Clinical aspects of endogenous hypothyroidism and subclinical hyperthyroidism in patients with differentiated thyroid carcinoma
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Type 2 Iodothyronine Deiodinase in Human Skeletal Muscle: Effects of Hypothyroidism and Fasting

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Abstract

Context: The iodothyronine deiodinases D1, D2 and D3 enable tissue-specific adaptation of thyroid hormone levels in response to various conditions, such as hypothyroidism or fasting. The possible expression of D2 mRNA in skeletal muscle is intriguing as this enzyme could play a role in systemic as well as local T3 production.

Objective: We determined D2 activity and D2 mRNA expression in human skeletal muscle biopsies under control conditions and during hypothyroidism, fasting and hyperinsulinemia.

Design: Prospective study.

Setting: University hospital.

Patients: We studied 11 thyroidectomized patients with differentiated thyroid carcinoma (DTC) on and after 4 weeks off thyroxine replacement, and 6 healthy lean subjects in the fasting state and during hyperinsulinemia after both 14 and 62 h of fasting.

Mean outcome measures: D2 activity and D2 mRNA levels were measured in skeletal muscle samples.

Results: No differences were observed in muscle D2 mRNA levels in DTC patients on and off thyroxine replacement therapy. In healthy subjects, muscle D2 mRNA levels were lower after 62 h compared to 14 h of fasting. Insulin increased mRNA expression after 62 h, but not after 14 h of fasting. Skeletal muscle D2 activities were very low and not influenced by hypothyroidism and fasting.

Conclusion: Human skeletal muscle D2 mRNA expression is modulated by fasting and insulin, but not by hypothyroidism. The lack of a clear effect of D2 mRNA modulation on the observed low D2 activities questions the physiological relevance of D2 activity in human skeletal muscle.

Introduction

Peripheral thyroid hormone metabolism is mainly regulated by the iodothyronine deiodinases D1, D2 and D3 (1;2). D1 is expressed in liver, kidney, thyroid and at a lower level in the pituitary. This enzyme converts the prohormone T4 to active T3 and is very active in the breakdown of rT3 (1;3). Although D1 contributes significantly to peripheral T4 to T3 conversion, it is probably not the major source of extrathyroidal T3 production in humans (1;2;4;5). D2 also catalyzes the production of T3 through outer ring deiodination of T4, and is present in brain, pituitary, thyroid, brown adipose tissue (BAT) and, perhaps, skeletal muscle (1;6-10). In brain, pituitary and BAT, D2 is very important for local T3 production. D3 inactivates T3 and T4 by inner ring deiodination (2) and is present in brain, skin, placenta and fetal tissues (1). These deiodinases allow the adaptation of thyroid hormone levels of individual tissues in response to various conditions.

During hypothyroidism, the conversion of T4 to T3 by D2 is increased, whereas the activities of D1 and D3 are decreased (1;11;12). D2 mRNA was found to be expressed in skeletal muscle samples from healthy subjects (9;13). Since skeletal mass is a major body compartment, muscle could therefore play a role in systemic and local T3 production (9). Maia *et al.* proposed that D2 is a major source of circulating T3 in euthyroid subjects and even more so during hypothyroidism (14). In line with this assumption, we hypothesized that during hypothyroidism caused by withdrawal from thyroxine substitution therapy in thyroidectomised patients treated for differentiated thyroid cancer (DTC), D2 activities might be up-regulated in skeletal muscle.

Short-term fasting induces a decrease in plasma T3 that is most probably due to a decreased activity of D1 and/or D2 and/or an increased activity of D3 (1;1;15;16). Indeed, the fasting-induced decrease in serum T3 levels has been attributed to lower peripheral conversion of T4 to T3 (17;18). Since the fall in T3 levels (50%) may be larger than can be accounted for by a drop in D1 activity, and because D2 has an extremely short half-life, D2 activity may have an important role in the reduction in serum T3 as well (1). In contrast to fasting, insulin has been shown to increase both D2 activity and mRNA expression in BAT in animal studies (19-21). Moreover, a recent study demonstrated that incubation of human myoblasts and myotubes with peroxisome proliferator-activated receptor (PPAR)-γ-agonists resulted in increased D2 activity and also suggested a possible role for D2 in insulin signalling (22). We therefore hypothesized that conditions of fasting as well as hyperinsulinemia would affect skeletal muscle D2 expression and activity in vivo since these conditions affect insulin signaling (23).

