Regulation of *Dio2* gene expression by thyroid hormones in normal and type 1 deiodinase-deficient C3H mice

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Abstract

The C3H/HeJ mouse presents an inherited type 1 deiodinase (D1) deficiency that results in elevated serum thyroxine (T_4), whereas TSH and tri-iodothyronine (T_3) concentrations are normal when compared with those in the C57BL/6J strain. Here, we evaluated the expression of the type 2 (D2), the other T_4 -activating enzyme, in C3H mice. A comparative analysis revealed that D2 mRNA levels in C3H are similar to those in C57 animals. The D2 activity in C3H pituitary and brain are reduced when compared with those in the C57 strain (3.75 ± 1.08 vs 5.78 ± 0.33 and 0.17 ± 0.05 vs 0.26 ± 0.07 fmol/min per mg protein respectively). However, no differences on D2 activity levels were observed in the brown adipose tissue (BAT) between both strains (0.34 ± 0.06 vs 0.36 ± 0.09 fmol/min per mg protein). Experiments using different T_4 doses showed that higher levels of serum T_4 than those of

the C3H mouse are required to downregulate D2 activity in this tissue. T_3 administration to euthyroid mice resulted in a two- to four-fold increase on D2 activity in BAT and brain of both strains, despite a marked decrease in BAT D2 transcripts and no changes in brain D2 mRNA levels. The increase in D2 activity was preceded by a decrease in serum T_4 levels, which appears to reduce D2 degradation. Indeed, administration of T_3 plus T_4 abolished the T_3 -induced D2 upregulation. In conclusion, our results demonstrated that D2 activity is mainly regulated at posttranslational level in a tissue-specific manner. These observations further characterize and provide insights into the complex and dual regulatory role of the iodothyronines in D2 regulation.

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Introduction

Thyroxine (T_4), a major secretory product of the thyroid gland, needs to be converted to tri-iodothyronine (T_3) to exert its biological activity. Two isoenzymes, types 1 and 2 iodothyronine deiodinase (D1 and D2), catalyze T_4 to T_3 conversion (Bianco *et al.* 2002). Because high levels of D1 activity were identified in the liver and kidney of rats and humans, it had been assumed that this enzyme was the source of most of the serum T_3 (Visser 1996). However, recent studies in mice with genetically inactivated hepatic *Dio1* demonstrated that the D1 is not essential to maintain normal serum T_3 level, at least in the euthyroid state (Streckfuss *et al.* 2005). D2 plays a critical role in maintaining intracellular T_3 level in specialized tissues, such as the anterior pituitary, central nervous system, and brown adipose tissue (BAT; Silva *et al.* 1978, Crantz *et al.* 1982, Bianco & Silva 1987) and, recently, it has been suggested that D2 might also provide a significant fraction of serum T_3 in euthyroid humans (Maia *et al.* 2005).

Although several factors such as hormones, growth factors, adrenergic agents, environmental and nutritional conditions influence deiodinase activities, these enzymes are mainly regulated by thyroid hormones (Bianco *et al.* 2002).

In response to severe iodine deficiency or hypothyroidism, serum T_3 and T_4 are reduced, thyroid-stimulating hormone (TSH) is increased, and the peripheral T_3 production from T_4 is maintained by upregulation of D2 and downregulation of D1 expression. Conversely, in the hyperthyroid state D1 is increased, whereas D2 is decreased. D1 activity is regulated by thyroid hormones almost exclusively at the transcriptional level (Berry *et al.* 1990, Maia *et al.* 1995a). In contrast, the control of the D2 expression is more complex, occurring by transcriptional, posttranscriptional, and posttranslational mechanisms (St Germain 1988, Burmeister *et al.* 1997, Gereben *et al.* 2002). At transcriptional level, D2 is downregulated by its end product T_3 , whereas its substrate, T_4 , controls enzyme activity at posttranslational level.

