Skeletal muscle deiodinase type 2 regulation during illness in mice

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Abstract

We have previously shown that skeletal muscle deiodinase type 2 (D2) mRNA (listed as Dio2 in MGI Database) is upregulated in an animal model of acute illness. However, human studies on the expression of muscle D2 during illness report conflicting data. Therefore, we evaluated the expression of skeletal muscle D2 during illness in mice. We evaluated the association of D2 expression with serum thyroid hormones, (de-)ubiquitinating enzymes and D2-regulating factors in two mouse models of illness that differ in timing and severity of illness: 1) turpentine-induced inflammation, and 2) Streptococcus pneumoniae infection. During turpentine-induced inflammation, D2 mRNA and activity increased compared to pair-fed controls, most prominently at day 1 and 2, whereas after S. pneumoniae infection D2 mRNA decreased. We evaluated the association of D2 expression with serum thyroid hormones, (de-)ubiquitinating enzymes ubiquitin-specific peptidase 33 and WD repeat and SOCS box-containing 1 (Wsb1), cytokine expression and activation of inflammatory pathways and cAMP pathway.

Introduction

During illness, the central and peripheral thyroid hormone metabolism changes profoundly. This is known as non-thyroidal illness syndrome (NTIS). NTIS is characterized by decreased serum tri-iodothyronine (T\textsubscript{3}) levels, while TSH remains unchanged or even decreases. Furthermore, the expression of deiodinating enzymes changes in various tissues (Wiersinga 2005). Deiodinase type 2 (D2; listed as Dio2 in MGI Database) is one of the three known deiodinases. It converts the prohormone thyroxine (T\textsubscript{4}) into the active hormone T\textsubscript{3} by outer-ring deiodination. D2 is expressed in brain, pituitary, skeletal muscle, brown adipose tissue, and placenta and is present as an active dimer in the endoplasmic reticulum (Kohrle 2000, Bianco & Kim 2006).

Recently it has been shown that skeletal muscle D2 is involved in the peripheral production of T\textsubscript{3} under normal circumstances (Maia et al. 2005), which makes D2 a possible factor contributing to the low serum T\textsubscript{3} levels during illness. A study of Rodriguez-Perez et al. (2008) indeed reports decreased muscle D2 mRNA and activity in septic patients. In contrast, D2 mRNA and activity were upregulated in muscles of intensive care unit (ICU) -patients compared to healthy controls (Mebis et al. 2007), in line with our previous finding that muscle D2 mRNA is increased after LPS administration in mice (Kwakkel et al. 2008).

D2 expression is known to be influenced by thyroid hormone levels. T\textsubscript{3} downregulates D2 mRNA expression (Burmeister et al. 1997), and T\textsubscript{4} and reverse T\textsubscript{3} (rT\textsubscript{3}) (the substrates of D2) subsequently increase D2 ubiquitination and proteasomal degradation, resulting in decreased D2 activity. The ubiquitin ligase adaptor WD repeat and SOCS box-containing 1 (Wsb1) is involved in the ubiquitination process of D2, whereas ubiquitin-specific peptidase 33 (Usp33) is a de-ubiquitinating enzyme (Sagar et al. 2007).

cAMP activation stimulates D2 expression on mRNA and activity level. D2 mRNA is upregulated via the CREB responsive element present in the D2 promoter and cAMP inhibits Wsb1 mediated ubiquitination of D2 (Bartha et al. 2000, Dentice et al. 2007).
Proinflammatory cytokines are also capable of affecting D2; Hosoi et al. (1999) showed that tumor necrosis factor α (TNF-α) reduced the forskolin-induced increase of D2 mRNA and activity in skeletal muscle cells. Proinflammatory cytokines exert their actions via specific signal transduction pathways, such as nuclear factor (NF)-κB, extracellular-signal related kinase (ERK) 1/2 and activator protein-1 (AP-1). NFκB and AP-1 sites have been characterized in the D2 promoter (Gereben & Salvatore 2005, Zeold et al. 2006), suggesting that activation of these pathways results in changes in D2 expression.

The aim of the present study was to evaluate the association between D2 expression and D2 regulating factors during illness. To this end, we used two animal models that differ in acute phase response, timing, and severity of illness. 1) Turpentine-induced abscess in the hindlimb, a model of local chronic inflammation. 2) Streptococcus pneumoniae infection, a lethal model that results in severe pneumonia and sepsis. Muscle D2 expression and D2 regulatory factors were evaluated in both models.

