Hypothyroidism induces type 2 iodothyronine deiodinase expression in mouse heart and testis

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Abstract

In the present study we show the expression profiles of both type 1 and type 2 iodothyronine deiodinases (D1 and D2) in a wide spectrum of mouse tissues, and D2 regulation by thyroid status. A characteristic tissue-specific expression for each isoform was observed. D2 transcripts were detected in most tissues with variable levels of expression. The observed D2 mRNA tissue distribution was similar to that described in rats and is in agreement with the view of different patterns of expression between rodents and humans. However, it is interesting to note that despite the low levels of D2 transcripts in mouse heart and testis in the euthyroid state, the induction of hypothyroidism caused a significant increase in D2 activity in these tissues. Similar results were also obtained in adult rats. These results suggest a previously unrecognized role for type 2 deiodinase in controlling intracellular triiodothyronine levels in rodent heart and testis during states of thyroid hormone deficiency.

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Introduction

The outer ring deiodination of the pro-hormone, thyroxine (T_4), is an essential step for the production of the active thyroid hormone, 3,5,3'-triiodothyronine (T_3). This activation reaction may be catalyzed by two different enzymes, type 1 and type 2 iodothyronine deiodinases (D1 and D2) (Larsen 1996, Leonard & Koehle 1996). D1 and D2 are distinguished by their kinetic properties, substrate specificity, inhibition by thyrostatic drugs such as 6n-propyl-2-thiouracil (PTU) and aurothioglucose, and response to thyroid hormones (Larsen 1996, Bianco *et al.* 2002). D1 is believed to provide the major portion of the circulating plasma T_3 in vertebrates. In contrast, D2 seems to play a critical role in maintaining intracellular T_3 levels in specialized tissues such as the anterior pituitary, central nervous system, and brown adipose tissue (BAT) (Bianco *et al.* 2002). The regulation of deiodinase activities is a complex process that involves hormonal, nutritional and environmental factors (Leonard & Visser 1986), and among these the most important seems to be thyroid hormone (TH) status (Kaplan 1986). Most of these concepts have been largely based on studies of enzyme

activity in rat tissue homogenates, and the cloning of D1 (Berry *et al.* 1991, Mandel *et al.* 1992, Maia *et al.* 1995a) and D2 (Davey *et al.* 1995, Croteau *et al.* 1996, Valverde *et al.* 1997, Davey *et al.* 1999, Gereben *et al.* 1999) from different species has confirmed results of earlier studies and provided important new information concerning their structural characteristics, expression profiles and regulation.

The deiodinase isoforms show a pattern of tissue-specific expression which varies among species (Croteau *et al.* 1996, Salvatore *et al.* 1996a, Valverde *et al.* 1997, Gereben *et al.* 1999) and the reason for this is not clear. In vertebrates, D1 is expressed in most tissues (Leonard & Koehle 1996), although the enzyme content varies widely among them. In the rat and humans, the highest levels of D1 activity are found in thyroid, liver, kidney and pituitary gland. In humans, D2 expression appears to be more widespread than previously supposed with large amounts of D2 transcripts being found not only in the pituitary and brain, but also in the thyroid gland (Salvatore *et al.* 1996b), cardiac and skeletal muscles (Croteau *et al.* 1996, Salvatore *et al.* 1996a) and, recently, it has also been reported in kidney and pancreas

(Bartha *et al.* 2000). Mice and rats seem to present a very similar pattern of deiodinase distribution among tissues, with D2 being predominantly expressed in the central nervous system, pituitary gland, and BAT. D2 activity has also been found in thymus (Soutto *et al.* 1996), pineal and Harderian gland (Garcia-Macias *et al.* 1997, Araki *et al.* 1998, Kamiya *et al.* 1999) and uterus of the pregnant rat (Galton *et al.* 2001) and in mouse cochlea and mammary gland (Campos-Barros *et al.* 2000, Song *et al.* 2000). Since both 5' deiodinases are primarily activating enzymes, it would be interesting to compare their tissue expression levels concurrently in order to understand their possible physiological roles and contribution to tissue and plasma T₃ homeostasis. Also, the effect of thyroid status on these newly identified transcripts, which has not yet been completely investigated, may add important insights.