The C3H/HeJ (C3H) inbred mouse has an inherited D1-deficiency which results in approximately tenfold reduction in hepatic levels of D1 mRNA and activity when compared with the C57BL/6J (C57) inbred strain, which presents higher *Dio1* expression (Berry *et al.* 1993, Schoenmakers *et al.* 1993). In addition to the inherited D1 deficiency, the C3H mouse exhibits a higher susceptibility to chemically induced hepatocarcinogenesis and lower susceptibility to atherosclerotic plaque formation in response to a high-fat diet and larger spleen, when

compared with C57 mice (Paigen *et al.* 1987, Buchmann *et al.* 1991, Manning & McDonald 1997). Despite all these known genetic traits, the C3H mouse presents a mild phenotype in that it appears healthy, and reproduction and growth are unimpaired. The reduced D1 activity in C3H mice correlates with a CGT repeat insertion into the 5'-flanking region of the *Dio1* gene that seems to impair C3H promoter potency (Maia *et al.* 1995b). The serum T₃ and TSH concentrations in C3H mice are at normal range, whereas total and free T₄ levels are elevated when compared with those in C57 mice. The normal serum T₃ is partially explained by the reduction in T₃ clearance, due to the lower D1 levels, and increased serum T₄ concentration that would compensate for the reduced fractional conversion of T₄ to T₃. However, the expression of D2, another T₄-activating enzyme, has not been entirely assessed in D1-deficient mice. Comparative analysis of D2 activity levels between C3H and C57 mice demonstrated that pituitary and brain D2 activity in C3H mice was about 50% lower than that in C57 animals (Berry *et al.* 1993), which is attributed to the twofold increase in serum T₄ concentration. The D2 mRNA expression has not been evaluated in the C3H mouse. In the recently described D1-deficient mouse (D1KO), created by targeted disruption of the *Dio1* gene, D2 activity was assessed in pituitary, brain, liver, skin, and thyroid (Schneider *et al.* 2006). However, D2 expression in BAT, which can be an important source of peripheral T₃ under certain circumstances (Silva & Larsen 1985), was not evaluated.

The aim of the present study was to further investigate *Dio2* gene expression and regulation in C3H D1-deficient mice.

Materials and Methods

Materials

All reagents were of analytical grade and obtained from commercial sources. T₄ and T₃ were obtained from Sigma Chemical Co. High specific activity [¹²⁵I]T₄ (1500 µCi/µg) was purchased from Amersham Biosciences. Reagents to determine protein concentration were obtained from Bio-Rad Laboratories.

Animals

Male C57BL/6J and C3H/HeJ mice (22–28 g), ~7 weeks old, obtained from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS, Porto Alegre, RS, Brazil), were housed under controlled lighting and temperature conditions, and fed a commercial diet and water available *ad libitum*. The animals were maintained in accordance with the guidelines of the Hospital de Clínicas de Porto Alegre Ethics Committee for the Use and Care of Experimental Animals.

In initial studies, the experimental groups ($n=4-6$ mice/group) included euthyroid control and euthyroid animals treated with L-T₃ (10 µg/animal, i.p. injected, daily) for 3 days before death to induce hyperthyroidism.

Subsequently, the time course of thyroid hormone effects on D2 mRNA and activity in mice tissues was determined using shorter periods of T₃ administration. Mice were treated with either saline solution or 10 µg L-T₃ for 4, 12, 24, and 72 h.

A second series of experiments was conducted to determine the time course of the reduction of D2 activity by maximal doses of T₄ alone or in combination with T₃ in tissues of C3H and C57 animals. Euthyroid mice were i.p. injected with L-T₄ 1, 3 or 9 µg/100 g BW alone or a combination of L-T₄ 3 µg/100 g BW + L-T₃ 10 µg for 6, 24, and 48 h before death. Mice treated only with vehicle served as euthyroid controls. The chosen doses of administered thyroid hormones were based on previous reports to induce graded thyrotoxicosis (Escobar-Morreale *et al.* 1997, Schneider *et al.* 2001). After treatments, mice were euthanized under CO₂ and tissues were rapidly removed, frozen in liquid nitrogen, and stored at -70 °C until RNA extraction or homogenization for activity analysis.