Materials and Methods

Animal experiments

Female C57Bl6 mice (Harlan Spraque–Dawley, Horst, The Netherlands) were used at 6–12 weeks of age. The mice were kept in 12 h light:12 h darkness cycles in a temperature-controlled room. A week before the experiment the animals were housed in groups according to the experimental setup.

Turpentine injection Local chronic inflammation was induced by s.c. injection of 100 μl steam-distilled turpentine in each hindlimb. Control mice received 100 μl saline in each hindlimb and were pair-fed, because the decreased food-intake 1 and 2 days after turpentine injection affects thyroid hormone metabolism. The pair-fed control mice (5 mice per cage) received 5 g/cage at day 1, 5 g/cage at day 2, 8 g/cage at day 3, 12 g/cage at day 4, and 16 g/cage at day 5. Normal food intake is ~20 g/cage. The mean decrease in weight was 9.3% (turpentine) and 9.4% (pair-fed controls) at day 1; 12.4% (turpentine) and 12.7% (pair-fed controls) at day 2; 2.3% (turpentine) and 8.6% (pair-fed controls) at day 5. At days 0, 1, 2, and 5 four to five mice per group were anesthetized with isoflurane. Blood was taken by cardiac puncture and the mice were subsequently killed by cervical dislocation. Serum was stored at −20 °C until analysis. Turpentine injection resulted in a sterile abscess, infiltrating cells at the site of injection and a decrease in serum thyroid hormone levels as previously described (Boelen et al. 2006a). Because the abscess and infiltrating cells in the hind-limb muscle tissue might influence the results, the forelimb–muscle tissue was used for D2 analysis in this study. Muscle tissue was obtained and immediately stored in liquid nitrogen.

S. pneumoniae infection Acute pneumonia was induced as previously described (Boelen et al. 2008). Briefly, S. pneumoniae serotype 3 (American Type Culture Collection, Manassas, VA, USA) was grown in Todd Hewitt broth (Difco, Detroit, MI, USA) at 37 °C, harvested at mid-logarithmic phase, and washed twice in sterile saline. Bacteria were then resuspended in sterile saline at a concentration of 5×10^8 colony-forming units (CFU)/50 μl. Mice (n=6) were lightly anesthetized by inhalation of isoflurane, and 50 μl containing 5×10^8 CFU was inoculated intranasally (i.n.). Control mice received 50 μl sterile saline i.n. The amount of S. pneumoniae bacteria inoculated was determined by plating serial dilutions of the inoculum onto sheep-blood agar plates and incubated at 37 °C and 5% CO₂. CFUs were counted after 16 h. Serum was stored at −20 °C until analysis. Hind-limb muscle tissue was obtained after 48 h and immediately stored in liquid nitrogen. Both the studies were approved by the local animal welfare committee.

Thyroid hormone levels

Serum T₃ and T₄ were measured with in-house RIAs (Wiersinga & Chopra 1982). To prevent inter-assay variation (T₃: 6-2% and T₄: 7-3%), all samples of one experiment were measured within the same assay (intra-assay variability T₃: 3-6% and T₄: 6-6%).