The present study was undertaken to determine the co-expression patterns of 5' activation enzymes in different mouse tissues and to examine the effects of thyroid hormone in the regulation of D2 expression. The data presented show that both 5'-deiodinases, D1 and D2, are distributed in a wide spectrum of mouse tissues. Of particular interest was the finding that hypothyroidism induces D2 expression in adult mouse heart and testis.

Materials and methods

Animals

Male C57BL/6J mice (22–28 g) approximately 7-weeks-old were housed under conditions of controlled lighting and temperature and fed a commercial diet and water available *ad libitum*. To induce the hyperthyroid condition, animals were i.p. injected with 5 or 10 µg L-T₃ (Sigma; diluted in 4 mM NaOH solution in a final volume of 50 µl) at 24-h intervals for 3 days. Simultaneously, a control group was injected with the same volume of vehicle (alkaline physiological saline). Mice were rendered hypothyroid by adding 0.03% methimazole (MMI) to the drinking water for 8 weeks. After treatments, animals were killed under CO₂ anesthesia by exsanguination through heart puncture, and BAT, cerebrum, cerebellum, pituitary gland, heart, liver, kidney, skeletal muscle, lung, spleen and testis were

rapidly removed and frozen in liquid nitrogen and stored at –70 °C until the RNA isolation.

A second series of experiments was conducted using 7-week-old male Wistar rats (150–200 g). Hypothyroid rats were produced by administering 0.03% MMI to the drinking water for 6 weeks.

Serum hormone measurements

Assays were performed on batched serum samples (duplicates) that had been stored at –20 °C awaiting study completion. Total serum T₄ and T₃ levels were measured by RIA (Diagnostic Products, Los Angeles, CA, USA and Immunotech, Marseille, France respectively). Interassay coefficients of variation were 8% for T₄ and 10% for T₃.

5'-Deiodinase assay

D2 assay was performed as previously described (Silva *et al.* 1982, Pachucki *et al.* 2001). Briefly, tissues samples were homogenized on ice in buffer containing 1 × PE (0.1 M potassium phosphate and 1 mM EDTA), 0.25 M sucrose and 10 mM dithiothreitol (DTT) (pH 6.9). The reaction mixtures containing 100–300 µg tissue protein were incubated in a total volume of 300 µl with 100 000 c.p.m. [¹²⁵I]T₄ (Amersham, Biosciences) purified by LH-20 column chromatography (Pharmacia, Uppsala, Sweden), 1 nM unlabeled T₄, 20 mM DTT, and 1 mM propylthiouracil (PTU) in PE buffer at 37 °C for 2 h. Reactions were terminated by the addition of 200 µl horse serum and 100 µl 50% trichloroacetic acid. After centrifugation at 3000 r.p.m. for 10 min, free ¹²⁵I[–] in the supernatant was counted with a gamma-counter. All reactions were performed in duplicate and experiments were repeated twice.

RNA isolation and Northern analysis

Total RNA was isolated from different tissues using Trizol reagent (Life Technologies Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Approximately 30 or 10 µg (pituitary) total RNA from different treatment groups were denatured and electrophoresed on a 1% agarose gel containing 5% formaldehyde, 2 µg ethidium bromide and 1 × MOPS buffer at room temperature. RNA was then transferred by capillary action with 10 × SSC to nylon

membranes (Hybond N+, Amersham) and cross-linked with a UV Stratalinker (Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA). After prehybridization for 2 h at 42 °C in a solution containing 1% SDS, 10% Denhardt's solution, 100 µg/ml denatured salmon sperm DNA and 6 × SSC, hybridization was performed overnight at 42 °C with a solution containing 50% deionized formamide, 1% SDS, 5% dextran sulfate, 6 × SSC, 100 mg/ml denatured salmon sperm DNA and 1 × 10⁶ c.p.m./ml hybridization mix of denatured labeled D2 cDNA probe (random primed). Membranes were then washed twice in 1 × SSC, 0.1% SDS at 25 °C for 10 min and twice in 0.1 × SSC, 0.1% SDS at 52 °C for 30 min. Filters were exposed to X-Omat Kodak films at -70 °C for 3 to 18 days according to signal intensity. D2 transcript levels were quantified by densitometry using Image Master VDS (Pharmacia Biotech) and normalized for the 18S ribosomal RNA signal in each tissue.