Serum hormone measurements

Assays were performed on batched serum samples that had been stored at -20 °C awaiting study completion. Serum total T₄ was measured by radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA) and interassay coefficient of variation was 8%. Serum total T₃ was also determined by radioimmunoassay in the first series of experiments (Immunotech, Marseille, France) and by electro-quimioluminescence immunoassay (Roche Diagnostics) in experiments shown in Tables 2 and 3. Interassay coefficients of variation were 9 and 10% respectively.

Isolation of RNA and northern blot analysis

Total RNA was isolated using TRIzol reagent (Invitrogen Corp.) according to the manufacturer's instructions. Samples of total RNA (~30 µg) were examined for the presence of D2 transcripts by northern analysis, using the rat D2 cDNA as probe. Northern blots and radioactive probes were prepared as previously described (Wagner *et al.* 2003). D2 hybridization signals were quantified by densitometry using Image Master VDS (Pharmacia Biotech). Blots were rehybridized with 18S ribosomal RNA probe and 18S signals were used as a control to normalize for differences in the amount of total RNA in samples. All experiments were repeated twice.

Real-time PCR analysis

RNA was reverse-transcribed with the SuperScript Pre-amplification System for First Strand cDNA Synthesis (Invitrogen, Corp.) using 3 µg total RNA and 100 ng random hexamers. Reactions for the quantification of target mRNAs were performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Warrington, UK) using the SYBR Green PCR Master Mix (Applied Biosystems) and cyclophilin as a housekeeping internal

control. Samples were run in duplicate. The cycle conditions were 94 °C×5 min (Hot Start), 35 cycles of 94 °C×30 s; 58 °C×30 s; 72 °C×45 s and a final 5 min extension period. Initially, standard curves representing five-point serial dilution of mixed cDNAs of the control and experimental groups were analyzed and used as calibrators to determine the relative quantification of product generated in the exponential phase of the amplification curve. Comparable efficiency was observed presenting $r^2 > 0.99$. Samples were measured by relative quantification (change in expression C3H versus C57 mice; untreated versus treated animals). The data generated by the ABI Prism 7500 system SDS software (Applied Biosystems, Warrington, UK) were then transferred to an Excel spreadsheet and the experimental values corrected by that of the cyclophilin standard. Oligonucleotides for mouse D2 and cyclophilin respectively, were as follows: (5'-TTCTCCAAGTGCCTCTTCCTG-3' and 5'-CCCAT-CAGCGGTCTTCTCC-3'); (5'-GCCGATGACGAGCCCTTG-3' and 5'TGCCGCCAGTGCCATTATG-3').

Deiodinase assays

5'-Deiodinase assays were performed as previously described (Wagner *et al.* 2003). 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. [3',5'-¹²⁵I]T₄ purified by LH-20 column chromatography (Pharmacia), 1 nM (D2) or 1 µM (D1) unlabeled T₄, 10, or 20 mM DTT, in the presence or absence of 1 mM propylthiouracil 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 g for 2 min, the free ¹²⁵I in the supernatant was counted with a gamma-counter. Deiodination was linear with both protein concentration and time, and the quantity of enzyme assayed was adjusted to consume <30% of substrate. Activity is expressed as femtomoles iodide generated/min per mg protein. In determining deiodination activity, the percent iodide generated was multiplied by two to account for the random labeling and deiodination at the 3' and 5' positions in the

[3',5'-¹²⁵I]T₄ (Kuiper *et al.* 2002). All reactions were performed in duplicate.

Statistical analysis

Results are presented as mean ± S.D. of two experiments. Four to six animals were used per group per experiment. Data were log-transformed prior to analysis. Comparisons among groups were assessed by one-way ANOVA followed by Dunnett's *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

D1 activity in C3H mice

To confirm that the C3H mice used in this study present the described D1 deficiency, we determined the level of hepatic D1 activity and compared it with that of the C57 mice. D1 activity in the C3H mouse was significantly lower than that observed in the C57 strain (0.08 ± 0.04 vs 0.41 ± 0.12 pmol/min per mg protein respectively). Accordingly, the serum T₄ concentration in C3H mice was approximately twice that in C57 animals (6.0 ± 0.9 vs 3.4 ± 0.5 µg/dl), whereas T₃ levels were comparable between both mice strains (51.3 ± 22.2 vs 51.5 ± 8.9 ng/dl).

