Effect of excessive iodine exposure on the placental deiodinase activities and Hoxc8 expression during mouse embryogenesis

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Excessive iodine induces thyroid dysfunction. However, the effect of excessive iodine exposure on maternal–fetal thyroid hormone metabolism and on the expression of genes involved in differentiation, growth and development is poorly understood. Since a thyroid hormone receptor response element was found in the Hoxc8 promoter region, Hoxc8 expression possibly regulated by excessive iodine exposure was firstly investigated. In the present study, Balb/C mice were given different doses of iodine in the form of potassium iodate (KIO₃) at the levels of 0 (sterile water), 1.5, 3.0, 6.0, 12.0 and 24.0 µg/ml in drinking water for 4 months, then were mated. On 12.5 d postcoitum, placental type 2 and type 3 deiodinase activities and fetal Hoxc8 expression were determined. The results showed that excessive iodine exposure above 1.5 µg/ml resulted in an increase of total thyroxine and a decrease of total triiodothyronine in the serum of maternal mice, which was mainly associated with the inhibition of type 1 deiodinase activity in liver and kidney. Placental type 2 deiodinase activity decreased, showing an inverse relationship with maternal thyroxine level. Hoxc8 mRNA and protein expression at 12.5 d postcoitum embryos were down regulated. Because Hoxc8 plays an important role in normal skeletal development, this finding provides a possible explanation for the skeletal malformation induced by excessive iodine exposure and also provides a new clue to study the relationship between iodine or thyroid hormones and Hox gene expression pattern.

Excessive iodine: Deiodinase: Hoxc8

Adequate iodine intake is essential for fetal and postnatal development. Iodine deficiency during pregnancy may result in in utero hypothyroidism and increase the rates of miscarriage, stillbirths, as well as congenital abnormalities such as cretinism, a grave irreversible form of mental retardation (Giinoer, 1997; Utiger, 1999). The goal of eliminating iodine-deficiency disease has been achieved since universal salt iodisation policy has been widely carried out in many nations including China (Delange & Lecomte, 2000). On the other hand, reports are increasingly appearing on the toxic effects caused by high amounts of iodine intake. Exposure to excessive iodine occurs via food (Konno et al. 1994), drinking water (Zhao et al. 1998), medication (Martino et al. 2001) and iodised salt or iodinated oil (Wolf, 2001). Recent studies have reported that iodine excess also causes either hypothyroidism or hyperthyroidism (Markou et al. 2001; Roti & Uberti, 2001), which may induce embryo toxicity, especially skeletal anomalies. Though little is known about the cellular and molecular basis for these abnormalities, disruption of thyroid hormone metabolism and modulation of the expression pattern of genes involved in differentiation, growth and metabolism mediated by thyroid hormones may play a pivotal role in this process.

Excessive iodine has a complex disruptive effect on thyroid hormone metabolism. Animal studies (Bednarczuk et al. 1993) have suggested that excessive iodine-induced thyroid hormone abnormalities are related to the inhibition of the activity of type 1 deiodinase (D1), which catalyses the deiodination of both the outer and inner rings of thyroxine (T₄) and is responsible for most of the circulating triiodothyronine (T₃) (Bianco et al. 2002). Fetal thyroid hormones must come from the maternal circulation before the fetal thyroid gland and pituitary–thyroid axis become functional (Obregon et al. 1984). The maternal transfer of T₄ constitutes a major fraction of fetal thyroid hormones, even after the onset of fetal thyroid secretion (Morreale de Escobar et al. 1985; Burrow et al. 1994; Santini et al. 1999). Therefore, the changes of maternal thyroid hormone level have an affirmative effect on the fetus by deiodination of iodothyronines through the placenta. Placental type 2 deiodinase (D2), which mainly catalyses the outer ring deiodination of T₄, and type 3 deiodinase (D3), which inactivates T₄ and T₃, may have an important function in regulating fetal thyroid hormone levels. However, the effect of maternal excessive iodine exposure on placental D2 and D3 activity has not been reported.