RT-PCR analysis

A semiquantitative RT-PCR technique was used to determine the presence of D1 and D2 transcripts in samples of RNA from different mouse tissues. RT-PCR was performed using the SuperScript Preamplification System for First Strand cDNA Synthesis (Invitrogen, Life Technologies) with 3 µg total RNA as template. Specific oligonucleotides derived from the coding region of the mouse D1 (sense: 5' GCC ACT TCT GCC CCG TGC TGA G 3' and antisense: 5' CTG CCT TGA ATG AAA TCC CAG ATG T 3') and rat D2 (sense: 5' ACT CGG TCA TTC TGC TCA AG 3' and antisense: 5' TTC AAA GGC TAC CCC ATA AG 3') were used to prime target cDNA resulting in the predicted 368 and 590 bp (base pairs) PCR products respectively. A mouse β-actin primer set (Clontech, Palo Alto, CA, USA) that generates a 315 bp product was used as an internal control. PCR was performed separately for D1 and D2 that were coamplified with β-actin within the same reaction in order to evaluate inter-sample variation in cDNA contents and PCR efficiency. For each cDNA, a preliminary PCR amplification was carried out to determine the range of cDNA concentrations and number of cycles over which the species should be examined before reaching a plateau. The PCR reactions included 2 µl RT

products and were carried out with Taq DNA polymerase (Gibco BRL) in a 50 µl volume under the following conditions: 94 °C × 3 min, 30 or 35 cycles of 94 °C × 1 min, 58 °C × 1 min, 72 °C × 2 min and a final 5 min extension period. RT-PCRs without reverse transcriptase and/or cDNA samples were carried out as negative controls. To confirm that the 590 bp bands were D2 amplicons, the PCR products were isolated with a Wizard Purification Kit (Promega Corp., Madison, WI, USA) and double digested with AccI and HincII. Digestion produced 280, 100, and 210 bp fragments as predicted, indicating the authenticity of the D2 amplicons. After amplification, the PCR products were separated on a 1.5% ethidium bromide agarose gel and densitometric results of D1 and D2 bands were normalized against the corresponding values of the β-actin band intensities. Densitometric quantification of the ethidium bromide stained bands was carried out using the ImageMaster VDS (Pharmacia Biotech).

Statistical analysis

Results are presented as means ± S.D. of two experiments. Four to six animals were used per group per experiment. Comparisons between two groups were performed using Student's *t*-test or ANOVA when there were three or more groups simultaneously involved. Duncan's test was used for multiple comparisons. All densitometry data were rank transformed prior to analysis. *P* < 0.05 was considered statistically significant.

Results

D1 and D2 expression patterns in different mouse tissues

D1 mRNA was highly expressed in liver and kidney, and less abundantly in testis and pituitary gland (Fig. 1A). In addition, detectable levels of transcripts were observed in almost all tissues analyzed by RT-PCR, which is what would be expected for housekeeping genes (Maia *et al.* 1995c).

D2 transcripts were readily demonstrable by RT-PCR analysis predominantly in BAT, cerebrum, cerebellum, pituitary gland and testis (Fig. 1A). D2 mRNA has also been identified at lower levels in mouse heart, lung and kidney. Northern blot analysis of total RNA (Fig. 1B)

Figure 1 D1 and D2 mRNA expression in different mouse tissues. (A) RT-PCR analysis of PCR products from 11 tissues (as indicated) and a control reaction without template (neg. control) were separated on a 1.5% agarose gel. The D1 368 bp, D2 590 bp and β -actin 515 bp products are indicated. (B) Northern blot analysis of D2 transcripts in different mouse tissues. The transcript sizes are indicated in kilobases.

