inhibits pituitary D2 activity and induces elevation of serum TSH levels. These findings may provide a potential mechanism of iodine excess-induced overt and subclinical hypothyroidism.

Key words: Iodine: Type 2 deiodinase: Thyroid-stimulating hormone: Hypothyroidism: Pituitary gland

Iodine is an essential element for thyroid hormone synthesis, and iodine deficiency disorders seriously threaten human health. The WHO and other international health professional societies have recommended universal salt iodisation as an efficient strategy to prevent iodine deficiency disorders. However, some evidence indicates that excessive iodine intake has emerged as a result. Our previous epidemiological studies demonstrated that serum thyrotropin (thyroid-stimulating hormone, TSH) levels were increased in the normal population and that this was correlated with increased iodine intake. Furthermore, the present study results suggested that non-autoimmune factors might play an important role in the underlying mechanisms of excess iodine-induced subclinical and overt hypothyroidism^(1,2).

It is well known that negative feedback of thyroid hormone at the level of the pituitary plays an important role in the

regulation of thyroid function^(3,4). Thyroid hormones modulate pituitary TSH level with the involvement of several important proteins in the pituitary, providing a negative feedback loop. Type 2 deiodinase (D2) is an obligate outer ring 5'-deiodinase that is crucial in thyroxine (T₄) activation in several organs including the pituitary, brown adipose tissue (BAT), brain and placenta. D2 activity has also been seen in rat skin⁽⁵⁾, human thyroid (but not in rat thyroid)^(6–8) and human skeletal muscle^(9,10), while D2 mRNA is also expressed in human heart^(9,11). Triiodothyronine (T₃) is a much more potent biologically active form of thyroid hormones and is produced locally in the anterior pituitary. D2 mediates the conversion of T₄ to T₃ in the anterior pituitary. Earlier studies have shown, in thyrotropic cells of rats, that more than 50% of T₃ is locally produced^(12–16). By binding to the thyroid hormone receptor (TR) isoforms (mostly TR β 2 in rats), T₃ acts on

Abbreviations: 3HI, 3-fold iodine; 6HI, 6-fold iodine; 10HI, 10-fold iodine; 50HI, 50-fold iodine; BAT, brown adipose tissue; D1, type 1 deiodinase; D2, type 2 deiodinase; D3, type 3 deiodinase; IRD, inner ring deiodination; MCT8, monocarboxylate transporter 8; NI, normal iodine; ORD, outer ring deiodination; T₃, triiodothyronine; T₄, thyroxine; TR, thyroid hormone receptor; TSH, thyroid-stimulating hormone.

* **Corresponding author:** Professor W. Teng, fax +86 24 83283294, email twpendocrine@yahoo.com.cn

† Ningna Li and Yaqiu Jiang contributed equally to this work.

thyroid hormone response elements to inhibit TSH mRNA expression^(17,18). Monocarboxylate transporter 8 (MCT8), a thyroid hormone transporter, plays an important role in the pituitary by providing D2-expressing cells with thyroid hormone T4 as a substrate promotes T3 release from these cells^(19,20). We hypothesise that excessive iodine intake may induce elevation of plasma TSH levels by inhibition of D2 activity/expression and/or expression of MCT8 and TR β 2. In the present study, we investigated the time and dose effect of iodine intake on serum TSH level and explored the non-autoimmune regulation of serum TSH by pituitary D2, MCT8 and TR β 2.

Materials and methods

Animals

We used Wistar rats in this study because they have low autoimmunity. A total of 360 Wistar rats (male/female 1:1) weighing 120 (SEM 20) g and aged 3–4 weeks were provided by Silaike Experimental Animal Cultivation Center (Shanghai, China). The rats were kept in temperature-controlled rooms ($20 \pm 2^\circ\text{C}$) with a 12-h light–12-h dark cycle in the animal facility at the China Medical University (Shenyang, People's Republic of China). A week before and during the experiment, the rats were housed in groups according to the experimental protocol. All animal care and experimental procedures were performed according to the Guideline for Animal Experimentation with the approval of the animal ethics committee of China Medical University.

Experimental protocol

Rats were randomly divided into five groups depending on the dosage of iodine intake (fold of physiological dose): normal iodine (NI), 3-fold (3HI), 6-fold (6HI), 10-fold (10HI) and 50-fold (50HI). The dosages of NI, 3HI and 6HI groups were based on our previous epidemiological study that investigated iodine intake in three rural communities (equal to normal, three and six times of iodine physiological dose). The dosages for the 10HI and 50HI groups were established by identifying the different tolerance levels of iodine excess between rat and human subjects. Rats in all groups were fed normal chow (AIN-93 purified diets) with average iodine content of 200 $\mu\text{g}/\text{kg}$. Average iodine content of tap water in Shenyang is 5 $\mu\text{g}/\text{l}$. Considering the average dosage of normal chow to be 20 g/d and the average dosage of water to be 30 ml/d, we calculated the iodine intake dosage per d of rats in the high-iodine groups. Rats in the NI group were allowed tap water *ad libitum*, which contained an average iodine content of 5 $\mu\text{g}/\text{l}$. Rats in the high-iodine groups drank tap water with different concentrations of sodium iodide containing an iodine content of 277 $\mu\text{g}/\text{l}$ (3HI), 692 $\mu\text{g}/\text{l}$ (6HI), 1245 $\mu\text{g}/\text{l}$ (10HI) or 6778 $\mu\text{g}/\text{l}$ (50HI). Rats were killed at 4, 8, 12 and 24 weeks after iodine administration using 10% chloral hydrate (0.4 ml per 100 g body weight) by intraperitoneal injection (n 18 per time point). Blood was taken by cardiac puncture and sera were stored at -20°C until analysis. Pituitaries were also excised.

Thyroid hormone measurement

Serum TSH, T3 and T4 were measured using an automated Immulite[®] 2000 analyser with a chemiluminescent immunoassay kit (Diagnostic Products Corporation, Los Angeles, CA, USA), following the manufacturer's instructions. The between-run CV and the within-run CV were $<5\%$.

RNA preparation and real-time quantitative RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and was quantified by absorption at 260 nm. The optical density 260/280 absorbance ratio was between 1.9 and 2.0 in each RNA sample. Total RNA (500 ng) was used to prepare complementary DNA, which was synthesised (37°C for 15 min, followed by 85°C for 5 s on an ABI 9700 PCR thermal cycler; Applied Biosystems, Foster City, CA, USA) by RT using PrimeScript[™] RT reagent kit (Perfect Real Time, code DRR037A; TaKaRa, Tokyo, Japan).

