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ORIGINAL CONTRIBUTION

Dose and time-dependent hypercholesterolemic effects of iodine excess via TRβ1-mediated down regulation of hepatic LDLr gene expression

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Abstract
Background With the global improvement of iodine nutrition, iodine excess is emerging as a new concern.
Aim of study The aim of this study is to illustrate the physiological effects and potential molecular mechanisms of excessive iodine intake on lipid metabolism.
Methods Balb/c mice were given drinking water containing different levels of iodine for 1 month and treated with 1.2 μg/mL iodine for different periods of time, respectively. Plasma lipid parameters and serum thyroid hormones were measured. Expressions of hepatic genes were detected by real-time polymerase chain reactions and Western blot.
Results Dose-dependent hypercholesterolemic effects were detected in mice (TC, $r = 0.615$; $p < 0.01$). Drinking 1.2 μg/mL iodine water for 1 month had no significant effect on serum lipid metabolism, while prolonged exposure induced an increase of serum cholesterol. Serum thyroid hormones were not affected by iodine throughout the study. At the molecular level, we detected a dose-dependent attenuation of hepatic low density lipoprotein receptor (LDLr) and thyroid hormone receptor β1 (TRβ1) expression in parallel to the change of serum cholesterol. Treatment with 1.2 μg/mL iodine water for 1 month did not affect LDLr and TRβ1 expression, while 3 or 6 months exposure resulted in a decrease of their expression.
Conclusion Present findings demonstrated dose- and time-dependent hypercholesterolemic effects of iodine excess. Furthermore, our data suggests that TRβ1-mediated down regulation of hepatic LDLr gene may play a critical role in iodine excess-induced hypercholesterolemic effects.

Keywords Iodine excess · Hypercholesterolemic · Thyroid hormone receptor β1 (TRβ1) · Low density lipoproteins receptor (LDLr) · Scavenger receptor class B type-I (SR-BI)

Introduction

Iodine is an essential microelement for human health. Historically, iodine deficiency-caused disease (IDD) has been the focal point of public health. Comprehensive studies on IDD led to the implementation of universal salt iodization (USI) policy in many countries. With rapid global progress in correcting iodine deficiency, excessive iodine intake is emerging as a new concern [2, 5, 12, 27, 29]. Iodine excess is associated with a spectrum of thyroidal effects, including hyperthyroidism, hypothyroidism, thyroid autoimmune diseases, thyroid endemic goiter and thyroid cancer [13, 24, 25, 35]. Recent studies from this laboratory and other groups indicated that iodine excess during pregnancy may cause stillbirths, abortions, embryo toxicity, and congenital abnormalities [10, 15, 34].

Excessive iodine intake from different sources has been reported to affect plasma lipid metabolism**.** Plasma cholesterol was elevated in laying hens fed with high-iodine diets [14, 22]. Moreover, a regression of data from Perry et al. [23] showed that cholesterol concentration in the

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plasma increases 0.3 mmol/L for each 100 mg/kg dietary iodine. Wistar rats drinking iodine water for 3 months resulted in an obvious increase of serum cholesterol level in a dose-dependent manner [32]. These studies demonstrated a potential physiological connection between iodine excess and lipid metabolism; however, the underlying mechanisms are still elusive. Though little is known about the cellular and molecular basis for these abnormalities, genes involved in lipid metabolism mediated by thyroid hormones may play a pivotal role in this process.

All aspects of lipid metabolism including synthesis, mobilization and degradation are influenced by thyroid hormones. T3 exerts its diverse metabolic effects by binding to nuclear thyroid hormone receptors (TRs). TRs can bind to the thyroid hormone response element (TRE) of target genes [21] with or without ligand binding and regulate the expression of many key genes involved in hepatic cholesterol metabolism. Thyroid hormones are important regulators of lipid and glucose metabolism in the liver. Liver also plays an important role in thyroid hormone metabolism, Thyroid hormone receptor $\beta 1$ (TR$\beta 1$) is the most abundant TR isoform in liver.

Liver plays a major role in regulating systemic cholesterol homeostasis by converting cholesterol into bile and secreting both bile acid and free cholesterol into gut lumen via the bile duct. Hepatic lipoprotein receptors including low density lipoproteins receptor (LDLr), scavenger receptor class B type-I (SR-BI) are gateways of hepatic cholesterol metabolism and lipoprotein metabolism. Approximately 70% of cholesterol in the circulation is transported in low density lipoproteins (LDL) in human. LDL receptor (LDLr)-mediated endocytosis in the liver is the primary pathway for the clearance of circulating LDL. Reductions in hepatic LDLr activity result in elevated plasma LDL-cholesterol concentrations and the deposition of cholesterol in body tissues [4, 7]. The scavenger receptor class B type-I (SR-BI) is an 82-kDa protein, identified as an HDL receptor is most highly expressed in hepatocytes, where it helps regulation of high density lipoproteins (HDL) metabolism and the transport of cholesterol secretion in biliary salts [1]. Normal expression and moderate transgene-mediated overexpression of hepatic SR-BI have been shown to protect against atherosclerosis in murine model [6].