To our knowledge, no studies in human skeletal muscle samples have been performed to investigate the effect of hypothyroidism or fasting and insulin on skeletal muscle deiodinase mRNA expression or D2 activity. To address this issue, we analyzed D2 activity and mRNA expression of D2 and D3 in skeletal muscle samples in thyroidectomised patients with differentiated thyroid carcinoma (DTC) on and after 4 weeks off thyroxine replacement therapy, and in healthy subjects in the fasting state and during hyperinsulinemia after both 14 and 62 h of fasting.

Material and methods

Subjects with Differentiated Thyroid Carcinoma
Patients were recruited from the outpatient clinic of the Department of Endocrinology of Leiden University Medical Center, which is a tertiary referral center for differentiated thyroid carcinoma. Patients were included who had been diagnosed with DTC and had received initial therapy consisting of near-total thyroidectomy and radioiodine ablation therapy.

Additional therapies were allowed, as long as they resulted in cure. Cure was documented by the absence of measurable serum thyroglobulin (Tg) during TSH stimulation as well as by a negative total-body scintigraphy with 4 mCi 131I. The patients had to be on TSH suppressive therapy, defined as TSH levels below the lower reference value for TSH (0.4 mU/l). The adequacy of the TSH suppressive therapy was documented by yearly TSH measurements.

 14 and 62 h of fasting. Patients who had diabetes mellitus or other endocrine diseases or had a BMI >30 kg/m^2 were excluded. Patients who used any drugs known to influence thyroid hormone metabolism were also excluded. The ethics committee of Leiden University Medical Center approved the study, and written informed consent was obtained from all subjects.

Study design in DTC patients

Patients with DTC undergoing TSH-stimulated ^{131}I scintigraphy were asked to participate in the study. Four weeks after thyroxine withdrawal and 8 weeks after subsequent thyroxine replacement, patients were admitted to the clinical research unit at 8 a.m.. All subjects fasted from the preceding evening (6 p.m.) until the end of the study day. Length (m), weight (kg) and BMI (weight/length^2 [kg/m^2]) were measured. Patients were studied in a semi-recumbent position. A catheter was inserted in a dorsal hand vein to collect plasma samples for measurement of TSH, FT4, T3 and rT3. Muscles biopsies were taken from the quadriceps muscle (vastus lateralis) under local anesthesia (Lidocaine 20 mg/ml; Fresenius, Kabi, Den Bosch, The Netherlands) as described earlier (23). One skeletal muscle biopsy obtained during hypothyroidism was lost. Biopsies were quickly washed in HEPES-buffered saline to remove blood, inspected for fat or fascia content, dried on gauze swabs, and subsequently stored in liquid nitrogen until analysis. Serum samples were handled immediately and stored at –20 C.

Fasting subjects

Six lean healthy men with a normal thyroid status who participated in a study on fasting-induced peripheral insulin resistance were included in this study (23). Written informed consent was obtained from all subjects after explanation of purpose, nature, and potential risks of the study. The study was approved by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam.

Study design in fasting subjects

The experimental protocol has been briefly described earlier (23). In short, subjects were studied twice: after 14 and 62 h of fasting. Study days were separated by at least a week. Subjects were fasting from 8 p.m. until 10 a.m. the next day or 3 days later. They were allowed to drink water ad libitum.

After admission at the metabolic research unit, a catheter was inserted into an antecubital vein for sampling of venous blood for determination of plasma TSH, T4, FT4, T3 and rT3. Hereafter, a muscle biopsy (vastus lateralis of the quadriceps muscle) was performed as described above. Thereafter a continuous infusions of insulin (60 mU/m^2/min) (Actrapid 100 IU/ml; Novo Nordisk Farma B.V., Alphen aan den Rijn, The Netherlands) and glucose 20% (to maintain a plasma glucose level of 5 mmol/L) were started. Plasma glucose levels were measured every 5 min at the bedside. After 5 h of insulin infusion, muscle biopsies were repeated.