Briefly, real-time PCR was performed for quantification of TSH β /D2 and glyceraldehyde-3-phosphate using the following primers: TSH β primers, 5'-AGTGTGCCTACTGCCTGAC-CATC-3' (forward) and 5'-TCCACCGTTCTGTAGGTGAAGTC-TC-3' (reverse); D2 primers, 5'-GCTCTATGACTCGGTCATTCT-GCTC-3' (forward) and 5'-GACACGTGCACCACACTGGA-3' (reverse). Glyceraldehyde-3-phosphate was used as a house-keeping internal control with the following primers: 5'-TGGTG-AAGGTCG GTGTGAAC-3' (forward) and 5'-CCATGTAGTTGA-GGTCAATGAAGG-3' (reverse). TaKaRa (Customer Service, Japan) for each mRNA assayed, a standard curve was generated using fivefold serial dilutions of the target PCR product, and the same primers were used to amplify the complementary DNA. The complementary DNA equivalent to 20 ng RNA was used for real-time PCR. Reactions were performed using SYBR[®] Premix Ex Taq[™] (Perfect Real Time, code DRR041A; TaKaRa) and cycled in a LightCycler 480 (Roche Molecular Biochemicals, Indianapolis, IN, USA). The cycle conditions were 30 s at 95°C (hot start), 5 s at 95°C and 30 s at 60°C for forty cycles followed by the melting curve protocol to verify the specificity of the amplicon. The LightCycler software (Lightcycler 480 software 1.5; Roche Applied Science, Indianapolis, IN, USA) generated a standard curve and the R^2 was greater than 0.99 for all samples. Data were analysed using Rotor-Gene Real-Time Analysis Software 6.0. (QIAGEN, Doncaster, VIC, Australia)

Preparation of pituitary homogenates for assay of type 2 deiodinase activity

After anaesthetising the rats, they were perfused with 150 ml normal saline. Rats were decapitated and pituitaries were removed. Pituitaries were snap-frozen in isopentane cooled to -40°C on dry ice, and then stored at -70°C for subsequent assays. Before use, the pituitaries were homogenised using a TissueLyser (QIAGEN, Hilden, Germany) set to 25 Hz for

3 min in 100 μ l homogenisation buffer (250 mM-sucrose, 20 mM-HEPES, 1 mM-EDTA and 2 mM-dithiothreitol (pH 7.0)).

Measurement of type 2 deiodinase activity

The D2 assay was established previously based on the release of radiiodide from the $^{125}\text{I}^-$ -labelled substrate⁽²¹⁾. Considering that T4 is a better substrate for D2, we used 100 000 cpm (5'- ^{125}I) T4 (Puerweiye, Beijing, China) as the substrate. The mixture containing 25 μ l tissue homogenate in 0.1 mM-potassium phosphate buffer (pH 7.0) and 1 mM-EDTA, and 650 μ l of substrate for a final concentration of 1 nM-T4 (Sigma Chemical Company, St Louis, MO, USA), 20 mM-cofactor dithiothreitol and 1 mM-propylthiouracil (pH 7.0) was incubated at 37°C for 1 h. The reactions were stopped by the addition of 50 μ l ice-cold 5% bovine serum albumin followed by 350 μ l 10% ice-cold TCA, and mixtures were centrifuged at 4000 g for 20 min. The supernatant was further purified by cation exchange chromatography using 1.6 ml Dowex 50 W-X2 (100–200 mesh; Sigma Chemical Company). The iodide was then eluted twice with 1 ml 10% glacial acetic acid and counted in a γ -counter. The control pituitary homogenate (buffer was used as a substitution) and the amount of $^{125}\text{I}^-$ yielded were subtracted from the sample results. Enzymatic activity was expressed in fmol $^{125}\text{I}^-$ released/h per mg protein.

Protein determination and Western blotting

For Western blotting analysis of D2 and MCT8, membrane proteins were extracted using Mem-PER[®] Eukaryotic Membrane Protein Extraction Reagent Kit (no. 89 826; Rockford, IL, USA) according to the manufacturer's instructions. For Western blotting of TR β 2, the cytoplasmic protein and the nuclear protein were extracted using NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific; no. 78 833). The protein content of tissue homogenates was determined using a Bradford assay with bovine serum albumin as a standard. SDS-PAGE was performed using 30% acrylamide (Sigma Chemical Company). After electrophoresis, gels were equilibrated for 20 min in transfer buffer (25 mM-Tris, 190 mM-glycine and 20% methanol). Proteins were transferred onto polyvinylidene difluoride membranes (0.5 h, 30 V), which were then incubated with a blocking solution (5% dried

skimmed milk in 100 mM-Tris (pH 7.5) with 140 mM-NaCl and 0.01% Tween 20) for a minimum of 1.5 h. The blots were then incubated overnight at 4°C with antibodies against D2 (DIO2, H-165, catalogue no. sc-98 716, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), MCT8 (C-18, catalogue no. sc-47 124, 1:200; Santa Cruz Biotechnology) and TR β 2 (N-16, catalogue no. sc-10 824, 1:100; Santa Cruz Biotechnology) with diluted specific antisera. After washing three times with the blocking solution, the blots were incubated with diluted horseradish peroxidase-conjugated second antibodies (1:1000) for 1 h at room temperature. Blots were washed four times and developed using an enhanced chemiluminescence kit (Thermo Scientific, catalogue no. NC15079). Reactive bands were quantified by means of the FluorChem FC2 Chemiluminescent, fluorescent and visible light gel imaging system using the vendor's software (Alpha Innotech, San Leandro, CA, USA) and β -actin was used as an internal control.

Immunohistochemistry

The pituitaries were placed in a cryomold, covered with ornithine carbamyl transferase (Tissue-Tek, Torrance, CA, USA) and snap-frozen on dry ice. Serial 10- μ m thick coronal sections were cut on a cryostat (Leica CM3050 S; Leica Microsystems GmbH, Nussloch, Germany) and adhered to SuperFrost glass slides (Haimen, Jiangsu, China). The tissue sections were stored at -70°C until immunohistochemistry was performed. Sections were fixed by immersion in 4% paraformaldehyde for 1 h at room temperature, and endogenous peroxidase was quenched with 3% H₂O₂ for 10 min. All sections were washed with 0.01 mM-PBS and incubated overnight at 4°C with the primary antibody (TSH β , FL-138, catalogue no. sc-28 917, 1:200; Santa Cruz Biotechnology). The binding of antibodies was detected using a two-step immunohistochemistry detection reagent (PV-6001, ZSGB-BIO, Beijing, China) following the manufacturer's instructions, including 30 min in a 37°C water bath. The streptavidin-peroxidase activity was revealed with a 3-3'diaminobenzidine tetrahydrochloride Substrate Kit (ZLI-9031; ZSGB-BIO). Sections were counterstained with Mayer's haematoxylin, rinsed and mounted in neutral gum (China National Medicines Corporation Limited, Beijing, China). To verify the binding specificity, we incubated some sections with the second antibody alone as a no primary control.