Hox genes, namely homeobox-containing genes, are a cluster of genes which encode transcriptional factors regulating
many aspects of development. Expression patterns of Hox genes are characterised by spatial collinearity, temporal collinearity and retinoic acid sensitivity collinearity (Lufkin, 1996; Martinez & Amemiya, 2002). Thyroid hormone receptor (TR) and retinoic acid receptor have been shown to share an identical P-box sequence, which implies that they can bind the same DNA sequences and can interact physically (Kumar & Thompson, 1999). In frog embryogenesis, TR can modulate retinoic acid-mediated axis formation, and small changes in levels of TR in early embryos may directly affect the retinoic acid responsiveness of Xho.lab2. In addition, PCR assays have shown that T3 can induce the expression of Xho.lab2 in embryos which ectopically expressed TRα (Banker & Eisenman, 1993). However, little information is available in the literature on Hox genes expression regulated directly by thyroid hormones in mammals. The structural analysis of Hox3.1 (Hoxc8) transcription unit and the Hox3.2-Hox3.1 intergenic region found that there is a thyroid hormone response element in the transcriptional regulation region of the mouse Hoxc8 gene (Awgulewitsch et al. 1990), which implicated that thyroid hormones may regulate Hoxc8 expression during mouse embryogenesis. Hoxc8 belongs to the Hox gene family and expresses in limbs, backbone rudiments, the neural tube of mouse mid-gestation embryos, and in the cartilage and skeleton of newborns (Kwon et al. 2005). Skeletal abnormalities in ribs, sternum and vertebrae have been observed in Hoxc8 knockout mice (Akker et al. 2001; Juan & Ruddle, 2003). These findings suggested that Hoxc8 is an important regulator of pattern formation during the development of the vertebrate skeleton. Our laboratory has previously illustrated (Yang et al. 2006) that maternal excessive iodine exposure result in defects in skeletal patterning in fetuses, such as supernumerary ribs, agenesis of sternbrae, poor ossification of metacarpals and metatarsals and distortion of vertebrae. Such alternations induced by excessive iodine may be related to the modulation of Hoxc8 expression by thyroid hormones. However, little in the literature was available on this hypothesis. Therefore, we conducted the present study to determine whether maternal and fetal thyroid hormone metabolism was influenced, and whether Hoxc8 expression pattern was regulated by excessive iodine exposure during mouse embryogenesis.

Materials and methods

Animals and treatment

Weaning Balb/C mice obtained from the Laboratory Animal Centre of Hubei Provincial Centre for Disease Control and Prevention (Wuhan, China) were maintained in constant temperature-controlled rooms (22 ± 2°C) with controlled lighting (12 h light–dark cycle). 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 and water was 365 μg/kg and 8 μg/l respectively. 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 acclimatisation to the laboratory environment for 1 week, animals were randomly assigned to six groups of twelve animals each (eight females and four males) according to body weight and given different doses of iodine in the form of potassium iodate (KIO3) in the drinking water at the levels of 0, 1·5, 3·0, 6·0, 12·0 and 24·0 μg/ml by using sterile water as the vehicle. Water consumption of each group was recorded. Female mice were placed into the metabolism cages, 4 months later, of four mice each and urine samples of 3 h in the morning were collected for 3 d for urinary iodine concentration determination. Then females were paired with a male in a 1:1 ratio overnight and examined for the presence of a vaginal plug in the following morning, which was defined as 0·5 days postcoitum (dpc). The treatment with high doses of iodine continued through the period of gestation. Dams were killed by cervical dislocation on 12·5 dpc and blood was collected for thyroid hormone analysis. Placentas were collected immediately, frozen in liquid N2 and stored at −80°C for D2 and D3 activity determination. Embryos were dissected free of the maternal and extra-embryonic tissue in PBS, then frozen in liquid N2 and stored at −80°C for RT-PCR and Western analysis.

Iodine concentration and thyroid hormone analysis

Iodine concentration in diet, water and urine was measured by the Cer-Arsenite colorimetric method as modified by Fischer et al. (1986). Urinary creatinine concentrations were determined by the alkaline picrate method. The urinary iodine:creatinine ratio (μg/g Cr) was used to estimate urinary iodine concentration. Serum total T4 and total T3 were measured by RIA kits obtained from the Chinese Academy of Atomic Energy (Beijing, China).