We conducted this study to provide further insights into iodine excess on lipid homeostasis at physiological and molecular levels. In a model of iodine excess in which animals were challenged with different dose and durations, biological samples were collected and subjected to histological physiological and molecular analysis. This study will further expand our knowledge on the side effect of iodine excess on health and the results will be instructive for reasonable iodine intake.

Materials and methods

Animal and experimental design

Weaning female Balb/c mice obtained from the Laboratory Animal Centre of Shanghai were maintained in constant temperature-controlled rooms (22–28 °C) with controlled lighting (12 h light–dark cycles). All animals were housed in stainless steel cages and given a commercial laboratory chow and sterile water ad libitum. The content of iodine in the diet was 365 mg/kg. The animals were cared for according to the Guiding Principles in the Care and Use of Animals. The experiments were approved by the Tongji Medical College Council on Animal Care Committee. After acclimatization to the laboratory environment for 1 week, animals were randomly assigned to ten groups of ten animals each according to body weight and given iodine in the form of potassium iodate (KIO_3) in the drinking water with different dose and durations. Six groups were given iodine at different levels (0, 0.3, 0.6, 1.2, 2.4, 4.8 μg/ml) for 1 month. Two groups were given 0 and 1.2 μg/ml iodine in the drinking water for 3 months, respectively. Two groups were given 0 and 1.2 μg/ml iodine in the drinking water for 6 months, respectively. Food consumption, water consumption of each group and weight gain of each mouse was recorded. At end of the experiment, mice were fasted for 8 h before being killed by cervical dislocation and blood was collected for serum biochemistry and thyroid hormone analysis. Hepatic tissues were collected immediately, frozen in liquid N_2 and stored at −80 °C for real-time polymerase chain reactions and Western analysis.

Iodine concentration

Iodine concentration in diet, water and urine was measured by modified Cer-Arsenite colorimetric method [8]. Female mice were placed into metabolism cages of three or two mice each and urine samples of 3 h in the morning were collected for 3 days for urinary iodine determination.

Thyroid histology and thyroid hormone analysis

Serum total T4, total T3 and thyroid stimulating hormone (TSH) were measured by RIA kits obtained from the Chinese Academy of Atomic Energy (Beijing, China).Thyroid glands were placed in 10% buffered formalin, embedded in methacrylate, step sectioned, mounted on glass slides, and stained with hematoxylin and eosin.

Lipid parameters

Plasma concentrations of total cholesterol (TC), triglyceride and HDL-cholesterol were measured by enzymatic

colorimetric assays (BIOSINO Biotechnology and science INC, Beijing, china). Non-HDL-cholesterol level was calculated by subtracting HDL-cholesterol level from total cholesterol level.

Real-time polymerase chain reactions

Total RNA from tissues was isolated with Trizol agent (Invitrogen Life Technologies, US.). RNA concentration was determined by a spectrophotometer at A260 and the purity designated valid if the ratio of A260/A280 ranged from 1.8 to 2.0. Using the RT system (Promage, Madsion, WI), cDNA was synthesized from 3 μg RNA at 37 °C for 60 min followed by 95 °C for 5 min. Real-time PCR was performed with SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa BIO INC, Dalian) using the ABI 7900HT real-time thermocycler (Applied Biosystems, Forster, CA) with the following program: 95 °C/10 s, (95 °C/5 s, 60 °C/30 s) × 40. The dissociation curve was performed and analyzed using the ABI 7900HT software. Target gene was analyzed and normalized to β-actin. The pairs of primers were listed in the following respectively: TRβ1 sense 5′ GT GCCAGGAATGTCGCTTTA 3′ antisense 5′ CGCCTCTT CTCACGGTTCTC 3′; LDLr sense 5′ TCAGTCCCAG GCAGCGTAT 3′ antisense 5′ CTTGATCTTGGCG GGTGTT 3′; SR-BI sense 5′ CCCAGACATGCTTCCC ATAA 3′antisense 5′ GTCCGTTCCATTTGTCCACC 3′; β-actin sense 5′ ATCGTGCGTGACATCAAAGA 3′ antisense 5′ ATGCCACAGGATTCCATACC 3′. The C_T value represents the number of cycles required for the fluorescence signal reach at threshold for each reaction [$\Delta C_T = C_T$ (target gene) − C_T (β-actin)]. The relative expression level of target gene and β-actin were calculated as 2 ($-\Delta\Delta C_T$) [17].