Thyroid parameters

Plasma and serum thyroid hormone levels of DTC patients and fasting volunteers were determined as described previously (reference (24) and (25) respectively).

D2 activity

Skeletal muscle samples were homogenized on ice in 10 volumes of PED10 buffer (0.1 M phosphate, pH 7.2, 2 mM EDTA, and 10 mM dithiothreitol) using a polytron (Kinematica AG, Lucerne, Switzerland). Protein concentrations were measured with the Bio-Rad protein assay (Bio-Rad, Veenendaal, The Netherlands) using BSA as the standard according to the manufacturer's protocol.

Skeletal muscle D2 activities were measured as previously described (9). Duplicates of 200 µg homogenate protein were incubated for 60 min at 37 C with 1 nM (1-2x10^5 cpm) [3',5'-^{125}I]T4 in a final volume of 0.1 ml PED10 buffer. The incubations were done in the absence or presence of 0.1 µM unlabeled T3, to prevent inner ring deiodination of the labeled T4 substrate by D3, if present, and in the absence or presence of 0.1 µM unlabeled T4, which is sufficient to saturate D2. Deiodination of labeled T4 in the absence minus that in the presence of excess labeled T4 represents D2 activity. Reaction products were analyzed by determination of the [^{125}I]T3 generated by HPLC analysis of ethanol extracts of the reaction mixtures as previously described (26). The samples from the DTC patients were also analyzed by isolation of the released ^{125}I- from the supernatant after addition of albumin and protein precipitation with 10% TCA.

To rule out interfering effects of local anesthesia on D2 activity in the human muscle samples, we analyzed the effects of increasing lidocaine concentrations on D2 activity expressed in COS1 cells transfected with a human D2 construct (D2-COS1 cells) in pcDNA3 as previously described (27). To rule out the presence of factors in skeletal muscle homogenates that could inhibit D2 activity, we measured D2 activity in D2-COS1 cell lysates with addition of increasing volumes (12.5-50 µl) of homogenate (50-200 µg of protein).

Quantitative mRNA analysis

RNA was isolated from skeletal muscle samples using High Pure RNA kit (Roche Diagnostics, Almere, The Netherlands) following the manufacturer's instructions. RNA concentrations were determined using the RiboGreen RNA quantification kit (molecular Probes, Leiden, The Netherlands). All samples were diluted to 0.1 µg/µl, and 1 µg was used for cDNA synthesis using TaqMan RT kit (Roche Diagnostics, Almere, The Netherlands). D2 and D3 cDNA were analyzed on an ABO PRISM 7700 sequence detection system (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), which uses TaqMan chemistry for highly accurate quantitation of mRNA levels. Sequences and concentrations of the primers are given in Table 1. The D2 and D3 mRNA levels are expressed relative to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or cyclophilin A. Probes and primers for these housekeeping genes were provided as preoptimized control system (Applied Biosystems).

Table 1. Primers and probes used for the determination of D2 and D3 mRNA levels by quantitative real-time RT-PCR

Primers and probes	Sequence
D2 forward	CAAGTCCACTCGCGGAGA
D2 reverse	GACATGCACCACACTGGA
D2 probe	ACGCAGGCGCAGTCCCTCT
D3 forward	TTCCAGAGCCAGCACATC
D3 reverse	ACGTCGCGCTGGTACTTA
D3 probe	TGCACCTGACCACCGTTC

Reactions were done for 2 min minimal at 50 C and for 10 min at 95 C, followed by 40 cycles of 15 sec at 95 C and 1 min at 60 C. Following the manufacturer's guidelines, the cycle threshold (Ct) was determined, which represents the cycle number at which probe-derived dye absorbance reaches the calculated threshold value. Data were expressed as 2ΔCt, where ΔCt represents the Ct value of the housekeeping gene minus the Ct value of the target gene.