confirmed this tissue distribution demonstrating a predominant hybridization signal of approximately 7.5 kb in size, compatible with the size of D2 mRNA previously described (Croteau *et al.* 1996, Salvatore *et al.* 1996a). However, in testis, the relative levels of the usual size 7.5 kb D2 transcripts detected by Northern hybridization were surprisingly much lower than would be expected from expression shown by RT-PCR. A second major ~ 1.8 kb transcript (Fig. 1B), also identified in a number of other Northern hybridization analyses (data not shown), appears to be the predominant D2 mRNA transcript in mouse testis. The high levels of D2 expression observed by RT-PCR are likely to reflect amplification of both D2 transcripts in this organ. The low levels of D2 gene expression in heart, lung and kidneys are undetectable by Northern analysis.

Effect of thyroid status on D2 expression

To determine the effects of altered thyroid hormone status on D2 expression, tissues were collected from control and T_3 - or MMI-treated mice as described. As expected, serum T_4 was reduced and serum T_3 concentrations were elevated 24 h after the last T_3 injection in T_3 -treated animals (Table 1). Despite MMI treatment, T_4 levels were reduced to only about 50% of those in euthyroid mice, indicating that hypothyroidism had not been sufficiently severe to further reduce T_4 concentration.

The administration of 5 μ g L- T_3 to euthyroid mice caused a significant reduction of D2 mRNA levels in BAT and heart, whereas no significant alteration in D2 expression was observed in cerebrum and testis by RT-PCR (Fig. 2A). Even

Table 1 Thyroid hormone concentrations in sera from control, MMI-treated or T₃-treated mice and rats. Data are presented as means±S.D. from determinations in four animals

	Mice		Rats	
	T ₃ (ng/dl)	T ₄ (µg/dl)	T ₃ (ng/dl)	T ₄ (µg/dl)
Thyroid status				
MMI-treated		2.0±0.4*	<40.0	<1.0
Control	44.5±3.0	3.7±0.3	56.8±10.0	6.16±0.93
T ₃ -treated	173.5±158.1	<1.0		

Animals were treated as described in Methods. Sera were obtained 24 h after the last T₃ injection.

*Significantly different from control ($P<0.05$).

higher doses of L-T₃ (10 µg/animal/day) failed to decrease cerebral D2 transcript levels when evaluated by Northern analysis (Fig. 2B), findings compatible with those obtained by RT-PCR. Since in cerebral cortex the saturation of T₃ receptors is substantially higher than that of other tissues, such as BAT and heart, a possible explanation for the lack of effect of T₃ administration on D2 mRNA levels could be the higher degree of thyroid hormone receptor occupancy in the euthyroid state. To explore this hypothesis, D2 mRNA levels were compared in hypothyroid, euthyroid and hypothyroid mice treated with L-T₃ (10 µg/animal/day) for 3 days. As shown in Fig. 2C, hypothyroidism was associated with a significant increase in the cerebral cortex D2 mRNA levels compared with those in control animals ($P<0.05$). In contrast, T₃ administration for 3 days to MMI-treated mice resulted in a decrease in the cortical D2 transcript levels that fell close to the initial control levels.

Since the heart is one of the most sensitive target organs for thyroid hormone effects, we explored the physiological relevance of the low levels of the D2 transcript detected by RT-PCR in mouse. T₃ administration caused a significant decrease in D2 mRNA levels (Fig. 2A). Most interesting, however, was that D2 activity showed a 70% increase (0.052 ± 0.021 vs 0.088 ± 0.036 pmol I⁻ released/min per mg protein, $P=0.078$) in heart of MMI-treated mice (Fig. 3A), despite the mild hypothyroidism obtained with this treatment (Table 1). Furthermore, we examined D2 expression in heart obtained from rats with very low thyroid hormone levels (Table 1). Accordingly, these animals showed a marked fivefold increase (0.171 ± 0.040 vs 0.958 ± 0.083 pmol I⁻ released/min per mg protein, $P=0.005$) in heart D2 activity

when compared with euthyroid control animals (Fig. 3B), suggesting an important role for this enzyme in this tissue under thyroid hormone deficiency in rodents.