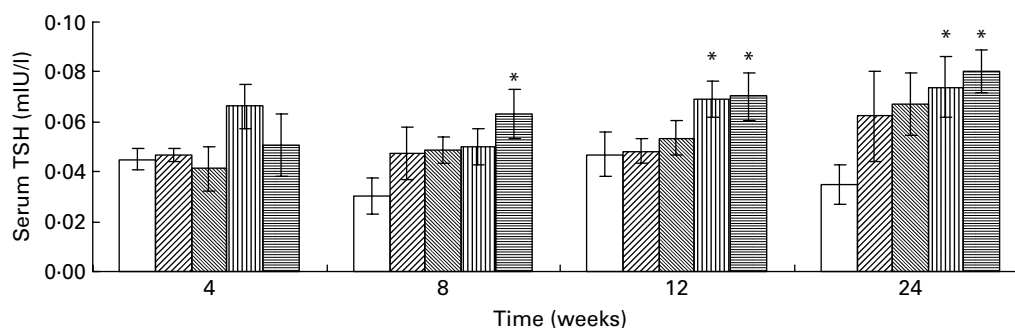


Fig. 1. Serum thyroid stimulating hormone (TSH) levels in Wistar rats administered normal iodine (NI, □) or excess iodine for 4, 8, 12 and 24 weeks. Values are means, with their standard errors represented by vertical bars. * Mean values were significantly different from those of control (NI; $P < 0.05$). 3HI (▨), 3-fold iodine; 6HI (▩), 6-fold iodine; 10HI (▮), 10-fold iodine; 50HI (▭), 50-fold iodine treatments.

Slides were viewed under a light microscope (BX51/BX52; Olympus, Tokyo, Japan). The contents of TSH β were expressed by integrated optical density value analysed using Image-Pro Plus 5.0 (Media Cybernetics, Inc., Bethesda, MD, USA).

Statistical analysis

Data were evaluated by one-way ANOVA followed by the Newman–Keuls *post hoc* test (or Dunnett's multiple comparison test, where indicated) and are presented as means and their standard errors. $P < 0.05$ was considered statistically significant.

Results

Prolonged high iodine intake elevated secretion and synthesis of thyroid stimulating hormone

As shown in Fig. 1, serum TSH level of rats increased in each high iodine intake group as expected, and the increases paralleled the increases in iodine intake time and dosage. Serum T₄ and T₃ did not change significantly in the long-term high iodine intake animals. Serum TSH levels did not change in rats that were administered iodine for 4 weeks. In the 8th week serum TSH levels doubled in the 50HI group, compared to the control group (NI, $P < 0.05$). Serum TSH of the 10HI and

50HI groups, which were administered iodine for 12 weeks, increased 1.47- and 1.49-fold, respectively, compared with that of the control group ($P < 0.05$). Serum TSH levels of the rats in the 10HI and 50HI groups, which were administered iodine for 24 weeks, had a 2.13- and 2.31-fold increase, respectively ($P < 0.05$).

We also measured the TSH β subunit (TSH β) expression in the pituitaries by immunohistochemistry and real-time quantitative RT-PCR analysis. The integrated optical density value of the TSH β immunostaining reaction in pituitaries significantly increased with high iodine intake, in accordance with the change of serum TSH (Fig. 2). In addition, TSH β mRNA expression levels exhibited the same statistically significant increasing pattern after 8, 12 and 24 weeks with high (10HI and/or 50HI) iodine intake (Fig. 3). These findings indicate that long-term high iodine intake induced not only TSH secretion into the circulation but also *de novo* synthesis by the pituitary.

We noticed that serum TSH levels of the rats in control groups changed at different time points during the experiment. Serum TSH levels in the control groups receiving iodine for 4 and 12 weeks were higher than those administered iodine for 8 and 24 weeks, but this difference did not reach statistical significance (when compared with other time points). This finding suggested that age might be associated with serum TSH levels.

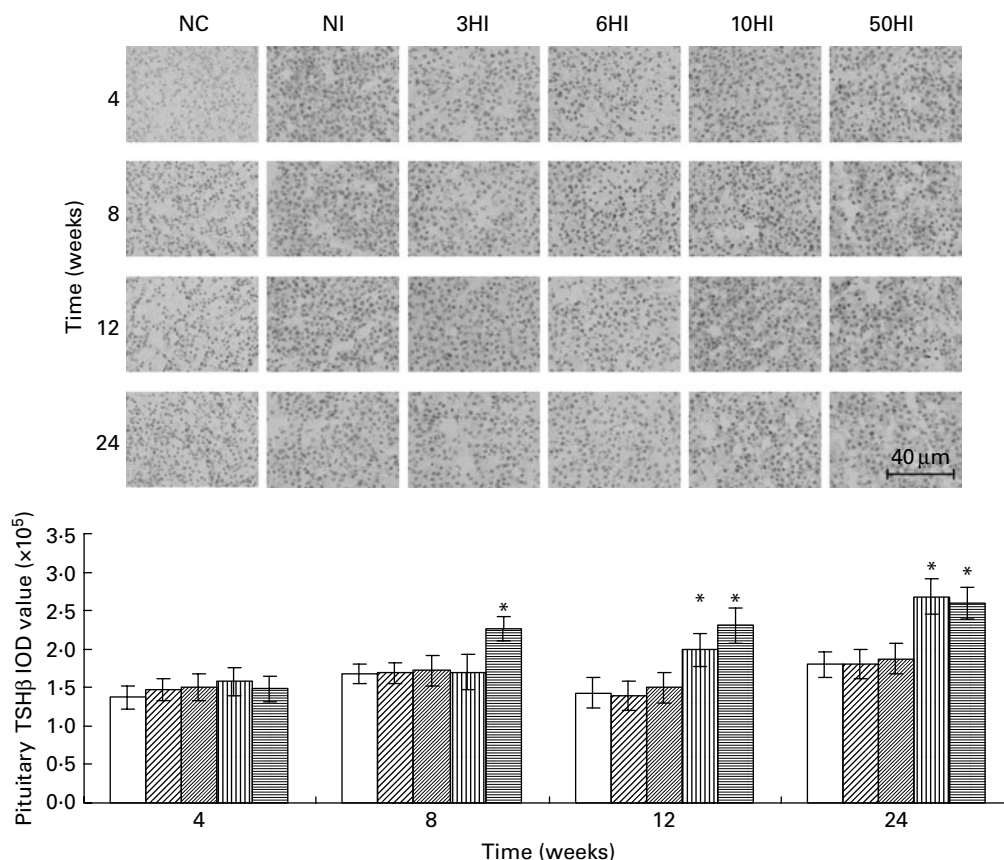


Fig. 2. Pituitary thyroid stimulating hormone (TSH) β in Wistar rats administered normal iodine (NI, \square) or excess iodine for 4, 8, 12 and 24 weeks. Scale bar = 40 μ m. Integrated optical density (IOD) value of pituitary TSH β in Wistar rats administered NI and excess iodine for 4, 8, 12 and 24 weeks. Values are means, with their standard errors represented by vertical bars. * Mean values were significantly different from those of control (NI; $P < 0.05$). NC, negative control; 3HI (\square), 3-fold iodine; 6HI (\square), 6-fold iodine; 10HI (\square), 10-fold iodine; 50HI (\square), 50-fold iodine treatments.