Statistical Analysis
Statistical comparisons were performed with the Wilcoxon Signed Rank test. Differences were considered statistically significant at $P<0.05$. The SPSS statistical software program version 12.0.2 (SPSS Inc, Chicago, IL) was used for statistical analysis. Data are presented as mean ± SE.

Results

DTC patients characteristics and thyroid hormone levels
Eleven patients were included in the DTC group (4 males/7 females). Mean ± SE age was 45.5 ± 2.1 years. Mean ± SE duration of TSH suppressive therapy was 5.0 ± 2.1 years (range 0.6-24.3 years). Mean ± SE thyroxine dosage was 197 ± 13 µg/day. Thyroid parameters are presented in Table 2.

Table 2 Thyroid hormone parameters in DTC patients

	Hypothyroidism (n=11)	Thyroxine treatment (n=11)	P-value
FT4 (pmol/L)	1.4 ± 0.2	24.8 ± 1.2	0.003
TSH (mU/L)	142.4 ± 10.4	0.8 ± 0.3	0.003
T3 (nmol/L)	0.3 ± 0.1	1.3 ± 0.1	0.005
rT3 (nmol/L)	0.02 ± 0.00	0.29 ± 0.02	0.003

Wilcoxon signed rank test. Data are presented as mean ± SE

After 4 weeks of thyroxine withdrawal, all patients were overtly hypothyroid. After 8 weeks of thyroxine replacement therapy, FT4, T3 and rT3 increased significantly (p=0.003), whereas TSH decreased significantly (p=0.003). Six patients had thyroid parameters within the reference range, whereas 5 patients had a TSH below the reference range with normal plasma T3 and T4 levels.

Skeletal muscle deiodinase expression and D2 activity in DTC patients
Results of the quantitative RT-PCR analysis of deiodinase mRNA levels in skeletal muscle biopsies are presented in Fig. 1A. D2 and D3 mRNA levels were present in all muscle biopsies, but there was no significant difference between the hypothyroid and thyroxine replacement states.

Very little D2 activity was detected in the muscle biopsies and it was not different between the thyroxine replacement and hypothyroid states (Fig. 2A). Similar results were obtained by HPLC analysis of T3 formation (Fig. 2A) and using the iodide release assay (data not shown). Little D3-catalyzed conversion of T4 to rT3 was observed in the skeletal muscle biopsies, and this was also not different between the hypothyroid and thyroxine replacement states (data not shown).
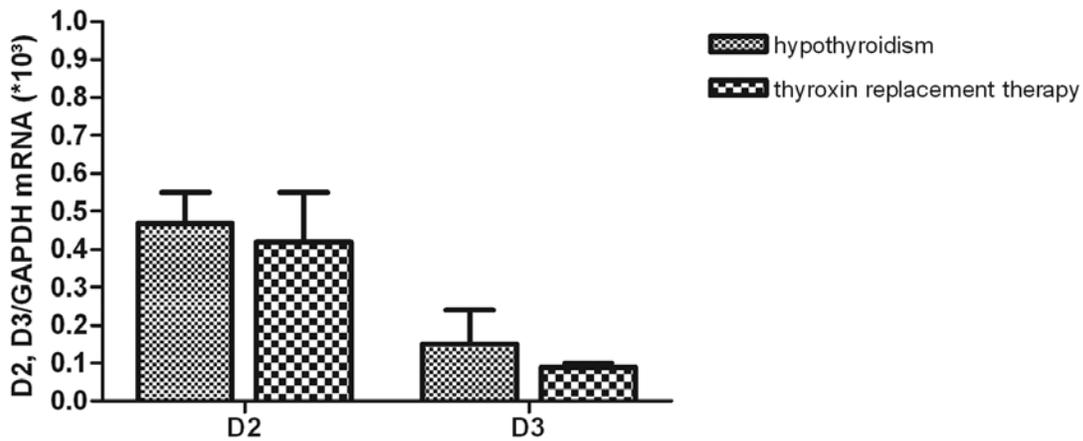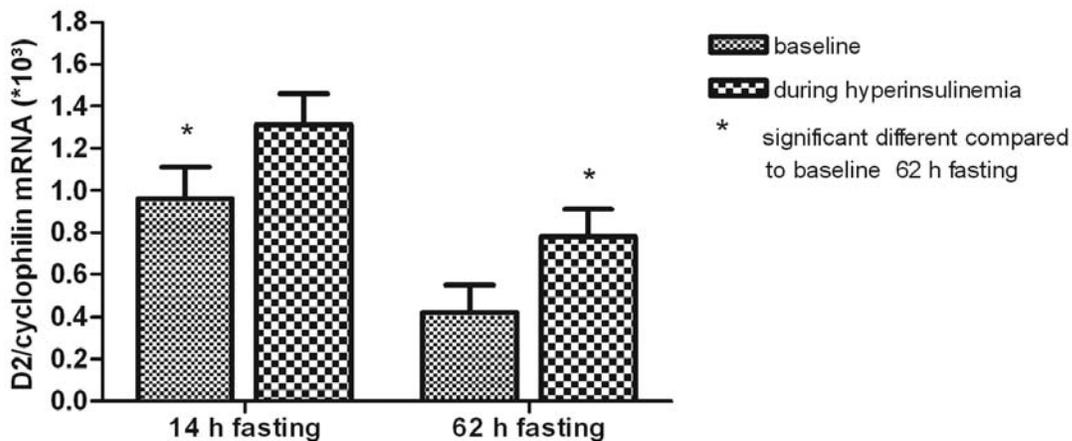
Figure 1. mRNA levels of deiodinase 2 and 3 vs. the household gene GAPDH or cyclophilin ($*10^3$) in skeletal muscle samples (mean ± SE).