Hepatic and renal type 1 deiodinase activity assays

Tissues were homogenised in cold hydroxyethyl piperazine ethanesulfonic acid buffer solution (dithiothreitol (1 mmol/l), hydroxyethyl piperazine ethanesulfonic acid buffer (10 mmol/l), pH 7·0, sucrose (320 mmol/l)) at a 1:39 and 1:24 dilution (w/v) for livers and kidneys, respectively. Homogenates were centrifuged at 1500 g for 5 min (10 mmol/l), pH 7·0, sucrose (320 mmol/l)) at a 1:39 and 1:24 dilution (w/v) for livers and kidneys, respectively. Homogenates were centrifuged at 1500 g for 5 min and 80°C for RT-PCR and Western analysis.

Placental type 2 and type 3 deiodinase activity assays

Placentas were homogenised at a 1:4 (w/v) dilution in hydroxyethyl piperazine ethanesulfonic acid (10 mmol/l; pH 7·2), sucrose (250 mmol/l) and dithiothreitol (10 mmol/l). Homogenates were stored at −80°C until further use. The measurement of D3 and D2 specific enzyme activities were performed...
as described previously (Koopdonk-Kool et al. 1996). In short, D3 activity was determined using \(^{[125]}\)I-T\(_4\) (0·6 nmol \(^{[125]}\)I-T\(_4\)/l; Sigma) as substrate, measured by the amount of \(^{125}\)I released in the conversion of \(^{[125]}\)I-T\(_3\) to diiodotyrosine by placental homogenates, and was corrected for non-enzymic 5-deiodination. D2 activity was determined using \(^{[125]}\)I-T\(_3\) (0·3 nmol \(^{[125]}\)I-T\(_3\)/l; Sigma) as substrate, measured by the amount of \(^{125}\)I released in the conversion of \(^{[125]}\)I-T\(_3\) to T\(_3\) and also corrected for non-enzymic deiodination; further deiodination was inhibited by adding excess non-radioactive T\(_3\) (Sigma). Enzyme activities were expressed as fmol of 125\(^{\text{I}}\) released from \(^{[125]}\)I-T\(_4\) (D2) or \(^{[125]}\)I-T\(_3\) (D3)/h per mg protein. Protein concentrations were determined using the method of Bradford (1976), with bovine serum albumin as standard.

**Semi-quantitative reverse transcriptase-polymerase chain reaction assay**

Total RNA of 12·5 d embryos was extracted by TriZol reagent (Gibco, Grand Island, NY, USA). RNA (2 \(\mu\)g) was reverse-transcribed with random hexamers by Moloney murine leukaemia virus RT, and then PCR were carried out using the following primers: Hoxc8 \(5'\)-GTCCAAGACTTCTCCACCA-3' (sense); \(5'\)-CTTGTCTTGTCTACTGTGTT-3' (antisense) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) \(5'\)-TACTCAAGAGTGTGCAGCA-3' (sense); \(5'\)-AGATCCACGACGCACATTT-3' (antisense) generating products of 215 and 308 bp respectively. All PCR reactions consisted of dNTP (0·2 mmol/l), 2 \(\mu\)l cDNA, 0·25 mmol/l of each primer, 1 \(\times\) PCR buffer and 0·8 \(\mu\)l Taq polymerase. The following cycling profile was used: 5 min of denaturation at 94°C followed by thirty-five cycles of each 1 min at 94°C denaturation, 1 min of annealing (GAPDH 58°C and Hoxc8 57°C) and 1 min extension at 72°C, and a final extension step of 10 min at 72°C in a PCR System thermocycler (Whatman, Biometra, Germany). The PCR products were separated on 1·5 % agarose gel and stained with ethidium bromide. Quantification of the Hoxc8 and GAPDH mRNA was performed by scanning the intensities of the ethidium bromide-stained PCR products using the BioDocAnalyse system (Whatman). The Hoxc8 mRNA levels were standardised relative to GAPDH mRNA.