RNA isolation and RT-PCR

Muscle mRNA was isolated on the Magna Pure (Roche Molecular Biochemicals) using the Magna Pure LC mRNA tissue kit and ~25 mg of tissue. The protocol and buffers supplied with the kit were followed. cDNA synthesis was performed using the first strand cDNA synthesis kit for RT-PCR with oligo d(T) primers (Roche Molecular Biochemicals). Real-Time PCR was performed using the Lightcyler (Roche Molecular Biochemicals). Lightcyler FastStart DNA Master Plus SYBR Green I kit (Roche Molecular Biochemicals) was used, adding 50 ng primers each (Biolegio, Manassas, VA, USA) was grown in Todd Hewitt broth (TBD Hewitt broth (Roche Molecular Biochemicals). Lightcyler FastStart DNA Master Plus SYBR Green I kit (Roche Molecular Biochemicals) was used, adding 50 ng primers each (Biolegio, Nijmegen, The Netherlands). Primer pairs for hypoxanthine phosphoribosyl transferase (Hprt1), interleukin-1 β (IL1β), TNF-α, and D2 and D3 were previously described (Bouaboula et al. 1992, Sweet et al. 2001, Boelen et al. 2004, Kwakkel et al. 2008). We designed primer pairs for Wsh1 and Usp33 (Wsh1-forward: 5'-GCC AGC CTT GCT GAT GAT A-3', Wsh1-reverse: 5'-CCC AGC AGC TAA AAC ACT GC-3', Usp33-forward: 5'-CTT TTC GAG GTT ATT CTC AGC AG-3', Usp33-reverse: 5'-GCC TCT TCC TTT CTT ACC AT-3'). Primers were intron-spanning or genomic DNA contamination was tested using a cDNA synthesis reaction without the addition of reverse transcriptase. PCR programs were as follows: denaturation 10 min 95 °C, 40–45 cycles of 0–10 s 95 °C, 10 s annealing temperature, 15–20 s 72 °C. Annealing temperatures were: 54 °C for Hprt1, 55 °C for D2,
62 °C for Tnf, D3, Whb1, and Usp33, and 60 °C for Il1b. For quantification a standard curve was generated of a sequence-specific PCR-product ranging from 0.01 to 100 fg/μl (measurements taken during the exponential phase of the amplification). Samples were corrected for their mRNA content using Hpirt as a housekeeping gene. Samples were individually checked for their PCR–efficiency (Ramakers et al. 2003). The median of the efficiency was calculated for each assay, samples that differed more than 0.05 of the efficiency median value were not taken into account. Aberrant PCR–efficiencies occurred randomly and therefore did not bias the results.

**Deiodinase activity**

Muscle D2 activity was measured as previously described (Mebis et al. 2007). Samples were homogenized on ice in 10 volumes of PED50 buffer (0·1 M sodium phosphate, 2 mM EDTA, and 50 mM dithiothreitol pH 7·2) using a Polytron (Kinematica, Luzern, Switzerland). Homogenates were used immediately. Protein concentration was measured with the Bio-Rad protein assay using BSA as the standard following phosphate, 2 mM EDTA (PE)/0·5% BSA. Reactions were added to 4·6 μl PED50 buffer (0·1 M sodium phosphate, 2 mM EDTA (PE)/0·5% BSA. Reactions were stopped by adding 0·15 ml ice-cold ethanol. After centrifugation, 0·125 ml of the supernatant was added to 0·125 ml 0·02 M ammonium acetate (pH 4), and 0·1 ml of the mixture was applied to 4·6 × 250 mm Symmetry C18 column connected to a Waters HPLC system (Model 600E pump, Model 717 WISP autosampler, Waters, Etten-Leur, The Netherlands). Mobile phase A: 0·02 M ammonium acetate (pH 4·0), mobile phase B: acetonitril. The column was eluted with a linear gradient (28–42% B in 15 min) at a flow of 1·2 ml/min. The activity of T4 and T3 in the eluate was measured online using a Radiomatic Flow–one/Beta scintillation detector (Packard, Meriden, CT, USA). Incubation with 500 nM T4 saturates D2, therefore D2 activity measured with the incubation with 1 nM T4 minus the incubation with 500 nM T4 represents true D2 activity. D2 activity was expressed as fmol generated 3,3'-diiodothyronine (T2) per minute per mg tissue.

**Western blotting**

Homogenates prepared for deiodinase measurement were immediately 1:1 mixed with freshly prepared protein dilution buffer (250 mM sucrose, 10% glycerol, 2 mM phenylmethylsulphonyl fluoride, 4 mM Na3VO4, 40 mM NaF, 2X Complete protease inhibitor cocktail (Roche Molecular Biochemicals). Protein content was measured and 25 μg was loaded on a 10% SDS-PAGE gel. Gels were blotted onto Immobilon-P transfer membrane (Millipore, Bedford, MA, USA). Blots were blocked with 3% casein in TBS/T, for 1 h at room temperature (RT). Primary antibodies were phospho–NFκB p65 (Ser536) (#3033), phospho–p44/p42 MAP kinase (Thr202/Tyr204) (#9101), phospho–c–jun (Ser63) (#9261), and phospho–CREB (Ser133) (#9191), goat-anti-rabbit–HRP (#7074) (Cell Signaling Technology, Danvers, MA, USA). Primary antibodies were incubated for 1 h at RT followed by an overnight incubation at 4 °C. Blots were washed three times 5 min with TBS/T. Following 1 h incubation at RT with secondary antibody goat-anti-rabbit–HRP, blots were washed again and detected with Lumi-Light chemiluminescent substrate (Roche Molecular Biochemicals). The emitted light was visualized and quantified on the Lumi-Imager (Roche Molecular Biochemicals). All antibodies were diluted 1:1000 in blocking buffer, except phospho–CREB, which is diluted 1:2000.