D2 mRNA and activity levels

D2 mRNA and activity were assessed in pituitary, brain, and BAT from C3H and C57 mice. The D2 mRNA levels in C3H tissues were similar to those observed in corresponding tissues in C57 animals. In the C3H mouse, D2 activity in pituitary and brain was approximately half of that found in the C57 strain (Table 1). However, interestingly, the level of D2 activity in BAT was similar between both mice strains.

Effects of the administration of different doses of T₄ on pituitary and BAT D2 activity levels in the C3H and C57 mice

Assuming that, in C3H mice, lower D2 activity levels in the brain and pituitary were due to their chronically elevated

Table 1 Basal levels of deiodinase (D2) expression in C3H and C57 mice. Data are presented as the means ± S.D. of values obtained in a minimum of four to six mice/group

	BAT		Brain		Pituitary	
	mRNA	Activity	mRNA	Activity	mRNA	Activity
Strain						
C3H/HeJ	5.95 ± 5.95	0.34 ± 0.06	1.82 ± 0.30	0.17 ± 0.05	3.50 ± 1.83	3.75 ± 1.08
C57BL/6J	5.29 ± 4.66	0.36 ± 0.09	2.06 ± 0.81	0.26 ± 0.07	3.96 ± 1.41	5.78 ± 0.33
<i>P</i>	0.34	0.72	0.45	0.01	0.64	0.04

Measurements of D2 mRNA (arbitrary units), determined by real-time PCR, and activity levels (fmols T₄/min per mg protein) were performed as described in Materials and Methods.

serum T_4 concentration, we wished to test whether higher levels of circulating T_4 would reduce BAT D2 activity in these animals. Therefore, we used a number of different T_4 doses to induce a graded increase in the level of serum T_4 and correlate them with the D2 activity in BAT and also in pituitary. The latter was used as a reference tissue.

The changes in serum T_4 concentration of mice injected with different doses of T_4 are shown in Fig. 1A. Serum T_4 was well above control levels by 24 h after the injection of either

Figure 1 Serum T_4 and pituitary and BAT D2 activities in euthyroid C3H mice injected with different doses of T_4 . A, Changes in serum T_4 concentration in mice injected with vehicle (C) or with increasing doses of T_4 (1 or 3 $\mu\text{g } T_4/100 \text{ g BW}$ for 24 h or 9 $\mu\text{g } T_4/100 \text{ g BW}$ for 6 or 48 h, as indicated). B and C, Effects of the different doses of T_4 on pituitary (B) and BAT (C) D2 activities. Data are reported as the means $\pm$ s.d. shown relative to controls. * $P < 0.05$ versus control.

1 or 3 $\mu\text{g } T_4/100 \text{ g BW}$ and reached the highest level by 6 h after animals were injected with 9 $\mu\text{g } T_4/100 \text{ g BW}$. A subsequent significant decrease in C3H pituitary D2 activity was observed with increasing circulating T_4 concentrations (Fig. 1B). On the contrary, no significant changes in BAT D2 activity were observed until serum T_4 was approximately threefold higher than C3H control level (Fig. 1C).

BAT and pituitary D2 activities were also measured in the T_4 -treated C57 mice to assess whether it presented the same relative insensitivity to changes in circulating T_4 as that observed in the C3H strain. Small increases in serum T_4 concentration in C57 mice induced a marked decrease on pituitary D2 activity (Table 2). However, similarly to what has been seen in the C3H mouse, no significant changes on BAT D2 activity was observed even when serum T_4 concentration was approximately threefold higher than control level (Table 2).

Effect of T_3 administration on D2 mRNA and activity levels in C3H and C57 mice

To evaluate the intrinsic responsiveness and regulation of the C3H *Dio2* gene by T_3 , we analyzed the D2 mRNA and activity in brain and BAT of euthyroid mice T_3 -treated for 3 days. T_3 administration resulted in a significant decrease in BAT D2 mRNA level in both strains (Fig. 2A and B), whereas no changes were observed in D2 mRNA concentration in the brain (Fig. 2C and D).