A.   In DTC patients (n=11) during hypothyroidism and during thyroxine replacement treatment.

B.   In healthy subjects (n=6) after 14 and 62 h of fasting before and after insulin infusion. $P < 0.05$ for D2 mRNA expression in the basal state after 62 h of fasting compared to the basal state after 14 h of fasting and D2 mRNA expression after insulin infusion after 62 h of fasting compared to the basal state after 62 h of fasting.

Fasting subjects characteristics and thyroid hormone levels

Six lean healthy men were included. Subject characteristics were: age: 23 ± 1.6 yrs; weight 69.4 ± 2.2 kg after 14 h and 67.5 ± 2.2 kg after 62 h of fasting, P = 0.002; BMI 21.2 ± 0.7 Kg/m^2 after 14 h and 20.5 ± 0.7 kg/m2 after 62 h of fasting, P = 0.001 (23).

Plasma FT4 and TSH levels were not different between 14 and 62 h of fasting (Table 3). T3 levels were significantly higher and rT3 levels were significantly lower after 14 h of fasting compared to 62 h of fasting. The T3/T4 (not shown) and T3/rT3 ratio's were significantly higher after 14 h of fasting compared to 62 h of fasting.

Skeletal muscle deiodinase expression and D2 activity after 14 and 62 h of fasting