Western analysis

Extraction of nuclear protein and tissue proteins from liver were performed as described previously [11]. The protein concentration was quantified with BIO-RAD Dc protein assay reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were mixed with SDS sample buffer and incubated for 3 min at 98 °C before loading. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunological blotting were performed according to the method of Amersham Biosciences. Immunoreactive bands were detected by means of an ECL plus Western Blotting Detection System (Amersham Biosciences, Little Chalford, UK) according to the manufacturer's instructions. The chemiluminescent signals were scanned from autoradiographic films (Nippon Polaroid KK, Tokyo, Japan).Quantitative analysis was performed by Gel Pro 3.0 software (Biometra, Goettingen, Germany). Antibodies used in the experiment were obtained from the following sources: purified rabbit polyclonal antibody anti-LDL receptor antibody (ab30532) rabbit polyclonal anti-SR-BI antibody (ab396) mouse monoclonal anti-TBP antibody (ab818) were purchased from Abcam company mouse monoclonal anti-TRβ1 antibody (Sc-738, Santa Cruz, USA). HRP-labeled anti-mouse IgG and HRP-labeled anti-rabbit IgG (Sigma, USA).

Statistical methods

The SPSS 12.0 software package was used for statistical analysis. Because of its skewed distribution, the medians were used to describe the central tendency of urinary iodine concentration. The Kruskal–Wallis method was used to test the differences in ranking of urinary iodine concentration. Other data were analyzed by a one-way ANOVA, t test and significance level was set at $p < 0.05$.

Results

Average daily water intake, food consumption, weight gain, urinary iodine concentration in mice

There was no obvious difference in average daily water intake and food consumption among groups. By the end of the treatment periods, the body weight gain were not different among the groups (data are not shown). After exposure to different doses of iodine, urinary iodine concentration were increased in a dose-dependent manner ($r = 0.944$, $p < 0.001$). Concentration of iodine in urine is currently the standard biochemical marker of iodine intake [28].

Dose–response and time course of effect of iodine on serum lipids and liver weight

A positive and statistically significant association was found between serum cholesterol and dosage of iodine in mice. (TC, $r = 0.615$; $p < 0.01$; Table 1).Compared to the control group, serum TC and non-HDL-C increased significantly in mice when iodine dose reached 2.4 μg/ml. Liver weights were not different among the groups by the end of the treatment periods.

Compared with control group, exposure of 1.2 μg/ml iodine increased serum total cholesterol by 29 and 29% at 3, 6 months, respectively (Table 2). Serum total cholesterol and non-HDL-C increased significantly in mice when the duration of exposure of 1.2 μg/ml iodine reached 3 months. Liver weights were not different between the 1.2 μg/ml iodine-exposed group and the control group by the end of the treatment periods.

Table 1 Dose-dependent effect of iodine excess on serum biochemistry and liver weights in mice

	0	0.3 μg/ml	0.6 μg/ml	1.2 μg/ml	2.4 μg/ml	4.8 μg/ml
TC (mmol/L)	1.81 ± 0.31	1.87 ± 0.32	1.91 ± 0.37	2.11 ± 0.37	2.40 ± 0.37*	2.58 ± 0.42*
TG (mmol/L)	1.12 ± 0.31	1.15 ± 0.24	1.11 ± 0.26	1.29 ± 0.29	1.22 ± 0.30	1.25 ± 0.22
HDL-C (mmol/L)	1.35 ± 0.20	1.39 ± 0.31	1.38 ± 0.35	1.40 ± 0.31	1.50 ± 0.31	1.52 ± 0.29
Non-HDL-C (mmol/L)	0.47 ± 0.18	0.48 ± 0.17	0.53 ± 0.17	0.70 ± 0.25	0.91 ± 0.26*	1.05 ± 0.30*
Liver weights (g)	0.91 ± 0.14	0.95 ± 0.12	0.96 ± 0.10	0.97 ± 0.19	0.98 ± 0.15	1.01 ± 0.18

Results are expressed as means ± SD; $n = 10$

TC Total cholesterol, *TG* total triglycerides, *HDL-C* high density lipoprotein cholesterol, *Non-HDL-C* non-high density lipoprotein cholesterol

* Statistically significant at $p < 0.05$ (t test)

Table 2 Time course of effects of iodine excess on biochemistry and liver weights in mice