In testis, RT-PCR analysis failed to demonstrate an effect of T₃ on testicular D2 mRNA levels (Fig. 2A). Similar to what has been found in mouse heart, MMI-treatment induced a significant increase in D2 activity in testis (Fig. 3A) with similar results obtained in hypothyroid rats. Figure 4 illustrates the Lineweaver-Burk plot of D2 activity in testis of hypothyroid rats. The kinetics parameters are in accordance with those of D2 described in other rat tissues (Visser *et al.* 1982, Silva & Larsen 1985).

Discussion

In the present study we show the expression profiles of both type 1 and type 2 iodothyronine deiodinases in a wide spectrum of mouse tissues, and also D2 regulation by thyroid status. A characteristic tissue-specific expression for each isoform was observed. D2 transcripts were detected in most tissues with variable levels of expression. The observed D2 mRNA tissue distribution was similar to that described in rats and corroborates the view that there are different patterns of D2 expression between rodents and humans. However, it is interesting to note that despite the low levels of D2 transcripts in mouse heart and testis in the euthyroid state, the induction of hypothyroidism caused a significant increase in D2 activity in these tissues. These results suggest a previously unrecognized role for type 2 deiodinase in controlling intracellular T₃ levels in rodent heart and testis during states of thyroid hormone deficiency.

Figure 2 Effect of T₃ treatment on D2 mRNA levels in mouse tissues. (A) RT-PCR analysis of D2 expression in tissues isolated from control (saline) and T₃-treated (5 µg/mouse, i.p.) mice. Bars indicate the mean±S.D. of values obtained in a minimum of four animals. *P* values are indicated under the bars. (B) Northern analysis of D2 mRNA levels in cerebrum of control and T₃-treated (10 µg T₃/mouse, i.p.), and (C) in MMI-treated, control and MMI-treated mice that were given 10 µg T₃, i.p. for 3 days. Each lane represents 30 µg total RNA obtained from an individual mouse. The blots were probed for D2, and then reprobbed for 18S ribosomal RNA as described. The transcript sizes are indicated in kilobases.

Figure 3 Effects of hypothyroidism on (A) 5' D2 mRNA levels (mRNA) and D2 activity (activity) in mouse heart and testis and (B) D2 activity in rat heart and testis. Bars indicate means $\pm$ S.D. of values obtained in a minimum of four animals. *P* values are indicated under the bars in (A).

Most aspects of the structural and functional characteristics of the iodothyronine deiodinases are well characterized but their relative physiological roles, particularly of D1 and D2, are still not well defined. It has been estimated that in euthyroid rats D1 activity generates T_3 primarily for export to the plasma, while D2 activity is thought to generate T_3 mainly to be used locally (Bianco *et al.* 2002). Nevertheless, the concept of separated functions for the two enzymes is questioned by the fact that pituitary and brain express both D1 and D2, and in addition, it has been demonstrated that in euthyroid rats D2 generates some of the extrathyroidal plasma T_3 (Nguyen *et al.* 1998). Our results demonstrate that there is a clear isoform preponderance in most tissues with the exception of the pituitary. This is in agreement with the classically defined deiodinase function and, at least in mice, higher D2 expression levels are present tissues where a tighter T_3 homeostasis is required.

Thyroid hormones are the most important recognized regulators of deiodinase expression, with well correlated changes in activities and mRNA levels (Berry *et al.* 1990, Maia *et al.* 1995a,b, Burmeister *et al.* 1997, Tu *et al.* 1997, Kim *et al.* 1998). The effects of T_3 on cerebral D2 mRNA levels is of particular interest because conflicting results relating to the suppressive effect of T_3 on D2

gene expression in rat cerebral cortex have previously been reported. Burmeister *et al.* (1997), demonstrated that T_3 administration to euthyroid rats significantly reduces D2 transcript levels in rat cerebral cortex while another study reports little or no effect on cortical D2 transcript levels in response to T_3 treatment (Croteau *et al.* 1996). In the present work, administration of high doses of T_3 to euthyroid mice did not change D2 expression, as assessed either by RT-PCR or Northern blot analysis. The near complete saturation of T_3 receptors in the brain could explain the lack of effect of thyroid hormone excess on the cortical D2 mRNA levels. On the other hand, reduction in T_3 circulating levels causes nuclear receptor desaturation, since the exposure of hypothyroid mice to high doses of T_3 caused a significant decrease in D2 transcripts nearly to the levels of the euthyroid controls. These findings are compatible with a predominant effect of thyroidal status on regulating D2 expression in the transition from the euthyroid to the hypothyroid state, whereas D2 mRNA levels seem to vary significantly, as previously suggested (Burmeister *et al.* 1997).