Despite the marked decrease in BAT D2 mRNA level, chronic T_3 treatment caused a significant increase in BAT and brain D2 activity of both C3H and C57 mice (Fig. 3A and B). The most likely explanation for the observed T_3 -induced increase in D2 activity was the reduction of serum T_4 concentration, due to the decreased thyroid T_4 secretion caused by T_3 -induced inhibition of TSH release. In this case, one could predict that D2 activity levels would increase earlier in tissues of C57 than in C3H animals in response to T_3 treatment, since the latter presents higher T_4 concentration and reduced fractional clearance by D1 (Table 3). Indeed, BAT D2 activity increased as early as 4 h after T_3 administration in the C57 mouse (Fig. 4A and Table 3). D2 activity reached the highest level at 24 h and remained elevated up to 72 h of treatment (Fig. 4A). In contrast, in C3H mice, BAT D2 activity did not change significantly at 4–24 h after T_3 administration (Table 2). By 72 h of T_3 exposure, BAT D2 activity was similarly higher in both mice strains.

The changes induced by 72 h T_3 treatment on D2 activity in brain resembled those in BAT. However, short times of exposure to T_3 (4–24 h) did not change significantly the D2 activity levels neither in this tissue in C57 nor in C3H animals (Fig. 4B).

Effects of combined T_3 plus T_4 treatment on D2 activity levels

In order to determine whether T_3 stimulation of D2 activity in mice tissues was caused by a T_3 -induced fall in serum T_4 concentration, C57 mice were injected with a

Table 2 Effects of thyroxine (T₄) administration on thyroid hormones concentrations and pituitary and brown adipose tissue (BAT) deiodinase (D2) activities in the C57 mice. Values are the means±s.d. from determinations in four to six mice

	Time (h)	T ₃ (nmol/l)	T ₄ (nmol/l)	Pituitary D2 activity (fmol/min per mg protein)	BAT D2 activity (fmol/min per mg protein)
(A) Control	6	1.90±0.43	53.8±6.43	3.72±2.65	0.86±0.40
T ₄ (1 µg)		1.59±0.13	100±20.6	1.27±0.58*	0.81±0.25
T ₄ (3 µg)		1.93±0.26	196±73.4	0.74±0.36*	0.64±0.25
(B) Control	24	1.43±0.04	55.3±5.15	1.74±0.74	0.87±0.30
T ₄ (1 µg)		1.24±0.12	52.8±5.15	1.59±0.45	0.96±0.10
T ₄ (3 µg)		1.34±0.05	86.3±5.15	0.38±0.07*	0.69±0.13

Mice were i.p. injected with 1 or 3 µg T₄/100 g BW and serum thyroid hormones concentrations and D2 activities were determined (A) 6 or (B) 24 h after each treatment. Control animals received the appropriate vehicle injections. *Significantly different from control ($P<0.05$).

combination of T₄ plus T₃. After 24 h of treatment with the combined doses of 10 µg T₃+3 µg T₄/100 g BW, circulating T₃ was approximately fivefold higher than the control, while T₄ did not decrease or exceed normal control values (Table 4). In contrast to the observed ~3.5-fold increase in C57 BAT D2 activity after T₃ treatment alone, the administration of T₃ plus T₄ did not change BAT D2 activity significantly (Table 4).

Discussion

We have evaluated the *Dio2* gene expression in D1-deficient C3H mice. Our data showed a similar D2 mRNA profile in C3H and C57 mice tissues. In euthyroid C3H animals, due to the increased level of serum T₄ concentration, pituitary and brain D2 activities are markedly reduced when compared with C57 mice. Nevertheless, BAT D2 activity is similar between the C3H and C57 animals. The results of T₄ dose-response experiments showed that higher levels of serum T₄ than those of the C3H mouse are required for D2 downregulation in this tissue. T₃ administration to euthyroid mice resulted in a ~2.5- to 4-fold increase in brain and BAT D2 activity respectively in both mice strains. The increase in D2 activity was not an effect of T₃ *per se* but the result of T₃-induced fall in serum T₄ concentration, since combined T₃ plus T₄ administration completely abolished it.