	1 month		3 months		6 months	
	0	1.2 μg/ml	0	1.2 μg/ml	0	1.2 μg/ml
TC (mmol/L)	1.81 ± 0.31	2.11 ± 0.37	1.94 ± 0.34	2.51 ± 0.43*	2.07 ± 0.33	2.67 ± 0.42*
TG (mmol/L)	1.12 ± 0.31	1.29 ± 0.29	1.32 ± 0.33	1.40 ± 0.32	1.34 ± 0.31	1.48 ± 0.48
HDL-C (mmol/L)	1.35 ± 0.20	1.40 ± 0.31	1.30 ± 0.21	1.47 ± 0.30	1.41 ± 0.26	1.41 ± 0.44
Non-HDL-C (mmol/L)	0.47 ± 0.18	0.70 ± 0.25	0.64 ± 0.16	1.04 ± 0.30*	0.67 ± 0.19	1.27 ± 0.26*
Liver weight (g)	0.91 ± 0.14	0.97 ± 0.19	1.35 ± 0.22	1.44 ± 0.19	1.79 ± 0.21	1.81 ± 0.27

Results are expressed as means ± SD; $n = 10$

TC Total cholesterol, *TG* total triglycerides, *HDL-C* high density lipoprotein cholesterol, *Non-HDL-C* non-high density lipoprotein cholesterol

* Statistically significant at $p < 0.05$ (t test)

Serum T4, T3, TSH concentration in mice and morphology of thyroid gland

No differences were observed between the control group and the iodine-exposed groups in terms of serum TT3, TT4, and TSH levels at any time point (Figs. 1, 2). The thyroid glands of the mouse were normally pink, soft, and as big as a grain of millet. With growth and age, the weight of the thyroid glands increased. An obvious colloid goiter was observed in thyroid of mice exposed to high doses of iodine (Fig. 3). The weight of the thyroid gradually increased as the dosage of iodine increased. Enlarged thyroid appeared slight skin color and were firm in texture. Follicular epithelial cells gradually became flattened, and the follicles gradually became distended with colloid with increasing iodine dose.

Dose–response and time course of effect of iodine on mRNA abundances and protein content of hepatic genes in mice

Effects of excessive iodine intake on hepatic LDLr, SR-BI and TRβ1 were depicted (Figs. 4, 5). LDLr expression were down regulated after treatment with 2.4 and 4.8 μg/ml iodine for 1 month, and iodine inhibited LDLr mRNA expression by 37 and 48%, LDLr protein expression by 30 and 37%, respectively. An inverse and statistically significant association was found between LDLr expression and dosage of iodine in mice. (LDLr mRNA: $r = -0.753$, $p < 0.01$; LDLr protein: $r = -0.748$, $p < 0.01$). No changes were observed in hepatic SR-BI mRNA and protein levels in mice; while although there were no significant changes observed in hepatic TRβ1 mRNA levels, a dose-dependent down regulation of TRβ1 protein levels were observed in mice (TRβ1 protein, $r = -0.847$; $p < 0.01$).

Time course of the effects of iodine on mRNA abundances and protein content of LDLr, SR-BI and TRβ1 were depicted (Figs. 4, 6), LDLr mRNA levels and protein content were down regulated as the duration of exposure was prolonged. Iodine inhibited LDLr mRNA expression by 36 and 51% for 3, 6 months of treatment ($p < 0.05$), respectively. The similar change was observed in LDLr content. Iodine inhibits TRβ1 protein expression by 16% ($p < 0.05$) and 25% ($p < 0.05$) for 3, 6 months of treatment although there were no significant changes were observed in hepatic TRβ1 mRNA levels. No significant changes were observed in hepatic SR-BI mRNA and protein levels in mice.