Prolonged high iodine intake inhibited type 2 deiodinase activity in the pituitary, but had no effect on type 2 deiodinase protein or mRNA levels

A strong decrease (approximately 25%) in D2 activity was observed in rats receiving 50HI for 8 weeks ($P < 0.05$ *v.* NI group). Remarkably, significant D2 inhibition was also detected in the 10HI and 50HI groups, which was greater than a 50% decrease at 12 weeks ($P < 0.001$), and nearly a 70% decrease at 24 weeks ($P < 0.001$ *v.* NI group) (Fig. 4).

To explore the possibility that high iodine intake may reduce the expression of D2 protein and/or mRNA levels, we tested the D2 expression in the pituitaries of rats by Western blotting and real-time quantitative RT-PCR analysis, respectively. Unexpectedly, there was no statistically significant difference in D2 mRNA and protein expression (Fig. 5) levels among excess iodine administration and NI group at each time point. The 50HI treatment group had a 70% loss of D2 activity at 24 weeks, but no significant change in D2 mRNA and protein expression. Interestingly, D2 protein levels trended downward in the 50HI group, treated for 24 weeks, but this difference did not reach statistical significance.

High iodine intake had no effect on monocarboxylate transporter 8 and thyroid hormone receptor $\beta 2$ protein levels in the pituitary

Considering the involvement of MCT8 and TR $\beta 2$ in negative regulation of thyroid hormone on TSH at the pituitary level, we examined these two proteins using Western blotting. There was no statistically significant change in MCT8 or TR $\beta 2$ protein levels during the 24-week course (Figs. 6 and 7), indicating that high iodine intake had no effect on TR $\beta 2$ and MCT8 protein expression in the pituitary.

Discussion and conclusions

In the present study, we found that rats receiving 10- and 50-fold increased iodine intake (10HI and 50HI) had

significantly higher pituitary and serum TSH levels from 8 to 24 weeks after administration ($P < 0.05$ *v.* NI). Excess iodine had no effect on D2 mRNA or protein expression; however, 10- and 50-fold increases in iodine (10HI and 50HI) significantly inhibited pituitary D2 activities from 8 to 24 weeks ($P < 0.05$ *v.* NI). Iodine had little effect on MCT8 or TR $\beta 2$ protein expression.

Our previous epidemiological study in three rural communities with different iodine intakes (median urinary iodine of 103, 374 and 615 $\mu\text{g}/\text{l}$) demonstrated that with increased iodine intake, serum TSH levels in normal populations increased⁽¹⁾. Furthermore, our other epidemiological study found that in 121 cases of subclinical hypothyroidism, only 30% of them had positive thyroid autoantibodies⁽²⁾. These data indicated that non-autoimmune factors might play an important role in the mechanism of iodine-induced subclinical and overt hypothyroidism^(1,2). To explore the non-autoimmune mechanisms of TSH elevations caused by iodine excess, Wistar rats were chosen as the study objects since they have no tendency for autoimmunity. The increases in serum TSH levels in rats receiving excess iodine were similar to the alterations in serum thyroid hormone levels that we found in human subjects⁽¹⁾. Unlike in human subjects, serum TSH levels in rats were elevated only at very high dosages of excess iodine intake, e.g. 10HI and 50HI groups. We speculate this may be a species-specific effect.

Although elevations in serum TSH levels in animal receiving excess iodine have been well documented, the underlying mechanism for these alterations is not clear⁽²²⁾.

The thyroid gland predominantly produces the prohormone T4 together with a small amount of the bioactive hormone T3. Most T3 is produced by enzymatic outer ring deiodination (ORD) of T4 in peripheral tissues. Alternatively, inner ring deiodination (IRD) of T4 yields the metabolite rT3; however, the thyroidal secretion of this metabolite is negligible. Different pathways, in particular glucuronidation and sulfation, metabolise the remainder of T4. T3 is further metabolised largely by IRD and rT3 largely by ORD, which yields the

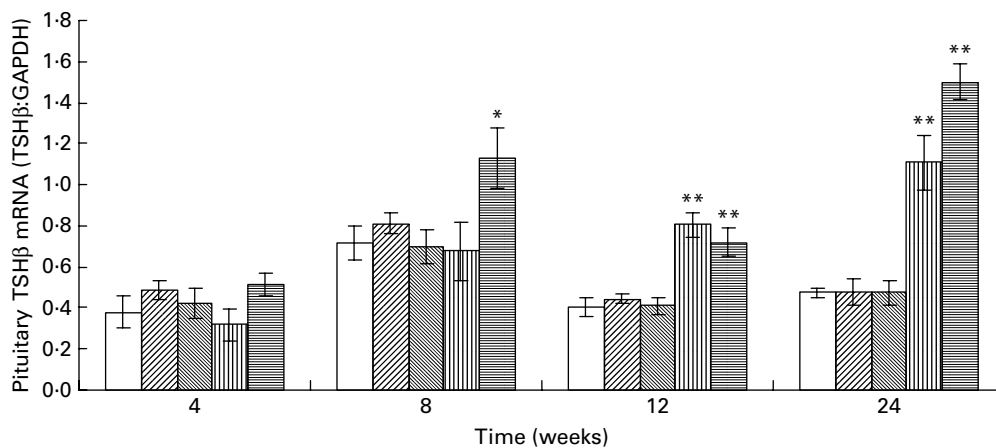


Fig. 3. Pituitary thyroid stimulating hormone (TSH) β mRNA in Wistar rats administered normal iodine (NI, □) or excess iodine for 4, 8, 12 and 24 weeks. Values are means, with their standard errors represented by vertical bars. Mean values were significantly different from those of control (NI): * $P < 0.05$, ** $P < 0.001$. 3HI (▨), 3-fold iodine; 6HI (▩), 6-fold iodine; 10HI (▧), 10-fold iodine; 50HI (▣), 50-fold iodine treatments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