**Western analysis**

Extraction nuclear protein from 12·5 dpc embryos was performed as described previously. The protein concentration was determined by DC protein assay (BioRad, Richmond, CA, USA). Nuclear protein samples (50 \(\mu\)g) were heated for 5 min at 95°C and separated on 12 % SDS-PAGE and transferred to NC membranes (Millipore, Bedford, MA, USA) in tri(hydroxymethyl)-aminomethane-glycine buffer (pH 8·5) plus 20 % methanol. The membranes were blocked overnight in 5 % non-fat milk in tri(hydroxymethyl)-aminomethane-buffer containing 0·1 % Tween-20 and then washed with tri(hydroxymethyl)-aminomethane-buffer. The blots were incubated for 2 h at room temperature with 1:500 mouse Hoxc8 monoclonal IgG (Covance, Princeton, NJ, USA) and 1:4000 rabbit polyclonal antibody anti-nucleolin (Abcam Ltd, Cambridge, Cambus, UK), respectively. The blots were washed and then incubated with anti-mouse IgG conjugated with peroxidase (Sigma, St Louis, MO, USA) at 1:10000 dilution. An Amersham ECLTM Detection Kit (GE HealthCare Life Sciences, Little Chalfont, Bucks, UK) provided the chemiluminescence substrate for horseradish peroxidase, and the targeted protein was visualised by autoradiography.

**Statistical methods**

The SPSS 12·0 software package (SPSS Inc., Chicago, IL, USA) 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 analysed by a one-way ANOVA and Duncan’s test. Significance level was set at \(P<0·05\).

**Results**

**Average daily water consumption, urinary iodine concentration and thyroid hormone level in maternal mice**

Average daily water intake was 4·9 (sd 0·8), 4·8 (sd 0·9), 4·8 (sd 1·2), 4·5 (sd 1·3), 4·2 (sd 0·7) and 4·2 (sd 1·2) ml in female mice of 0, 1·5, 3·0, 6·0, 12·0 and 24·0 \(\mu\)g iodine/ml groups, respectively. There was no obvious difference among groups. The mouse drinks about 5 ml daily. As for the groups given high doses of iodine, iodinated water was the main source of iodine. So, the daily iodine intake could be about 7·5, 15, 30, 60 and 120 \(\mu\)g in the treatment groups, which corresponded to 5-, 10-, 20-, 40- and 80-fold of the adequate iodine intake for mice. The concentration of iodine in urine is currently the most widely used biochemical marker of iodine intake. After exposure to excessive iodine for 4 months, the urinary iodine concentration of female mice increased in a dose-dependent manner (\(r=0·96;\ P<0·01\); Fig. 1). Compared with the control group, serum total T\(_4\) median values were significantly different from that of the control group (\(P<0·01\) (Kruskal–Wallis method)).
levels increased and serum total \( T_4 \) levels decreased significantly in dams when the iodine dose reached 3.0 \( \mu \text{g/ml} \), whereas exposure to 1.5 \( \mu \text{g} \) iodine/ml had no obvious effect on thyroid hormone level (Fig. 2).

**Hepatic and renal type 1 deiodinase activity, and placent al type 2 and type 3 deiodinase activity assays**

An obvious depression of D1 activity in liver and kidney was observed in groups when the exposure iodine dose reached 3.0 \( \mu \text{g/ml} \); this showed in a dose-dependent manner (liver: \( r^2 = 0.402, P < 0.01 \); kidney: \( r^2 = 0.276, P < 0.05 \); Fig. 3). High iodine intake had a predominant effect on D2 activity of 12.5 dpc placenta, and no effect on D3 activity (Fig. 4). A dose-dependent reduction of D2 activity was found in groups where the dose was 3.0 \( \mu \text{g/ml} \) or above (\( r^2 = 0.524; P < 0.01 \)). Meanwhile, D3 activity was obvious higher than D2 activity in 12.5 dpc placenta.

**Hoxc8 messenger ribonucleic acid and protein expression**

In the case of the temporal expression pattern, \( Hoxc8 \) was expressed in most of the stages of embryonic development from 8.5 to 17.5 dpc (Kwon *et al.* 2005). In the present study, a decreasing trend in mRNA abundance was semi-quantified by RT-PCR in 12.5 dpc embryos exposed to excessive iodine (Fig. 5 (A)). Western blot assay indicated that high iodine intake above 1.5 \( \mu \text{g/ml} \) induced down regulation of \( Hoxc8 \) protein (Fig. 5 (B)).