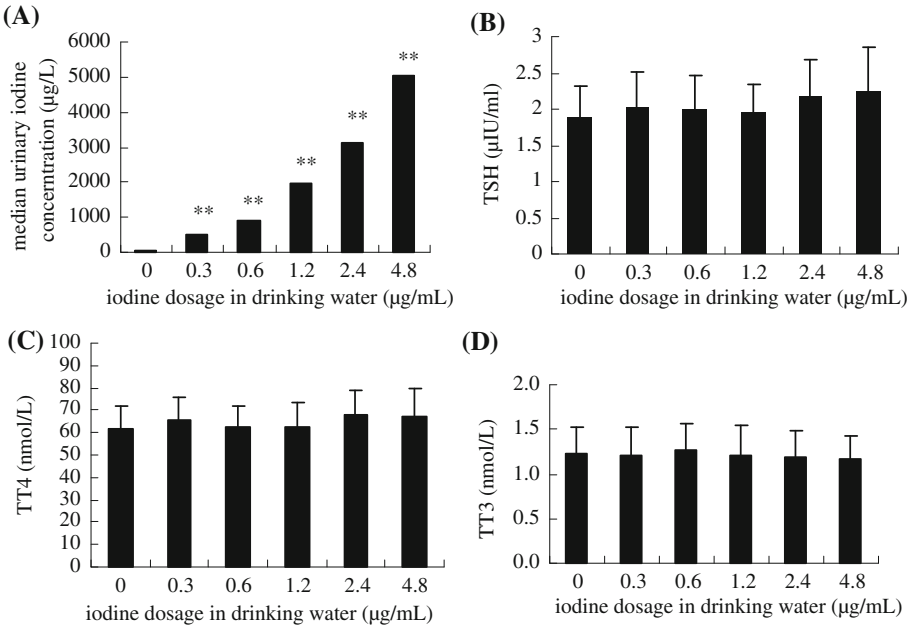

Fig. 1 Dose-dependent effect of iodine excess on urinary iodine level and serum thyroid hormone levels in mice. Values are medians for urinary iodine level (**a**), *each bar* representing the median of a group of seven samples. **Median values were significantly different from that of the control group ($p < 0.01$) (Kruskal–Wallis method).Values are means for serum TSH level (**b**), total thyroxine(TT4) level (**c**) and total triiodothyronine (TT3) level (**d**) ($n = 10$) with standard deviations represented by *vertical bars*. *Mean value was significantly different from that of the control group ($p < 0.05$) (ANOVA and Duncan's test)

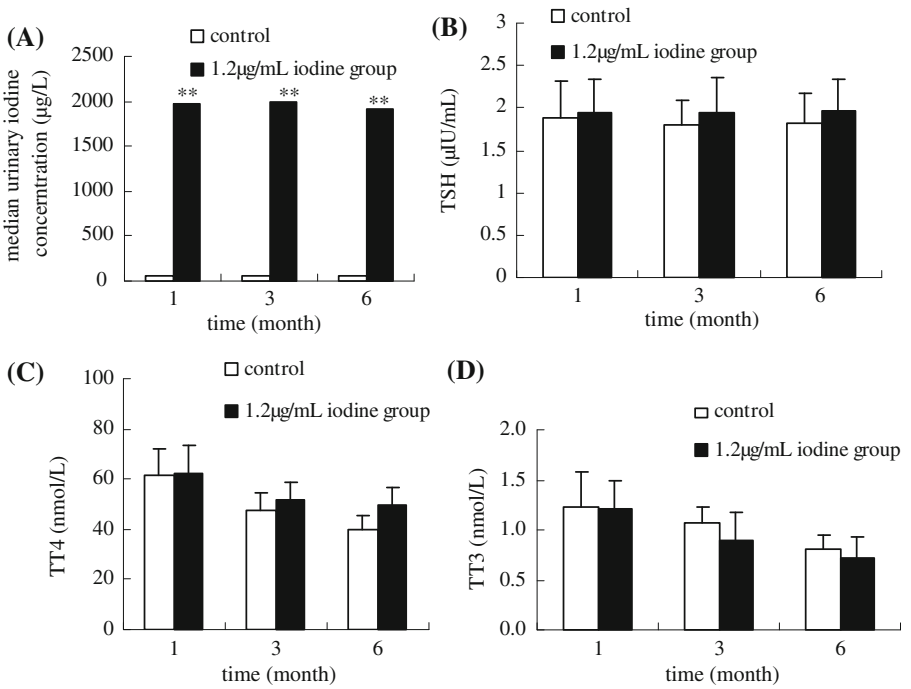

Fig. 2 Time course of effects of iodine excess on urinary iodine level and serum thyroid hormone levels in mice. Values are medians for urinary iodine level (**a**), *each bar* representing the median of a group of seven samples. **Median values were significantly different from that of the control group ($p < 0.01$) (Kruskal–Wallis method). Values are means for serum TSH level (**b**), total thyroxine (TT4) level (**c**) and total triiodothyronine (TT3) level (**d**) ($n = 10$) with standard deviations represented by *vertical bars*. *Mean value was significantly different from that of the control group ($p < 0.05$) (*t* test)

Discussion

In this study, mice were subjected to drinking water containing 0, 0.3, 0.6, 1.2, 2.4, 4.8 μg/mL iodine, corresponding to 0-, 1-, 2-, 4-, 8-, 16-fold of the adequate/normal iodine intake for 1 month, and 1.2 μg/mL iodine in drinking water for 1, 3 and 6 months, respectively. The results indicated that excessive iodine intake via drinking water may have a significant hypercholesterolemic impact on mice. The effect is dose- and time-dependent, and reflected mainly by serum TC levels. Iodine intake (≤4.8 μg/mL) in drinking water for 1 month or 1.2 μg/mL for 1, 3, 6 months had no effect on serum thyroid hormones (TT4, TT3, TSH). Compared with the control, hepatic TRβ1 expression was lower and accompanied by decreased expression of LDLr mRNA and protein. Down regulation of hepatic TRβ1 and LDLr may reduce cholesterol clearance in liver and partially explain excessive iodine-induced lipid metabolism disorder in mice.