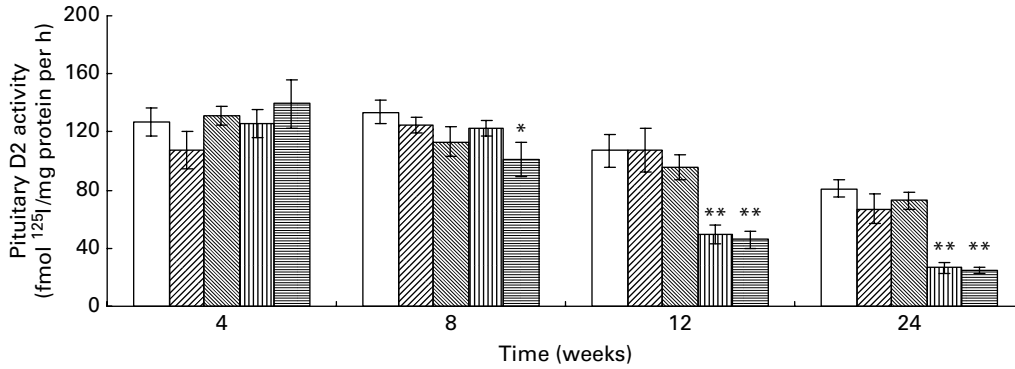


Fig. 4. Type 2 deiodinase (D2) activity in pituitaries from Wistar rats administered normal iodine (NI, □) or excess iodine for 4, 8, 12 and 24 weeks. Values are means, with their standard errors represented by vertical bars. Mean values were significantly different from those of control (NI): * $P < 0.05$, ** $P < 0.001$. 3HI (▨), 3-fold iodine; 6HI (▩), 6-fold iodine; 10HI (▧), 10-fold iodine; 50HI (▥), 50-fold iodine treatments.

metabolite 3,3'-T2 in both cases. Thus, ORD is regarded as an activating pathway and IRD as an inactivating pathway. Following three enzymes catalysing these deiodinations have been identified: type 1 (D1), type 2 (D2) and type 3 (D3) iodothyronine deiodinases. D1 has ORD and IRD activities, which are expressed mainly in the liver, kidneys and thyroid. D3 has only IRD activity, which mediates the degradation of thyroid hormone (and it expresses in human subjects, but not in rat anterior pituitary). D2 has only ORD activity, which is localised in the pituitary, BAT, brain and placenta^(12,13,19).

An earlier study reported that excessive intake of iodine arrested the cell cycle and inhibited the proliferation of FRTL-5 cells (Fisher rat thyroid line 5, a thyroid follicular cell line derived from normal rat thyroid) leading to apoptosis⁽²³⁾. In previous animal experiments, our group found that excessive iodine intake (twenty times of iodine physiological dose for 8 months) induced apoptosis of thyroid cells of rats through reactive oxygen species production, which in turn

affected the cell cycle (W. Chen, unpublished results). These findings suggest that the apoptotic effects of iodine on thyroid follicular cells might cause hypothyroidism.

In anterior pituitary of rats, T4 is taken up by D2-expressing cells (like folliculostellate cells) with the help of transporter MCT8 and is converted by D2 into T3. T3 then binds to TR especially TRβ2 in rat thyrotroph nucleus to down-regulate *TSHβ* gene expression^(20,24–26) or may be degraded locally by D1. As mentioned earlier, more than 50% of T3 is locally produced in thyrotropic cells in rats^(12–16). Moreover, in the present study, serum T4 and T3 did not change significantly in the long-term high iodine intake animals. At the same time, the serum TSH level in rats increased in each high iodine intake group as expected, and the increases paralleled the increases in iodine intake (considering both time and dosage). Taking all these factors into account, we conclude that even if high iodine intake might affect apoptosis of follicular cells in the thyroid, it might not be the major

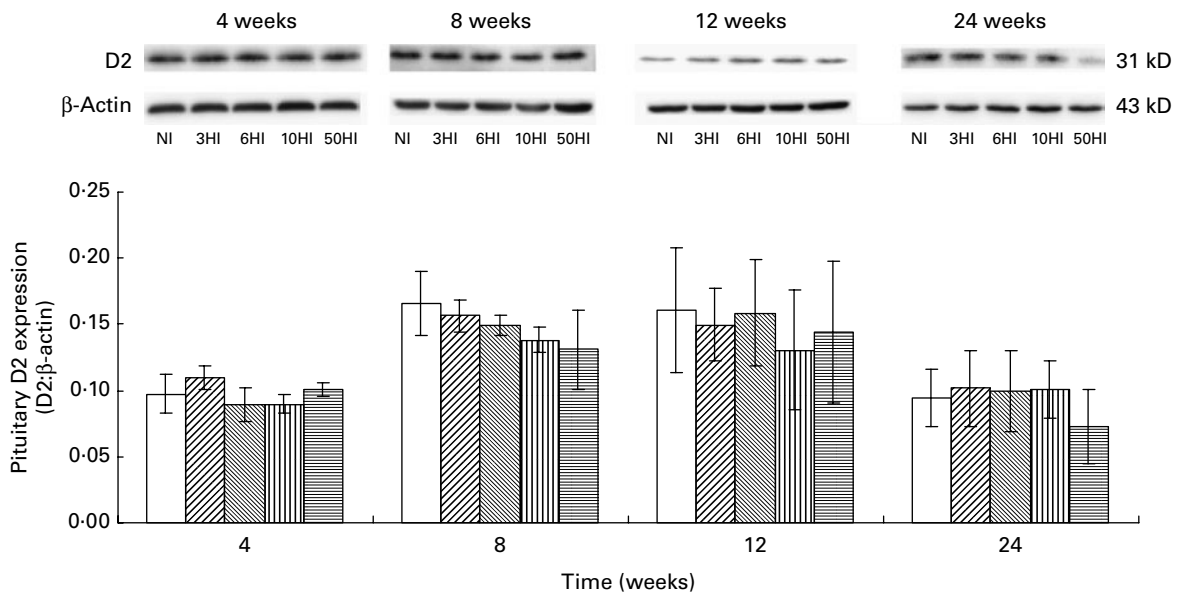


Fig. 5. Type 2 deiodinase (D2) protein expression in pituitaries from Wistar rats administered normal iodine (NI, □) or excess iodine for 4, 8, 12 and 24 weeks. Values are means, with their standard errors represented by vertical bars. 3HI (▨), 3-fold iodine; 6HI (▩), 6-fold iodine; 10HI (▧), 10-fold iodine; 50HI (▥), 50-fold iodine treatments.