**Statistical analysis**

Normal distribution of the data was tested using the Shapiro–Wilk test. Statistical significance between turpentine and control treatment were evaluated using two-way ANOVA with two grouping factors (time and treatment). When not normally distributed, data were ranked before performing ANOVA. P values in the figures represent the significant effect of the treatment. To test pair–wise comparisons ANOVA was followed by Students t-test when data was normally distributed or Mann–Whitney U tests when not normally distributed. Symbols in the figures represent the pair–wise P values. P values <0·05 were considered statistically significant. Spearman’s rank correlation tests were performed to test correlations. All tests were performed using SPSS (Chicago, IL, USA).

**Results**

**Muscle D2 expression**

Turpentine injection resulted in increased expression of muscle D2 mRNA and activity (P<0·01), compared to saline treated pair–fed controls, most prominent at day 1 and 2 after injection (Fig. 1A). In saline–treated pair–fed controls, D2 mRNA and activity decreased probably due to decreased food–intake. D2 activity in control groups was in some cases below detection limit. In contrast, muscle D2 mRNA decreased in muscle–tissue 48 h after S. pneumoniae infection,
compared to saline treated controls ($P<0.01$; Fig. 1B). Muscle $D_2$ activity was around the detection limit (2 above and 4 below the detection limit in each group) in muscle tissue of $S. pneumoniae$ infected and control mice.

**Serum thyroid hormone levels**

Serum $T_4$ and $rT_3$ levels decreased ($P<0.01$) 1, 2 and 5 days after turpentine injection, whereas serum $T_3$ did not change compared to pair-fed saline-treated controls (Fig. 2A). Muscle $D_2$ mRNA expression did not correlate to serum $T_3$. Serum $rT_3$ was associated with muscle $D_2$ activity ($\tau = -0.504$, $P<0.01$), while serum $T_4$ was not.

After $S. pneumoniae$ infection serum $T_4$ decreased ($P<0.01$). Serum $T_3$ and $rT_3$ did not change (Fig. 2B). Serum $T_3$ was not related to muscle $D_2$ mRNA expression.

**Muscle $Wsb1$ and $Usp33$ mRNA expression**

Ubiquitination promoting enzyme $Wsb1$ and de-ubiquitination enzyme $Usp33$ mRNA expression were evaluated. Muscle $Wsb1$ mRNA increased significantly 5 days after turpentine injection ($P<0.05$), whereas $Usp33$ mRNA did not change significantly (Fig. 3A). $Wsb1$ and $Usp33$ mRNA levels were not related to muscle $D_2$ activity during turpentine induced inflammation. After $S. pneumoniae$ infection muscle $Wsb1$ mRNA did not change, whereas $Usp33$ mRNA decreased compared to saline-treated controls (Fig. 3B).

**Muscle cytokine expression**

Muscle $Tnf$ mRNA increased day 5 after turpentine injection compared to saline-treated, pair-fed controls, whereas muscle $Il1b$ did not (Fig. 4A). Muscle $Il1b$ or $Tnf$
cytokine expression is not related to D2 mRNA expression. After *S. pneumoniae* infection muscle *Il1b* mRNA increased (*P* < 0.01; Fig. 4B) and was negatively correlated to D2 mRNA expression (*r* = −0.691, *P* < 0.05). Muscle *Tnf* mRNA expression did not change significantly after *S. pneumoniae* infection and was not correlated to muscle D2 mRNA expression.

**Activation of signalling pathways**

Phosphorylated NFκB, c-jun, ERK1/2, and CREB were evaluated in muscle tissue by western blotting. After turpentine injection CREB was highly phosphorylated at day 1 and 2 (Fig. 5A) while NFκB (p65) and ERK1/2 did not differ compared to saline-treated pair-fed controls. The phosphorylation of CREB coincides with the observed increase in D2 expression after turpentine injection. No difference was observed in phosphorylation of CREB, NFκB or ERK1/2 after *S. pneumoniae* infection compared to saline-treated controls (Fig. 5B). Phosphorylated c-jun was not detectable in muscle tissue (data not shown).