**Discussion**

In the present study, excessive iodine treatment resulted in an increase of total \( T_4 \) and a decrease of total \( T_3 \), consistent with previous studies (Harjai & Licata, 1997; Xiang *et al.* 1999). This change was mainly related to the inhibition of D1 activity in liver and kidney, resulting in a decrease in the generation of \( T_3 \) from \( T_4 \). Maternal thyroid hormone levels have an effect on fetal thyroid hormones by means of the placenta, which modulates the transfer of iodine and small but important amounts of thyroid hormones (especially \( T_4 \)) from the mother to the fetus (Burrow *et al.* 1994). The fetal thyroid gland becomes functional at about 17–18 dpc in rodents (Bianco *et al.* 2002). At 12.5 dpc, fetal thyroid hormones come from the maternal circulation by deiodination of \( T_4 \) through placenta D2. Placental D2 activity is negatively regulated by maternal \( T_4 \) level (Steinsapir *et al.* 2000). In the present study, placental D2 activity at 12.5 dpc decreased, showing an inverse relationship with maternal \( T_4 \) level (\( r = -0.301; P < 0.05 \)). Placental D2 activity is likely to be of considerable physiological importance for fetal thyroid hormone economy by contributing to the intraplacental \( T_3 \) content, and possibly to the plasma \( T_3 \). Placental D3 activity is much higher than that of D2, which could be important for the protection of fetal tissues from elevated \( T_3 \) levels (Bates *et al.* 1999). Placental D3 activity showed no significant change after exposure to excessive iodine; the underlying mechanism needs to be elucidated.
At 12.5 dpc, the mother is the only source of fetal thyroid hormones. After exposure to excessive iodine, T4 was higher but T3 was lower in the serum of maternal mice. At the same time, placental D2 and D3 activities were determined and D3 activities (B) were significantly different from that of the control group (P<0.01) (ANOVA).

Previously observed that excessive iodine exposure increased the incidence of skeletal malformation, especially supernumerary ribs. A similar phenomenon was observed in Hoxc8-/− mice (Akker et al. 2001). Other several lines of evidence also substantiate a role for Hoxc8 in the normal axial skeleton (Belting et al. 1998; Juan & Ruddle, 2003; Kwon et al. 2005). In the present study, maternal excessive iodine exposure down regulated mRNA and protein expression of Hoxc8 in 12.5 d embryos. Gaur et al. (2001) described a dramatic increase in the expression of the HoxA5 in the heart and aorta of the Mexican axolotl during the process of T4-induced metamorphosis. Disruption of Hoxc8 expression may associate with the fluctuation of thyroid hormone level induced by excessive iodine exposure. Moreover, with a thyroid hormone response element located in the Hoxc8 promoter region, hypothyroidism induced by excessive iodine could reduce Hoxc8 expression through this thyroid hormone response element-dependent pathway. This finding provided a possible explanation for the skeletal malformation induced by excessive exposure. Further studies are needed to provide more direct evidence of Hoxc8 expression regulation by thyroid hormones through this thyroid hormone response element-dependent pathway.

The mechanism of Hoxc8 modulating bone development has not been clarified. Recently, the identification of downstream targets of Hoxc8 genes found that osteopontin (OPN), also known as secreted phosphoprotein 1, is down regulated by Hoxc8 overexpression in microarray analysis and confirmed by chromatin immunoprecipitation (ChIP) analysis (Lei et al. 2005). OPN is the major non-collagenous bone matrix protein associated with osteoclastic cell adhesion and abundantly expressed during the early stages of osteoblast differentiation. More interestingly, analysis of thyroid hormone responsive gene expression found that OPN expression is also regulated by T3 in osteoblastic cells (Harvey et al. 2003). These findings provide more possible evidence of...
bone development modulated by thyroid hormones through Hox genes, which also need further investigation to verify.

In conclusion, we have demonstrated that excessive iodine exposure induced abnormalities of maternal-fetal thyroid hormone metabolism by affecting deiodinase activities, accompanying down regulation of Hoxc8 mRNA and protein expression. This mechanism may play a pivotal role in skeletal malformation induced by excessive iodine, and provide a new clue to study the relationship between nutrient-iodine or thyroid hormones and Hox gene expression pattern.

Acknowledgements

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