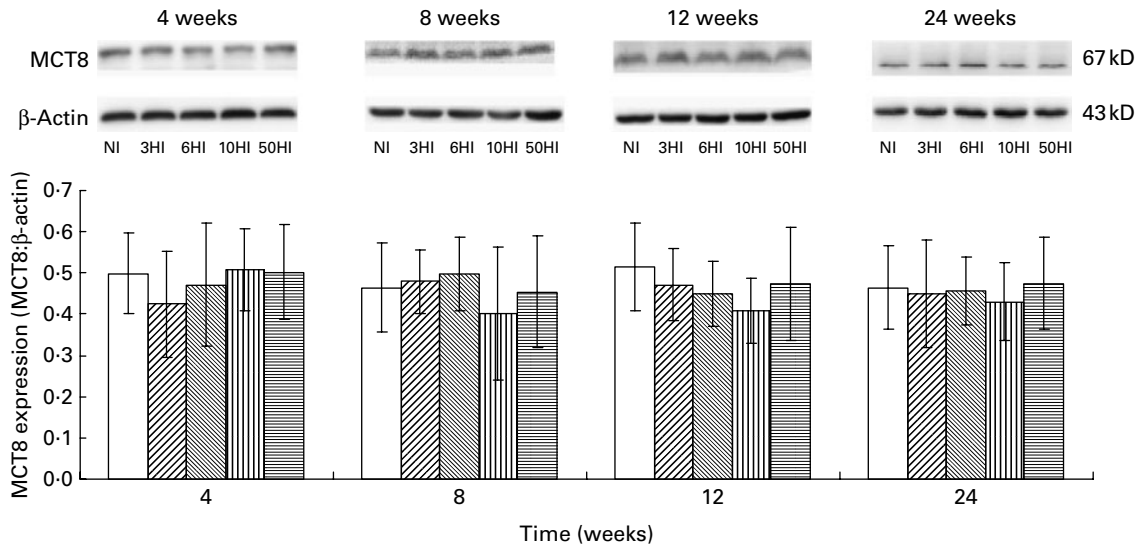


Fig. 6. Monocarboxylate transporter 8 (MCT8) expression in pituitaries from Wistar rats administered normal iodine (NI, □) or excess iodine for 4, 8, 12 and 24 weeks. Values are means, with their standard errors represented by vertical bars. 3HI (▨), 3-fold iodine; 6HI (▩), 6-fold iodine; 10HI (▧), 10-fold iodine; 50HI (▦), 50-fold iodine treatments.

reason of iodine excess-induced subclinical hypothyroidism. We suggest that high iodine intake might inhibit D2 ORD activity, and/or affect D2, MCT8 and TRβ2 protein levels in the pituitary, which will induce elevation of serum TSH levels.

In this study, D2 activity was significantly suppressed by high iodine intake in a dose- and time-dependent manner (Fig. 4). Therefore, the local intracellular level of T3 through conversion from T4 by D2 was reduced in the pituitary. As a result, the negative feedback regulation of T3 on TSHβ mRNA was attenuated. This was further supported by TSHβ mRNA and protein quantification in pituitary tissues (Figs. 2 and 3).

To our knowledge, a few experimental studies have provided a link regarding interaction of iodine deficiency and D2 enzyme. D2 activities are increased in the cerebral

cortex^(27–29), cerebellum, BAT^(27,30,31) and pituitary⁽³⁰⁾ of rats due to varying levels of iodine deficiency. Numerous experiments have confirmed decreases in D1 mRNA expression or activity in tissues (e.g. thyroid and liver) of iodine excess-treated animals^(32–35). Few experiments have focused on the influence of excess iodine intake on activity and/or mRNA expression of D2 in the pituitary, which plays a critical role in the negative feedback of TSH^(36,37). In our study, D2 activity was remarkably decreased without significant changes in D2 mRNA or protein levels. This is different from the effect of high iodine intake on D1 mRNA in many types of tissues. This discrepancy between D2 expression and activities suggests an important contribution of post-translational modulation by high iodine intake. The precise molecular mechanism by which iodine acts on D2 activity needs to be

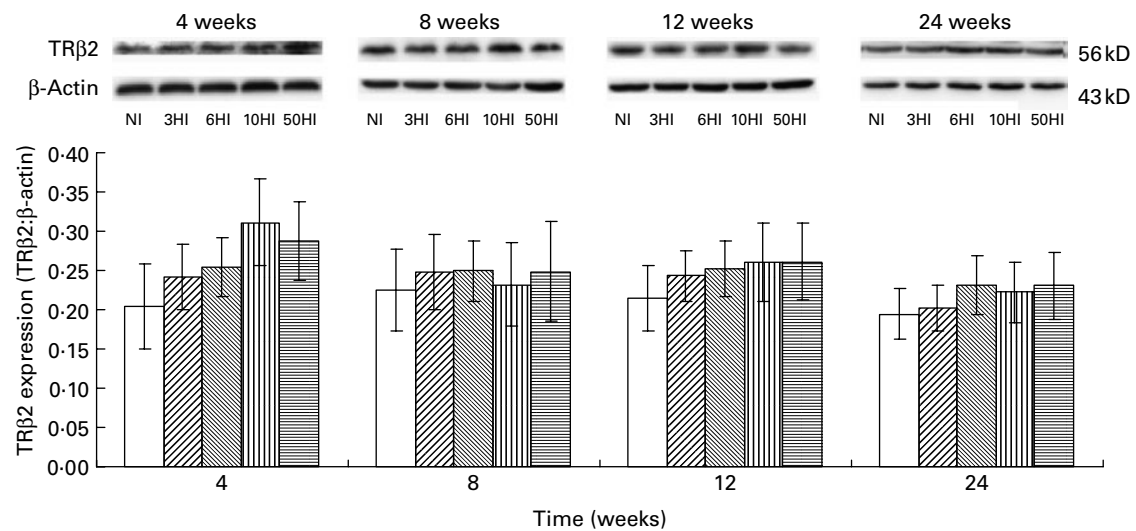


Fig. 7. Thyroid hormone receptor (TR)β2 expression in pituitaries from Wistar rats administered normal iodine (NI, □) or excess iodine for 4, 8, 12 and 24 weeks. Values are means, with their standard errors represented by vertical bars. 3HI (▨), 3-fold iodine; 6HI (▩), 6-fold iodine; 10HI (▧), 10-fold iodine; 50HI (▦), 50-fold iodine treatments.

fully elucidated in the future. The effects of excess iodine on D2 activity and protein levels in other tissues (i.e. BAT and brain) should be analysed in future experiments to clarify if this effect is pituitary specific.

However, the interaction between D2 and TSH or TH is complicated. In general, D2 activity is increased in hypothyroidism and decreased in hyperthyroidism^(13,26), and D2 mRNA levels in the brain, pituitary and BAT are up-regulated in hypothyroid rats and down-regulated in hyperthyroid animals^(38–40). In our study, D2 activity was remarkably decreased and serum TSH levels were significantly elevated in the same groups. It is likely that iodine has direct effects on D2 activity and inhibition of D2 activity by iodine intake results in elevated serum TSH.