D2 mRNA levels in skeletal muscle biopsies were significantly lower after 62 h of fasting compared to 14 h of fasting in the basal state (p=0.028). (Fig. 1B). No differences in D2 mRNA levels were observed during hyperinsulinemia after 62 h compared to 14 h of fasting.
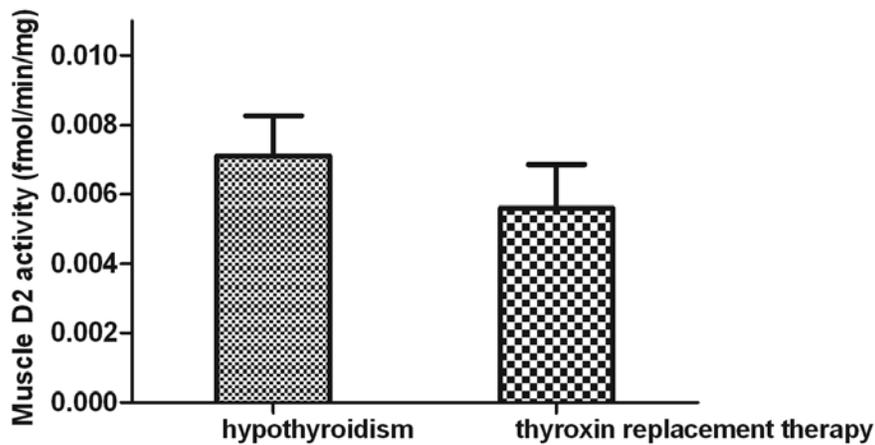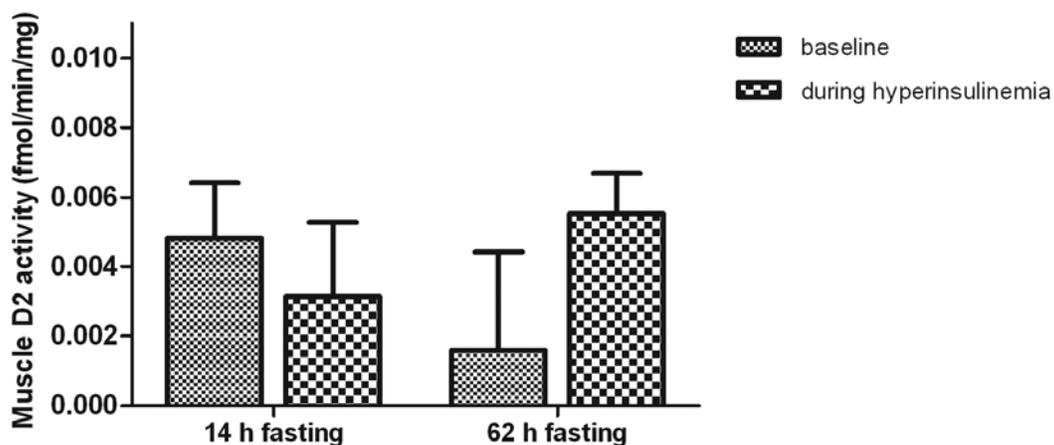
Figure 2a

Figure 2b

Figure 2. D2 activity in skeletal muscle samples (mean ± SE)
A.   In DTC patients during hypothyroidism and thyroxine treatment.
B.   In healthy subjects after 14 and 62 h of fasting.

Insulin infusion did not significantly increase D2 mRNA expression after 14 h of fasting, whereas insulin induced a significant increase in D2 mRNA levels after 62 h of fasting (p=0.028). D3 mRNA measurements were not reliable due to contamination with genomic DNA. D2 activity was low, but detectable in the muscle biopsies in the basal state and after 5 h of hyperinsulinemia both after 14 and 62 h of fasting. However, no significant differences were found (Fig. 2B). Very little D3 activity was demonstrated, and also here no differences were found (data not shown).

Different household genes were used for standardization of mRNA mesurements in DTC patients and healthy fasting subjects. However, their expression levels were constant in the patients and healthy subjects during the different conditions.

D2 activity, lidocaine, and possible inhibitors in muscle homogenate
Addition of increasing concentrations up to 1 mM of lidocaine did not inhibit D2 activity expressed in COS1 cells transfected with human D2 cDNA (Fig. 3A). Although a dose-dependent inhibition of D2 activity was observed after addition of increasing volumes of muscle homogenate up to of 50% of the total incubation volume, remaining activity still amounted to 60% of that expressed in D2-COS1 lysates (Fig. 3B).

Table 3 Thyroid hormone parameters in healthy subjects

	At baseline (n=6)			During hyperinsulinemia (n=6)		
	14h fasting	62h fasting	p-value	14h fasting	62h fasting	p-value
FT4 (pmol/L)	14.6 ± 1.0	14.6 ± 1.3	0.985	13.7 ± 0.8	14.4 ± 1.0	0.279
TSH (mU/L)	1.0 ± 0.2	0.5 ± 0.1	0.102	0.8 ± 0.1	0.5 ± 0.1	0.116
T3 (nmol/L)	1.6 ± 0.1	1.1 ± 0.1	0.027	1.4 ± 0.1	0.9 ± 0.05	0.027
rT3(nmol/L)	0.24 ± 0.02	0.44 ± 0.02	0.027	0.26 ± 0.01	0.38 ± 0.01	0.027
T3/rT3 ratio	6.9 ± 0.7	2.5 ± 0.2	0.028	5.3 ± 0.5	2.5 ± 0.2	0.028