The C3H inbred mouse strain has an inherited D1 deficiency. Besides the reduced hepatic and renal D1 activity, the most notable features of the C3H mouse are that, when compared with the C57 mouse, the circulating levels of T₄ and rT₃ are elevated, while those of TSH and T₃ are unchanged. It would therefore be anticipated that in C3H mice the higher serum free T₄ concentration would cause a decrease in D2 activity, which was confirmed by twofold lower D2 activity in pituitary and brain (Berry *et al.* 1993). Here, we demonstrated that the D2 mRNA levels are not different between C3H and C57 mice, providing additional evidence that the reduced D2 activity in C3H mice results from an increased rate of substrate-induced enzyme inactivation. Interestingly, in the recently described D1KO mouse,

the pituitary D2 activity is not reduced when compared with wild type animals, despite a significant increase in serum T₄ concentration (Schneider *et al.* 2006). Since serum T₄ in the D1KO mice is ~50% increased, while in the C3H is nearly doubled, a possible explanation for this paradoxical observation would be that this relatively smaller elevation in circulating T₄ would not be enough to downregulate pituitary D2 activity. Nevertheless, similar increase in serum T₄ concentration (~60%) in the C57 mice induces a marked decrease in pituitary D2 activity (Table 2). Thus, it is conceivable, as suggested by Schneider *et al.* (2006), that some modification in the set point of the feedback system might have occurred during the development of the D1KO mouse.

An unexpected finding of this study was the lack of difference in BAT D2 activity between C3H and C57 mice strains. A major D2 property that characterizes its homeostatic behavior is a short half-life (~40 min) that can be further reduced by exposure to its substrates, T₄ or rT₃ (Leonard *et al.* 1984, Silva & Leonard 1985). The cellular mechanism for substrate-induced inactivation of D2 involves ubiquitination, which accelerates enzyme degradation through the ubiquitin-proteasome pathway (Steinsapir *et al.* 1998, 2000, Gereben *et al.* 2000). This regulatory feedback loop efficiently controls T₃ production and intracellular T₃ concentration based on the amount of T₄ available. Indeed, as shown here (Fig. 4) and by others (Croteau *et al.* 1996), decreases in serum T₄ level upregulate BAT D2 activity several times. Hence, it was reasonable to anticipated, based on the higher C3H serum T₄ level, that BAT D2 activity would be lower in these mice. Experiments using high doses of T₄ administration demonstrated that T₄-induced D2 downregulation in BAT requires a much higher T₄ level than those observed in the C3H mice (approximately four- to fivefold over C57 serum T₄ concentration). The relative resistance of BAT D2 activity to serum T₄ levels, in the transition from euthyroidism to hyperthyroidism, was further confirmed in C57 mice (Table 2).

In rodents, BAT is a site of complex interactions between the sympathetic nervous system and thyroid hormone, which make difficult to interpret the already intricate D2 regulation in this tissue (Bianco *et al.* 2005). D2 plays a critical role in

Figure 2 Comparison of D2 mRNA levels in brown adipose tissue (BAT) and brain from control (C) and T₃ treated (T₃) C3H and C57 mice. A and C: northern blot analysis of D2 transcripts in BAT and brain. 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 in Materials and Methods. The ethidium bromide-stained image of the 18S RNA is shown for brain blot. B and D: Quantification of the relative intensity of each pair of bands (D2/18S) was performed by densitometry. Bars indicate the means $\pm$ s.d. of values obtained in a minimum of three animals. The experiments were performed twice.