Hypercholesterolemic effects of iodine excess were reported in other animal studies. Plasma cholesterol was elevated in laying hens fed with high-iodine diets and

rodents supplied drinking water containing different levels of iodine [14, 22, 23, 32]. These previous studies demonstrated a potential physiological connection between iodine excess and lipid metabolism. This study confirmed the hypercholesterolemic effect of iodine excess and demonstrated that the major effect is on non-HDL-cholesterol. HDL-cholesterol remained normal in treated animals.

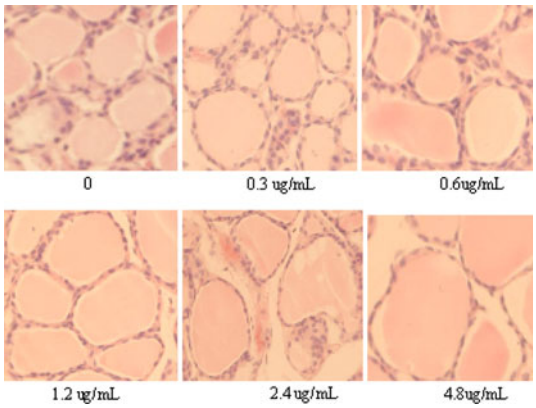

Fig. 3 Morphology of thyroid gland (H&E, magnification ×100). Chronic excessive iodine exposure induced colloid goiter, follicular epithelial cells gradually become flattened, and the follicles gradually became distended with colloid

To illustrate the mechanisms underling the hypercholesterolemic effects of excessive iodine, thyroid and thyroid function were analyzed. An obvious collide goiter was also observed in the thyroid of mice exposure to high doses of iodine (Fig. 3). Despite the morphological changes in thyroid gland, no changes of serum TT4, TT3, TSH was observed among groups throughout the experiment. Previous reports indicated that influence of iodine excess on thyroid function was decided by species, dose of iodine, duration of intake and existing thyroid gland conditions. Iodine excess could induce different clinical conditions such as euthyroidism, hypothyroidism or hyperthyroidism. Li and Carayanniotis [16] have reported a compensatory elevation in mean serum TSH concentration and decrease in TT4 in SJL mice but not in CBA/J mice. Teng et al. [30] have reported that no differences were observed between the control group at any time point in serum TT3, TT4, and TSH levels of NOD.H-2h4 mice treated with 5, 10, 100, or 1,000 times iodine for 4, 8, 16, and 24 weeks. In our previous studies [33], we found 1.5 μg/ml iodine (4 months)