Wilcoxon signed rank test. Data are presented as mean ± SE

Discussion

In this study, we investigated the D2 activity and expression of D2 and D3 mRNA in skeletal muscle samples in DTC patients on and off thyroxine replacement therapy and in healthy subjects after 14 and 62 h of fasting and during hyperinsulinemia. Hypothyroidism induced by withdrawal of thyroxine substitution in thyroidectomised patients did not affect muscle D2 mRNA expression, whereas fasting for 62 h reduced muscle D2 mRNA levels compared to fasting for 14 h.
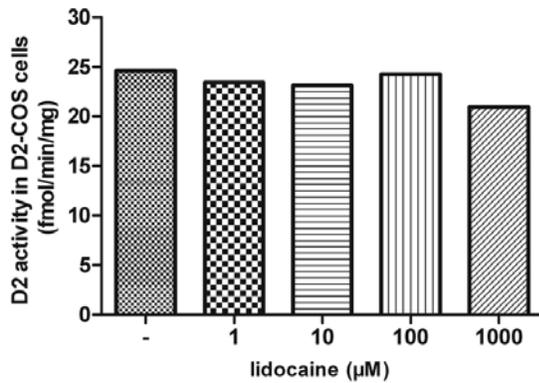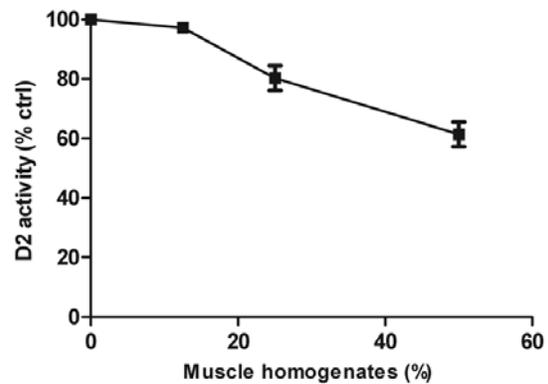
Figure 3a

Figure 3b

Figure 3
D2 activity in D2-COS cells with increasing levels of lidocaine.

Conversely, insulin increased mRNA expression after 62 h, but not after 14h of fasting. Nonetheless, skeletal muscle D2 activities were very low, and not influenced by hypothyroidism, fasting or insulin.
D2 activity is regulated by thyroid status both at the pre- and posttranslational level. In hypothyroidism, D2 activity is increased in different tissues predominantly by a decrease in substrate (T4)-induced degradation of D2 protein (1;28-30). Hypothyroidism also elevates D2 mRNA in rat brain and BAT (1;6;31;32). We found no differences in D2 mRNA expression in skeletal muscle of DTC patients between hypothyroid and thyroxine replacement states.
We measured D2 activity with a highly specific and sensitive D2 assay based on the measurement of radiolabeled T3 production (9;33), but found little D2 activity present in skeletal muscle samples of the DTC patients.

Furthermore, no differences were observed between the hypothyroid and thyroxine replacement states. The peripheral conversion of T4 to T3 is increased during hypothyroidism (1;11;12). In rats, extrathyroidal T3 production changes from PTU sensitive to PTU insensitive during hypothyroidism, representing an increase in the conversion of T4 to T3 by D2 and a decreased conversion by D1 (34). There is, however, no D2 expression and activity in rat skeletal muscle (6), whereas D2 activity has been reported in human skeletal muscle (14;35). Animal studies have shown significantly increased D2 activity in the cerebral cortex and pituitary during hypothyroidism (36-38). Collectively, our data on D2 mRNA expression and D2 activity in hypothyroid and thyroxine treated patients point out that changes in circulating plasma levels of thyroid hormones do not regulate muscle D2 activity or mRNA expression. Consequently, increased D2 activity in other tissues must be responsible for the increased conversion of T4 to T3 in hypothyroid subjects.