BAT adaptive thermogenesis and similarly to the pituitary and other D2-expressing tissues, BAT is quite dependent on D2-generated T₃ to supply nuclear T₃-receptor (de Jesus *et al.* 2001). Therefore, a decrease in D2 activity in this tissue may not be advantageous. Indeed, as recently demonstrated in mouse tumor cell line (T α T1 cells), some other physiological

mechanisms may interfere with T₄-induced D2 degradation (Christoffolete *et al.* 2006). In these cells, when a range of concentrations of T₄ was used, the loss of D2 activity was impaired at a concentration >50 pM. The potential explanation for this phenomenon is that the rate of D2 synthesis in these cells equals the maximal rate of T₄-induced

Figure 3 Effects of T₃ treatment on D2 activity in brown adipose tissue (BAT) (A) and brain (B) of C3H and C57 mice. Euthyroid mice were administered L-T₃ (10 µg/animal) or vehicle (controls) by i.p. injection for 3 days. Values are the means ± s.d. *P < 0.01 when compared with control.

D2 degradation, though the authors also considered a possible exhaustion of the ubiquitinating/ proteolytic machinery. Although the mechanism whereby BAT D2 activity remains elevated when marked increases in serum T₄ level are induced in euthyroid mice has not been determined in this study, it is conceivable that mechanisms similar to those observed in T α T1 cells may operate in this tissue. Furthermore, an increased rate of reactivation of ubiquitinated-D2 via von Hippel-Lindau protein-interacting deubiquitinating enzyme (VDU)-1,2-mediated deubiquitination could be involved, since it has been shown that this system is very important in regulating the supply of active thyroid hormone in BAT (Curcio-Morelli *et al.* 2003). According to this line of

reasoning, we can expect that in a setting of T₄-induced hyperthyroidism, other mechanisms than inhibition of D2 are present in order to prevent BAT thyrotoxicosis. Indeed, previous studies have shown that infusion of high doses of T₄ in rats did not decrease BAT D2 activity, whereas BAT T₃ concentration remained normal or only slightly elevated (Escobar-Morreale *et al.* 1997).

The effect of T₃ administration on D2 expression was also investigated in D1-deficient mice. Treatment of euthyroid mice with T₃ results in a marked decrease in BAT D2 mRNA levels, whereas it did not significantly change cerebral D2 mRNA. As demonstrated in rats, the negative control of D2 expression in hypothyroid brain and pituitary by T₃ is probably mediated by

Figure 4 Time course of the induction on BAT (A) and brain (B) D2 activities by T₃ administration in the C3H and C57 mice. Euthyroid mice were administered L-T₃ (10 µg/animal) or vehicle (controls) by i.p. injections for 4, 12, 24, and 72 h. Data are the means ± s.d. shown relative to controls. *P < 0.05 versus zero time-point.

Table 3 Serum thyroxine (T₄) and tri-iodothyronine (T₃) concentrations in control and T₃-treated C3H and C57 mice. Values are mean ± s.d. from determinations in three to five mice

		Control		T ₃ -treated	
	Time (h)	C3H	C57	C3H	C57
Hormone (nmol/l)					
T ₃	4	0.79±0.34	0.79±0.13	>9.22±0.0	>9.22±0.0
	12	0.87±0.34	0.79±0.18	>9.22±0.0	>9.22±0.0
	24	0.88±0.04	0.91±0.02	8.65±0.98	>9.22±0.0
	72	0.82±0.63	0.78±0.14	4.64±0.28	3.69±0.42
T ₄	4	77.2±11.6*	43.8±6.44	79.8±5.15*	36.1±15.4
	12	74.6±12.9*	46.3±18.02	59.2±11.6*	14.2±1.28
	24	75.9±15.4*	48.9±9.01	29.6±9.11*	16.7±2.58
	72	68.2±10.3*	39.9±2.60	<12.9±0.0	<12.9±0.0

Animals were treated as described in Materials and Methods. *Significantly different from C57 (*P* < 0.05).

the nuclear T₃ receptor, although a putative negative TRE in the promoter region of the *D2* gene remains to be identified (Burmeister *et al.* 1997, Kim *et al.* 1998). The lack of response of cerebral *D2* mRNA to T₃ treatment is in agreement with a previous study (Croteau *et al.* 1996) and is probably due to the near complete saturation of T₃ receptors in brain of euthyroid mice (Larsen *et al.* 1981). On the other hand, in cultured rat brown adipocytes, T₃ alone was shown to increase *D2* mRNA but did not change significantly *D2* activity levels (Martinez-deMena *et al.* 2002), indicating that discrepancies exist regarding the *in vivo* and *in vitro* effects of T₃ on the regulation of *D2* expression in BAT.