Most interesting were the findings obtained from thyroid hormone regulation on D2 expression in the heart. As previously mentioned, high levels of D2 expression seem to occur in tissues where a

Figure 4 Lineweaver-Burk plot showing 5'-deiodination of T_4 by rat testicular homogenates. Tissue protein (300 μg) was incubated for 2 h at 37 °C with 0.25, 0.50, 1, 3, and 6 nM T_4 , in the presence of 20 mM DTT and 1 mM PTU. The reaction rate was measured by the accumulation of $^{125}\text{I}^-$, as indicated in Methods. Kinetic constants were calculated to be: $K_m=1.56$ nM, $V_{\max}=0.52$ pmol/min per mg protein.

close T_3 homeostasis is required, and the finding that human heart expresses higher levels of D2 mRNA (Salvatore *et al.* 1996a) compared with rat heart (Davey *et al.* 1995), has been suggested as evidence of species-specific deiodinase tissue function. The higher expression of cardiac D2 in humans as compared with rodents has recently been attributed to the cardiac transcription factors Nkx-2.5/GATA-4 that, acting in synergy in humans but not in rats, would explain the different D2 levels between humans and rodents (Dentice *et al.* 2003). Therefore, it has been presupposed that rodents are not a faithful model to examine the contribution of D2 to the intracellular T_4 -to- T_3 conversion in the heart, with the possible implications in human pathological conditions. In the present work we show that in intact animals,

despite the low levels of D2 expression detected in euthyroid mouse and rat heart, hypothyroidism induces a marked increase in D2 activity. At least two inferences arise from these results. First, they indicate a role for D2 in rodent hearts and may reflect a protective mechanism aimed to maintaining normal T_3 concentrations in situations of naturally occurring low T_4 levels such as low iodine intake. Secondly, rodents, particularly rats, could be used to study the functional role of D2 in myocardium. Our results conflict with those obtained in transgenic mice expressing high levels of human D2 gene in which the induction of hypothyroidism caused a decrease in D2 activity (Pachucki *et al.* 2001).

The role of thyroid hormone on testis morphology and function has been discussed in the

literature since early studies suggested that the adult testes of experimental animals are metabolically unresponsive to thyroid hormones (Barker & Klitgaard 1952, Oppenheimer *et al.* 1974). However, many lines of evidence have demonstrated that thyroid malfunction is frequently associated with abnormalities in testicular morphology and function (Jannini *et al.* 1995, Manna *et al.* 2001). Thyroid hormone receptors (TR) isoforms have recently been detected in fetal, neonatal and adult human and rat testis (Buzzard *et al.* 2000, Jannini *et al.* 2000). The presence of TR α isoforms in the adult testis of these species suggests a direct effect of thyroid hormone on this organ and could explain some of the detrimental effects of altered thyroid status in testicular function (Jannini *et al.* 1995). Our results further support an important role for thyroid hormone regulation in this tissue. We found D2 mRNA and activity in adult mouse testis and demonstrated that even mild hypothyroidism induces a significant increase in this enzyme expression, indicating that this organ is highly sensitive to thyroid hormone deficiency.

In conclusion, our results demonstrate that D1 and D2 mRNA are broadly expressed in mouse tissues, and that D2 shows a pattern of tissue distribution similar to that described in rats. Most importantly, we found that hypothyroidism induces D2 expression in mouse and rat heart and testis, where it is normally expressed at low levels, causing a marked increase in the enzyme activity. These results suggest that, under conditions of low iodine intake, the increased activity of D2 might contribute to maintain normal T₃ concentrations in rodent heart and testis in the face of reduced T₄ levels in plasma.

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