**Muscle D3 expression**

Turpentine injection resulted in increased expression of muscle D3 mRNA and activity (*P* < 0.05), compared to saline treated pair-fed controls, most prominent at day 1 and 2 after injection (Fig. 6A). D3 activity in control groups was in most cases below detection limit. After *S. pneumoniae* infection muscle D3 mRNA did not change. Muscle D3 activity was below the detection limit in the saline controls, whereas three samples were positive after *S. pneumoniae* infection. However, the observed difference was not significantly different (*P* = 0.056).

**Discussion**

We studied the association between muscle D2 expression and D2 regulating factors in two different animal models of illness: turpentine-induced abscess in the hindlimb and *S. pneumoniae* infection. The animal models used differ in timing of the acute phase response and severity of illness. After turpentine injection in the hindlimb, an abscess is formed in the first 2 days, causing serious discomfort, fever, and decreased food-intake. Serum IL-6 and IL-1 are high during this early phase, whereas serum TNFα does not play a role. Five days after turpentine serum IL-1 and IL-6 decrease, temperature and food-intake return to normal and mice recover, although liver *Il1b* mRNA increases which is characteristic for the development of a systemic acute phase response (Boelen et al. 1996, 2005, Leon 2002, Elhija et al. 2006).

In contrast to turpentine induced inflammation, *S. pneumoniae* infection is lethal (Knapp et al. 2004). *S. pneumoniae* infection results in severe bronchopneumonia within 24 and 48 h after inoculation mice become septic (Boelen et al. 2008). Serum IL-1 and IL-6 increase rapidly after infection, whereas TNFα is produced at a later stage...
inflammation is in line with increased compared to pair-fed controls during turpentine-induced (black bars) or saline-treated (white bars) controls. Mean values mRNA and activity expression 48 h after S. pneumoniae (A) Muscle

Figure 5 Representative western blot of phosphorylated CREB expression of (A) 1, 2, and 5 days after turpentine or saline injection, (B) 48 h after S. pneumoniae infection or saline-treated controls.

(Bergeron et al. 1998). The difference in cytokine expression, acute phase response, and severity of illness between these two animal models suggests differential regulation of D2 expression. During turpentine-induced inflammation, D2 mRNA and activity increased compared to pair-fed controls, most prominently at day 1 and 2, whereas after S. pneumoniae infection D2 mRNA decreased. The D2 increase compared to pair-fed controls during turpentine-induced inflammation is in line with increased D2 expression recently observed in skeletal muscle of ICU patients and in LPS-treated mice (Mebis et al. 2007, Kwakkel et al. 2008), while decreased muscle D2 mRNA expression after S. pneumoniae infection corresponds with previously reported D2 decrease in muscle tissue of septic patients (Rodriguez-Perez et al. 2008). Although it might be possible that the observed differences in D2 expression during illness in our animal-models result from the different muscle origins, this seems unlikely because the D2 increase observed previously after LPS administration (Kwakkel et al. 2008) is similar in both muscle types (Kwakkel, unpublished observation).

D2 mRNA and activity are regulated by thyroid hormones; D2 mRNA and activity increases during hypothyroidism, while hyperthyroidism results in decreased D2 mRNA expression and activity, due to transcriptional (T3) and translational (T4 and rT3) regulation. However, thyroid hormone metabolism during illness differs from normal regulation (Wiersinga 2005). In both our animal models, no correlation between serum T3 and T4 levels and muscle D2 expression was observed. This is reminiscent to the T3-independent D2 activity increase in the mediobasal hypothalamus induced by LPS administration (Fekete et al. 2005). Factors involved in (de-)ubiquitination of D2 in our study did not correlate with D2 activity, although the observed increase in Wsb1 mRNA 5 days after turpentine coincides with a normalization of D2 activity. Inflammatory cytokines have profound effects on peripheral and central thyroid hormone metabolism (Boelen et al. 2006b). We studied muscle Il1b and Tnf mRNA expression and the activation of three inflammatory pathways in muscle tissue.

After turpentine injection, Il1b and Tnf mRNA expression tended to increase after 5 days, which is in accordance with the declining muscle D2 expression 5 days after turpentine injection as TNFα decreases muscle D2 mRNA expression in vitro (Hosoi et al. 1999). Furthermore, we observed increased Il1b mRNA expression in muscle tissue of S. pneumoniae infected mice, which was inversely correlated to D2 mRNA expression. However, the observed alterations in D2 expression appeared not to be mediated via activation of the inflammatory pathways NFκB, ERK1/2, or AP-1.