Since it has been shown that skeletal muscle expresses D3 mRNA and activity, we assessed D3 mRNA expression and activity as well. Furthermore, in the rat brain D3 mRNA was found to be decreased during hypothyroidism (1;39). However, there were no differences in D3 mRNA expression or activity during hypothyroidism and thyroxine replacement.

Between 14 and 62 h of fasting, plasma T3 levels decreased, whereas rT3 levels increased as has been shown earlier (15;40). Both the decrease in serum T3 and increase in serum rT3 may be explained by decreased D1 activity (in liver/kidney), decreased D2 activity (in muscle), or increased D3 activity (in the central nervous system) (2).

D2 mRNA levels in skeletal muscle samples were significantly lower after 62 h of fasting compared to 14 h of fasting. In mice, fasting decreased the expression of D2 in the pituitary (36). In other conditions of insulin deprivation, such as streptozotocin-induced diabetes in rats, the increase of D2 activity in BAT after insulin was exaggerated compared to the normal response (19).

A significant increase in D2 expression was found after 5 hours of hyperinsulinemia in healthy subjects after 62 h of fasting, whereas this was not the case after 14 h of fasting. It had been shown in rats D2 activity and expression are upregulated by insulin in BAT (17-19). Injections with insulin resulted in an increased D2 activity in BAT in diabetic and non-diabetic rats (19) and addition of insulin to rat brown adipocytes in vitro leads to an increase in V_{max} of D2 (18). Martinez-deMena *et al.* found that this induction is not a direct effect of insulin, but that insulin improves the adrenergic stimulation of D2 activity (17). A role of insulin in regulating D2 in skeletal muscle is conceivable. Moreover, the lack of a significant increase after 14 h of fasting suggests that minor increments of insulin (i.e. postabsorptive plasma insulin levels) are sufficient to induce D2 mRNA expression.

Little D2 activity was present in skeletal muscle samples after 14 and 62 h of fasting and no difference was observed between the two conditions, in contrast with the observed changes in D2 mRNA expression. There are several possible explanations for this. The particular level of D2 mRNA expression in skeletal muscle may not result in significant D2 activity. Therefore, D2 activity in other tissues may be responsible for the decrease in T3 levels. In rats, D1 activity in the thyroid and liver, and D2 activity in the thyroid were decreased after fasting (37;38), whereas D2 activity in the hypothalamus was increased (39). In other conditions where T3 levels decrease significantly, such as acute critical illness, no D2 activity could be measured in liver and skeletal muscle biopsies (30). However, Mebis *et al.* found low but significant skeletal muscle D2 activity during prolonged critical illness, indicating an adaptation to the low T3 levels (11). This may suggest that our volunteers had not been fasting long enough.

It is not likely that the local anaesthetics used for the sample collection could have influenced D2 activity, since we found no effect on D2 activity in D2-COS1 cells with increasing lidocaine concentrations. However, we cannot exclude a local effect of lidocaine resulting

in downregulation of D2 activity. On the other hand, Mebis *et al*. reported no differences in D2 expression and D2 activity in muscle samples taken under local anaesthetics or during laparotomy (11).

Recent findings show that D3 mRNA and activity (catalyzing T4 to rT3 deiodination and T3 degradation) may be increased in muscle and liver of patients hospitalized in the intensive care unit (40). However, we found no change in D3 activity in skeletal muscle during fasting or hyperinsulinemia.

In summary, no differences were observed in the expression of skeletal muscle D2 mRNA between hypothyroidism and thyroxine treatment, although a robust decrease was observed after 62 h of fasting. Moreover, insulin restored D2 mRNA expression after 62 h of fasting. Little D2 activity was measured in skeletal muscle samples and no differences were observed between hypothyroidism and thyroxine treatment or after 14 and 62 h of fasting. Our results therefore imply that skeletal muscle D2 mRNA expression is modulated by fasting and insulin, but not by hypothyroidism or thyroxine treatment. The lack of effect of changes in D2 mRNA on already low D2 activity questions the importance of a role for D2 activity in human skeletal muscle.

Acknowledgments

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