In addition, MCT8 and TR β 2 were investigated in this study. MCT8 has recently been identified as an important thyroid hormone transporter in the pituitary by providing cells D2^(19,20). Evidence indicates that negative regulation by thyroid hormone is mediated by nuclear TR acting on thyroid hormone response elements. TR β 2-selective knockout mice exhibited elevated levels of TH and TSH, suggesting that TR β 2 plays a major role^(17,18,41). However, neither protein changed significantly by high iodine intake.

Effects of age on serum TSH levels have not been elucidated at this time. Age was observed to be inversely associated with serum TSH level in some studies^(42,43), while other findings suggested a shift to higher serum TSH level with increase in age^(44,45). Our previous epidemiological study found that age was not associated with serum TSH levels in general. However, the mean in the youngest age group (14- to 19-year olds) was approximately 20–30% higher than those in other age decade groups⁽⁴⁶⁾. A few reports of increased TSH in ageing rats have been presented^(47,48). Animal experiments in recent years have indicated that serum TSH levels in young and old rats are not different⁽⁴⁹⁾. However, the present study was unable to confirm either of the aforementioned findings. The mechanisms regulating age effects on serum TSH levels should be studied in the future.

In conclusion, prolonged high iodine intake inhibits pituitary D2 activity without affecting D2 mRNA or protein expression, which will finally induce elevation of pituitary TSH synthesis and serum TSH levels in a dose- and time-dependent manner. It is indicated that high iodine intake inhibits D2 activity through a post-translational mechanism. These findings may provide a potential cause and mechanism of iodine excess-induced overt and subclinical hypothyroidism.

Acknowledgements

This work was supported by China Guanghua Foundation (grant no. 2007(02)). N. L., Y. J. and W. T. conceived and designed the study. N. L. and Y. J. carried out analysis and interpretation. N. L. carried out the data collection, statistical analysis, writing and critical revision of the article. Z. S. and W. T. finally approved the manuscript. Z. S. and W. T. obtained the funding. W. T. takes the overall responsibility. The authors have no conflicting interests.

References

1. Teng W, Shan Z, Teng X, *et al.* (2006) Effect of iodine intake on thyroid diseases in China. *N Engl J Med* **354**, 2783–2793.
2. Shan Z, Teng W, Li Y, *et al.* (2001) Comparative epidemiological study on the prevalence of iodine-induced hypothyroidism. *Chin J Endocrinol Metab* **7**, 71–74.
3. Gurr JA & Kourides IA (1983) Regulation of thyrotropin biosynthesis. Discordant effect of thyroid hormone on alpha and beta subunit mRNA levels. *J Biol Chem* **258**, 10 208–10 211.
4. Shupnik MA, Chin WW, Habener JF, *et al.* (1985) Transcriptional regulation of the thyrotropin subunit genes by thyroid hormone. *J Biol Chem* **260**, 2900–2903.
5. Schröder-van der Elst JP, van der Heide D, Morreale de Escobar G, *et al.* (1998) Iodothyronine deiodinase activities in fetal rat tissues at several levels of iodine deficiency: a role for the skin in 3,5,3'-triiodothyronine economy. *Endocrinology* **139**, 2229–2234.
6. Salvatore D, Tu H, Harney JW, *et al.* (1996) Type 2 iodothyronine deiodinase is highly expressed in human thyroid. *J Clin Invest* **98**, 962–968.
7. Murakami M, Araki O, Hosoi Y, *et al.* (2001) Expression and regulation of type II iodothyronine deiodinase in human thyroid gland. *Endocrinology* **142**, 2961–2967.
8. Imai Y, Toyoda N, Maeda A, *et al.* (2001) Type 2 iodothyronine deiodinase expression is upregulated by the protein kinase A-dependent pathway and is downregulated by the protein kinase C-dependent pathway in cultured human thyroid cells. *Thyroid* **11**, 899–907.
9. Croteau W, Davey JC, Galton VA, *et al.* (1996) Cloning of the mammalian type II iodothyronine deiodinase: a selenoprotein differentially expressed and regulated in human and rat brain and other tissues. *J Clin Invest* **98**, 405–417.
10. Hosoi Y, Murakami M, Mizuma H, *et al.* (1999) Expression and regulation of type II iodothyronine deiodinase in cultured human skeletal muscle cells. *J Clin Endocrinol Metab* **84**, 3293–3300.
11. Dentice M, Morisco C, Vitale M, *et al.* (2003) The different cardiac expression of the type 2 iodothyronine deiodinase gene between human and rat is related to the differential response of the Dio2 genes to Nkx-2.5 and GATA-4 transcription factors. *Mol Endocrinol* **17**, 1508–1521.
12. Kuiper GG, Kester MH, Peeters RP, *et al.* (2005) Biochemical mechanisms of thyroid hormone deiodination. *Thyroid* **15**, 787–798.
13. Bianco AC, Salvatore D, Gereben B, *et al.* (2002) Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr Rev* **23**, 38–89.
14. Bianco AC & Silva JE (1987) Nuclear 3,5,3'-triiodothyronine (T3) in brown adipose tissue: receptor occupancy and sources of T3 as determined by *in vivo* techniques. *Endocrinology* **120**, 55–62.
15. Silva JE & Larsen PR (1978) Contributions of plasma triiodothyronine and local thyroxine monodeiodination to triiodothyronine to nuclear triiodothyronine receptor saturation in pituitary, liver, and kidney of hypothyroid rats: further evidence relating saturation of pituitary nuclear triiodothyronine receptors and the acute inhibition of thyroid-stimulating hormone release. *J Clin Invest* **61**, 1247–1259.
16. Silva JE, Dick TE & Larsen PR (1978) The contribution of local tissue thyroxine monodeiodination to the nuclear 3,5,3'-triiodothyronine in pituitary, liver, and kidney of euthyroid rats. *Endocrinology* **103**, 1196–1207.