In contrast to the inhibitory effect of T₃ administration on *D2* mRNA expression, *D2* activity levels were increased ~2- to 3.5-fold in brain and BAT in both C3H and C57 strains. Consideration of these data led to the hypothesis that induction of *D2* activity in T₃-treated mice was due to the decrease in serum T₄ levels. Indeed, additional experiments demonstrated that the upregulation of *D2* activity induced by T₃ was offset by a concurrent decrease in serum T₄ level and combined T₃ plus T₄ administration abolished T₃-induce *D2* increase (Fig. 4 and Table 4). Although these results are quite predictable, they were somewhat unexpected because most studies that evaluated T₃ effect on *D2* activity were performed in hypothyroid animals and, in this setting, T₃ administration decreases *D2* mRNA and activity. In agreement with our results, elegant studies performed by Escobar-Morreale *et al.* (1997) have shown that

in thyroidectomized rats, *D2* activity returned to normal with T₄ infusion, whereas it was increased in animals infused with T₃, when compared with the activities found in animals infused with placebo. Taken together, these observations allow two other inferences. First, serum T₃ has a minor role in regulating *D2* at post-translational level. Second, the T₄ downregulation of *D2* activity is remarkable, since decreases in serum T₄ level upregulate activity several times, regardless of the T₃-induced suppression of *D2* mRNA synthesis.

T₃ treatment also increases *D2* activity in the brain of both strains. However, the time course and magnitude in response to T₃ was nearly identical in control and D1-deficient mice. Another interesting observation was that the increase in *D2* activity occurred later than that observed in the C57 BAT, which might indicate differences in the time required for the exchange of plasma/tissue T₄ among the different tissues.

In conclusion, we demonstrated that D1 deficiency does not affect *D2* mRNA levels, but differentially affects *D2* activity in pituitary, brain, and BAT in the C3H mice. While approximately twofold increase in serum T₄ concentration is enough to induce a significant decrease in *D2* activity levels in C3H pituitary and brain, the T₄-induced *D2* downregulation in BAT requires a much higher serum T₄ level. These results suggest that other intrinsic mechanisms prevent the loss of *D2* activity in this tissue, probably to avoid a decrease in the *D2*-generated T₃ to supply nuclear T₃-receptor. Furthermore, we showed that administration of T₃ to euthyroid mice

Table 4 Effects of combined thyroxine (T₄) + tri-iodothyronine (T₃) treatment on serum thyroid hormones concentration and brown adipose tissue (BAT) deiodinase (*D2*) activity. Values are the means ± s.d. from determinations in four mice

	Time (h)	T ₃ (nmol/l)	T ₄ (nmol/l)	BAT <i>D2</i> activity
(A) Control	6	1.92 ± 0.42	52.7 ± 6.43	0.99 ± 0.26
T ₃ + T ₄		9.99 ± 0.01*	86.2 ± 15.4*	0.85 ± 0.03
(B) Control	24	1.52 ± 0.04	51.4 ± 5.15	0.96 ± 0.10
T ₃ + T ₄		7.65 ± 2.24*	57.9 ± 10.31	0.97 ± 0.29

C57 mice were injected a combination of 10 µg T₃ + 3 µg T₄/100 g body weight and the circulating levels of thyroid hormones and *D2* activity (fmol/min per mg protein) were determined (A) 6 or (B) 24 h after treatment. Control animals received the appropriate vehicle injections. *Significantly different from control (*P* < 0.05).

causes a tissue-specific modulation of D2 mRNA levels and increases D2 activity in BAT and brain. The latter T₃ effect is rapid and marked, and seems to be the result of the decrease in serum T₄ levels, rather than a direct effect of thyrotoxicosis.

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