The decreased D2 expression after S. pneumoniae infection might also be due to diminished food-intake, as D2 expression decreases after fasting in healthy humans (Heemstra et al. 2009). In addition, D2 mRNA expression also decreased in the pair-fed controls of the turpentine experiment.

cAMP activation is a potent stimulator of D2, both pre- and post-transcriptionally (Hosoi et al. 1999, Bartha et al. 2000, Dentice et al. 2007). We evaluated phosphorylated CREB expression in muscle tissue as a marker for cAMP activation. CREB was highly phosphorylated in muscle tissue within 48 h after turpentine injection, which coincides with the marked increase of D2 expression compared with pair-fed controls, suggesting a dominant role of the cAMP pathway in the observed D2 increase.

Increased D2 activity theoretically results in increased T3 production, which is not reflected in increased serum

Figure 6 (A) Muscle D3 mRNA and activity expression 1, 2, and 5 days after turpentine (●) or saline (□) injection, (B) Muscle D3 mRNA and activity expression 48 h after S. pneumoniae infection (black bars) or saline-treated (white bars) controls. Mean values ± S.E.M. (n=4/6) are shown. P values indicate differences between groups by nonparametric ANOVA. Symbols indicate differences evaluated by Mann–Whitney U tests; *P≤0.05, **P≤0.01.

Journal of Endocrinology (2009) 203, 263–270
T₃ concentrations, suggesting that D₂ contribution to systemic T₃ levels is quantitatively limited. To evaluate the physiological implications of the observed D₂ alterations during illness, muscle D₃ expression was evaluated. After S. pneumoniae infection, D₃ mRNA did not change, while D₃ activity tended to increase, however the activity measured was very low. In contrast, muscle D₃ mRNA and activity increased after turpentine injection compared to pair-fed controls. The increase of muscle D₃ during illness has previously been reported (Rodriguez-Perez et al. 2008).

During turpentine induced inflammation, the increase in muscle D₂ and D₃ compared with pair-fed controls coincides with the increased body temperature and decreased food-intake observed during this phase of inflammation (Leon 2002). Cooper & Rothwell (1991) showed increased β-adrenergic-dependent oxygen-consumption in the first 36 h after turpentine injection. Sympathetic stimulation of cultured human skeletal muscle cells results in activation of the cAMP pathway and in upregulation of D₂ in muscle cells (Hosoi et al. 1999), subsequently followed by increased mitochondrial activity and thus increased oxygen-consumption (Watanabe et al. 2006).

Because both the measured D₂ and D₃ activities are very low, a local effect in the skeletal muscle seems more likely than an effect on serum thyroid hormone levels. The simultaneous upregulation of D₂ and D₃ theoretically only leads to the formation of 3,3′,5′-T₂, which might be able to activate the mitochondrial enzyme cytochrome c oxidase (Goglia et al. 1994, Lanni et al. 1994) thereby also increasing oxygen consumption. A shortage of 3,3′-T₂ might result in mitochondrial dysfunction, which is thought to play a role in the pathogenesis of sepsis (Fredriksson et al. 2006, Zang et al. 2007). Adding exogenous cytochrome c oxidase improves cardiomyocyte mitochondrial function in an animal model of sepsis (Levy & Deutschman 2007). These speculations need further investigation in order to consider the role of D₂ and D₃ in muscle tissue as a regulator of muscle mitochondrial activity during illness.

In conclusion, muscle D₂ expression is differentially regulated during illness, probably related to differences in the inflammatory response and type of pathology. D₂ mRNA and activity increase in skeletal muscle during the acute phase of chronic inflammation compared to pair-fed controls probably due to activation of the cAMP pathway. In contrast, muscle D₂ mRNA decreases 48 h after a severe bacterial infection, which is associated with local IL₁b mRNA expression and might also be due to diminished food-intake. The observed alterations in muscle D₂ and D₃ might result in changes in local 3,3′-T₂ concentrations and thereby affect mitochondrial activity.

Funding
This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements
We would like to thank J Daalhuizen (Department of Experimental Internal Medicine, Academic Medical Center, Amsterdam) for expert biotechnical assistance; Ing E Johannesma-Brian, and Drs E Endert (Department of Clinical Chemistry, Laboratory of Endocrinology, Academic Medical Center, Amsterdam) for measuring serum thyroid hormones.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Received in final form 3 July 2009
Accepted 5 August 2009
Made available online as an Accepted Preprint 5 August 2009