17. Yen PM, Sunday ME, Darling DS, *et al.* (1992) Isoform-specific thyroid hormone receptor antibodies detect multiple thyroid hormone receptors in rat and human pituitaries. *Endocrinology* **130**, 1539–1546.
18. Childs GV, Taub K, Jones KE, *et al.* (1991) Triiodothyronine receptor beta-2 messenger ribonucleic acid expression by somatotropes and thyrotropes: effect of propylthiouracil-induced hypothyroidism in rats. *Endocrinology* **129**, 2767–2773.
19. Fliers E, Unmehopa UA & Alkemade A (2006) Functional neuroanatomy of thyroid hormone feedback in the human hypothalamus and pituitary gland. *Mol Cell Endocrinol* **251**, 1–8.
20. Friesema EC, Ganguly S, Abdalla A, *et al.* (2003) Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter. *J Biol Chem* **278**, 40128–40135.
21. Diano S, Naftolin F, Goglia F, *et al.* (1998) Fasting-induced increase in type II iodothyronine deiodinase activity and messenger ribonucleic acid levels is not reversed by thyroxine in the rat hypothalamus. *Endocrinology* **139**, 2879–2884.
22. Gereben B, Zavacki AM, Ribich S, *et al.* (2008) Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling. *Endocr Rev* **29**, 898–938.
23. Smerdely P, Pitsiavas V & Boyages SC (1993) Evidence that the inhibitory effects of iodide on thyroid cell proliferation are due to arrest of the cell cycle at G0G1 and G2M phases. *Endocrinology* **133**, 2881–2888.
24. Alkemade A, Friesema EC, Kuiper GG, *et al.* (2006) Novel neuroanatomical pathways for thyroid hormone action in the human anterior pituitary. *Eur J Endocrinol* **154**, 491–500.
25. Christoffolete MA, Ribeiro R, Singru P, *et al.* (2006) Atypical expression of type 2 iodothyronine deiodinase in thyrotrophs explains the thyroxine-mediated pituitary thyrotropin feedback mechanism. *Endocrinology* **147**, 1735–1743.
26. St. Germain DL & Galton VA (1997) The deiodinase family of selenoproteins. *Thyroid* **7**, 655–668.
27. Obregón MJ, Ruiz de Oña C, Calvo R, *et al.* (1991) Outer ring iodothyronine deiodinases and thyroid hormone economy: responses to iodine deficiency in the rat fetus and neonate. *Endocrinology* **129**, 2663–2673.
28. Janssen KP, Van Der Heide D, Visser TJ, *et al.* (1994) Thyroid function and deiodinase activities in rats with marginal iodine deficiency. *Biol Trace Element Res* **40**, 237–246.
29. Meinhold H, Campos-Barros A, Walzog B, *et al.* (1993) Effects of selenium and iodine deficiency on type I, type II and type III iodothyronine deiodinases and circulating thyroid hormones in the rat. *Exp Clin Endocrinol* **101**, 87–93.
30. Mitchell JH, Nicol F, Beckett GJ, *et al.* (1997) Selenium and iodine deficiencies: effects on brain and brown adipose tissue selenoenzyme activity and expression. *J Endocrinol* **155**, 255–263.
31. Pazos-Moura CC, Moura EG, Dorris ML, *et al.* (1991) Effect of iodine deficiency and cold exposure on thyroxine 5'-deiodinase activity in various rat tissues. *Am J Physiol* **260**, (2 Pt 1), E175–E182.
32. Perret G, Yin YL, Nicolas P, *et al.* (1991) *In vivo* effects of macrolides on thyroid hormone serum levels and on hepatic type 1 5'-deiodinase in rat. A comparative study with amiodarone, phenobarbital and propranolol. *Fundam Clin Pharmacol* **5**, 583–593.
33. Wang K, Sun YN, Liu JY, *et al.* (2006) Type 1 iodothyronine deiodinase activity and mRNA expression in rat thyroid tissue with different iodine intakes. *Chin Med J (Engl)* **119**, 1899–1903.
34. Bednarczuk T, Pietrzykowski A, Słoń M, *et al.* (1993) Pharmacologic effect of excess iodine on type I thyroxine 5'-deiodinase activity in rat thyroid. *Endokrynol Pol* **44**, 405–412.
35. Aanderud S, Sundsfjord J & Aarbakke J (1984) Amiodarone inhibits the conversion of thyroxine to triiodothyronine in isolated rat hepatocytes. *Endocrinology* **115**, 1605–1608.
36. Safran M, Fang SL, Bambini G, *et al.* (1986) Effects of amiodarone and desethylamiodarone on pituitary deiodinase activity and thyrotropin secretion in the rat. *Am J Med Sci* **292**, 136–141.
37. Rosene ML, Wittmann G, Arrojo e Drigo R, *et al.* (2010) Inhibition of the type 2 iodothyronine deiodinase underlies the elevated plasma TSH associated with amiodarone treatment. *Endocrinology* **151**, 5961–5970.
38. Tu HM, Kim SW, Salvatore D, *et al.* (1997) Regional distribution of type 2 thyroxine deiodinase messenger ribonucleic acid in rat hypothalamus and pituitary and its regulation by thyroid hormone. *Endocrinology* **138**, 3359–3368.
39. Burmeister LA, Pachucki J & St. Germain DL (1997) Thyroid hormones inhibit type 2 iodothyronine deiodinase in rat cerebral cortex by both pre- and posttranslational mechanisms. *Endocrinology* **138**, 5231–5237.
40. Kim SW, Harney JW & Larsen PR (1998) Studies of the hormonal regulation of type 2 5'iodothyronine deiodinase messenger ribonucleic acid in pituitary tumor cells using semiquantitative reverse transcription-polymerase chain reaction. *Endocrinology* **139**, 4895–4905.
41. Abel ED, Boers ME, Pazos-Moura C, *et al.* (1999) Divergent roles for TH receptor beta isoforms in the endocrine axis and auditory system. *J Clin Invest* **104**, 291–300.
42. Kratzsch J, Fiedler G M, Leichtle A, *et al.* (2005) Newer reference intervals for thyrotropin and thyroid hormones based on National Academy of Clinical Biochemistry criteria and regular ultrasonography of the thyroid. *Clin Chem* **51**, 1480–1486.
43. Elmlinger MW, Dengler T, Weinstock C, *et al.* (2003) Endocrine alterations in the aging male. *Clin Chem Lab Med* **41**, 934–941.
44. Boucai L & Surks MI (2009) Reference limits of serum thyrotropin (TSH) and free thyroxine (Free T4) are significantly influenced by race and age in an urban outpatient practice of medicine. *Clin Endocrinol (Oxf)* **70**, 788–793.
45. Surks MI & Hollowell JG (2007) Age-specific distribution of serum thyrotropin and antithyroid antibodies in the U.S. population: implications for the prevalence of subclinical hypothyroidism. *J Clin Endocrinol Metab* **92**, 4575–4582.
46. Guan H, Shan Z, Teng X, *et al.* (2008) Influence of iodine on the reference interval of TSH and the optimal interval of TSH: results of a follow-up study in areas with different iodine intakes. *Clin Endocrinol (Oxf)* **69**, 136–141.
47. Pekary AE, Hershman JM, Sugawara M, *et al.* (1983) Preferential release of triiodothyronine: an intrathyroidal adaptation to reduced serum thyroxine in ageing rats. *J Gerontol* **38**, 653–659.
48. Co'nsole GM, Go'mez-Dumm CL & Goya RG (1995) Immunohistochemical and radioimmunological assessment of thyrotrophs in the pituitary of ageing rats. *Acta Anat (Basel)* **152**, 28–32.
49. Moreira DG, Marassi MP, Corrêa da Costa VM, *et al.* (2005) Effects of ageing and pharmacological hypothyroidism on pituitary-thyroid axis of Dutch-Miranda and Wistar rats. *Exp Gerontol* **40**, 330–334.