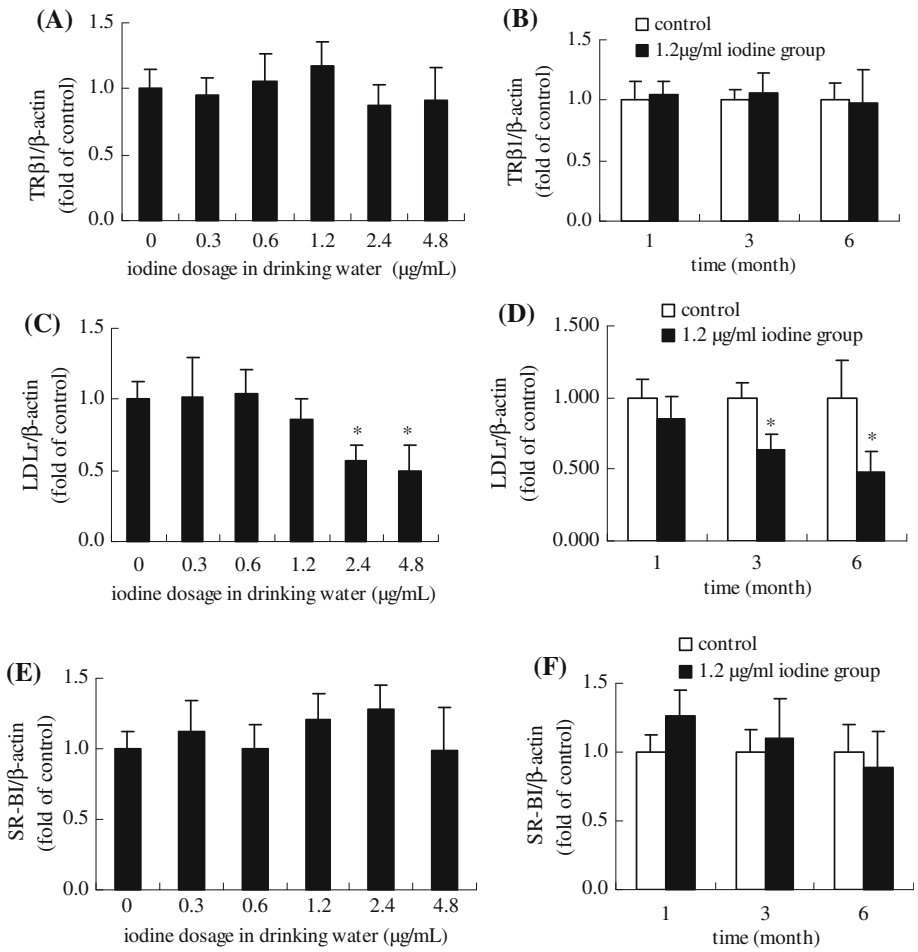

Fig. 4 Dose-dependent and time course of iodine excess on hepatic LDLR, SR-BI, and TRβ1 mRNA expressions. Relative mRNA amount to β-actin in the controls was set to 1.*Statistically significant at $p < 0.05$ (ANOVA) in pooled data of all experiments performed. Standard error of the mean is given from three experiments with three replicates each

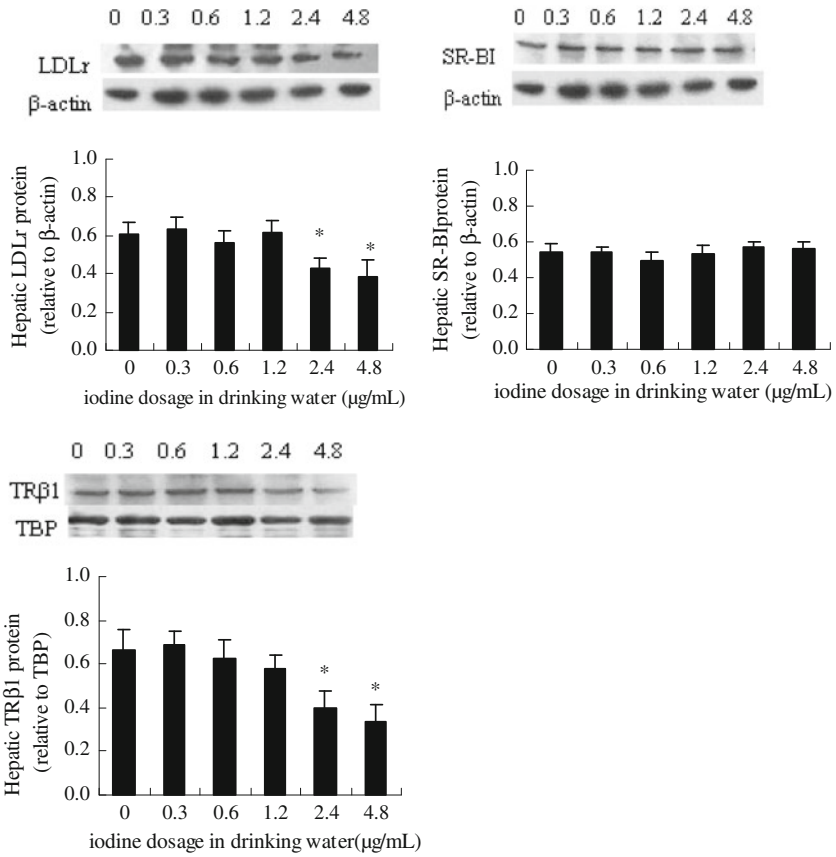

Fig. 5 Dose-dependent effects of iodine excess on hepatic LDLr, SR-BI and TRβ1 protein expressions. Mice were given different doses of iodine in drinking water for 1 month. Representative blots and data are expressed as means ± SD ($n = 3$) after normalization to the β-actin (LDLr, SR-BI) or TBP (TRβ1). Western analysis was carried out as described in "Methods". Statistical analyses were done according to Mann–Whitney U test. *$p < 0.05$ versus control

had no obvious effect on thyroid hormone level while 3 μg/ml (4 months) could result in an increase of TT4 and a decreased of TT3 in Balb/c mice. No significant change in serum thyroid hormone was detected in this study. This may be explained by the iodine dosage (1.2 μg/ml for 1, 3, 6 months) and duration (≤4.8 μg/ml for 1 month). In addition, TSH-independent thyroid autoregulatory processes could work effectively to maintain normal serum thyroid hormone.

The expression of key regulatory genes for lipid metabolism was analyzed. LDL receptor is a major regulator of circulating LDL-cholesterol. Our results indicated that diminished LDLr expression may have a negative impact on clearance of LDL from the circulation. The expression of hepatic SR-BI is regulated by a wide range of physiological stimuli and is believed to mediate the selective uptake of cholesterol ester from HDL in the reserve cholesterol transport system. In this study, no significant SR-BI expression was detected, suggesting that HDL-cholesterol clearance through SR-BI was not affected by excessive iodine intake. This may explain the finding that concentrations of HDL-cholesterol did not change throughout the experiment.

Expression of LDL is under the control of several hormones; one of these is thyroid hormone [3, 18, 26, 31]. Thyroid hormone receptor protein complex activates gene expression by binding to thyroid hormone response element (TRE). Researchers have found the promoters of the gene encoding the LDL receptor contain sequences resembling TRE [18, 20].Thyroid hormone receptor β is the predominant subtype found in liver and thyroid hormone receptor β is required in T3 mediated cholesterol metabolism in mice [9]. Under the conditions of this study, we illustrated that low TRβ1 protein content in liver parallels with high cholesterol in serum. Although the cellular and molecular mechanisms by which excessive iodine intake on hepatic TRβ1 expression is not clear at this stage, we showed that TRβ1 mRNA abundance was unchanged by excessive iodine, suggesting that the effect of iodine excess may be posttranscriptional.

In conclusion, this study demonstrated that excessive iodine intake may cause hypercholesterolemia via TRβ1 mediated down regulation of hepatic LDLr expression in mice. The results should be further verified by population-based epidemical studies. As elevated plasma lipids, particularly non-HDL-cholesterol, represent a critical risk factor for atherosclerotic vascular disease [19], public iodine intake should be watched and regulated to make sure public iodine intake is within the optimal levels for improving a person's overall health.

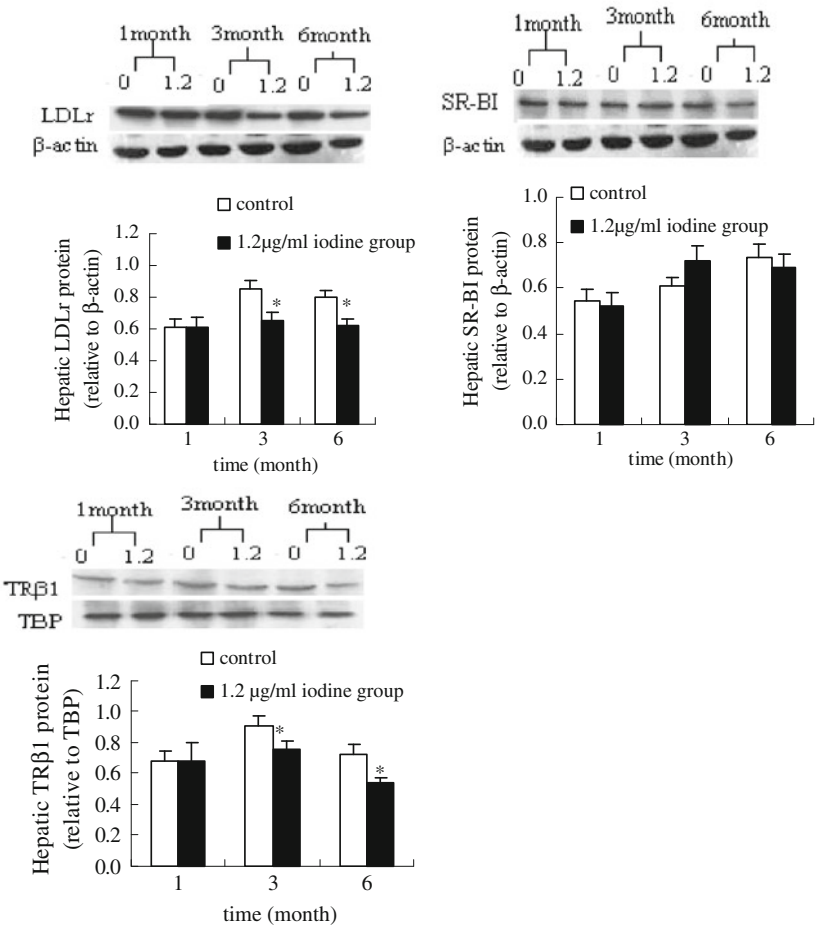

Fig. 6 Time course of effects of iodine excess on hepatic LDLr, SR-BI and TRβ1 protein expressions. Representative blots and data are expressed as means ± SD ($n = 3$) after normalization to the β-actin (LDLr, SR-BI) or TBP (TRβ1). Western analysis was carried out as described in "Methods". Statistical analyses were done according to ANOVA. *$p < 0.05$ versus control

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