Responses of skeletal muscle lipid metabolism in rat gastrocnemius to hypothyroidism and iodothyronine administration: a putative role for FAT/CD36

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Indeed, T3 increases 1) the mobilization of the triglycerides stored in adipose tissue, 2) the serum concentrations of free fatty acids (FFA), 3) lipoprotein lipase activity, and 4) the utilization of lipid substrates in metabolically active tissues (22, 39). Under some conditions, these effects are accompanied by a reduction in body weight (2, 24).

As a result, in the past T3 has been proposed and tentatively used as an antiobesity and lipid-lowering agent. However, its beneficial weight reduction effects were associated with the simultaneous induction of a thyrotoxic state and with undesirable side effects at the cardiovascular level, and so its use for such purposes has been stopped (2, 40). Now, in the hope of countering dyslipidemia and obesity, attention is being focused on the development of compounds that have the beneficial effects of excess thyroid hormone, but with minimal deleterious effects (2, 4). In particular, new perspectives in this field have been opened by the introduction of potent thyrmonic with thyroid hormone receptor-β isoform (TR-β) subtype-selective activities (40), and a few of them have recently entered clinical trials (25, 40). Importantly, TR-β receptors are scarcely expressed at all in cardiomyocytes and are involved mainly in the thyroid hormone-mediated regulation of lipid metabolism (5, 30, 34).

There is accumulating evidence to show that 3,5-diiodothyronine (T2), a thyroid hormone derivative, has biological effects both in vivo and in vitro (13). T2 is able to affect both energy and lipid metabolism without inducing the side effects associated with T3 (7, 30). Indeed, T2 administration to rats increases their resting metabolic rate (31) and prevents diet-induced obesity as well as liver steatosis, hypertriglyceridemia, hypercholesterolemia (7, 26), and insulin resistance (7, 32). The actions of T2 are mediated by mechanisms not involving TRs, and therefore, they are referred to as “non-nuclear-mediated” (7, 14).

Studies of the control exerted by T3 over fatty acid metabolism have focused principally on liver, adipose tissue, and heart (16, 42). Thorough investigations of the regulatory mechanisms involved in muscle fatty acid uptake and utilization in various thyroid states and of the effects of T3 and/or T2 administration would be expected to reveal in detail how these hormonal states and changes modulate the levels of fatty acid uptake and utilization in muscle tissue. Fatty acid translocase (FAT/CD36) seems to be the protein involved not only predominantly in the import of FFA into the tissue but also intracellularly (for review, see Refs. 12, 18, and 39). Reportedly, the functional presence of FAT/CD36 is not restricted to the plasma membrane, since it is also found within mitochondri-
dria, where it has a putative role in the modulation of fatty acid oxidation (17, 39). However, conflicting results are present in the literature. Indeed, regarding both the localization and the role played by FAT/CD36 at the mitochondrial level, the existing disagreements are based principally on 1) the absence of clear evidence of coimmunolocalization of FAT/CD36 with mitochondria (20–22) and 2) contrasting data concerning the reduced ability of skeletal muscle mitochondria isolated from FAT/CD36-null mice to oxidize lipid substrates (i.e., palmitate, palmitoyl-CoA, palmitoyl carnitine) (17, 22, 39). However, recent reports may resolve some of the above discrepancies insofar as functional data suggest that FAT/CD36 is located on the outer mitochondrial membrane upstream of long-chain acyl-CoA synthetase (39), and thus it may facilitate palmitate-supported and not palmitoyl-CoA- or palmitoyl carnitine-supported respiration. Moreover, evidence that, in skeletal muscle mitochondria from FAT/CD36-null mice, fatty acid oxidation was reduced but not abolished suggested that alteration of FAT/CD36 content at the mitochondrial level provides additional regulation that may be important for augmenting FFA oxidation rates (17, 39). This may be particularly important when the energy demands from FFA oxidation are substantially increased (17).

In fact, in cases of increased energy demand, the capacity of skeletal muscle to rapidly modulate the uptake of FFAs and their successive oxidation can be increased by rapid translocations of FAT/CD36 from its endosomal compartment to the plasma membrane (for FFA uptake) and mitochondria (for FFA oxidation) (3, 18).

In the present study, we tested our hypothesis that related changes in lipid handling and FAT/CD36 expression and subcellular distribution would occur 1) in hypothyroidism and 2) upon T3 or T2 treatment of hypothyroidism. To this end, we carried out a broad analysis of the metabolic parameters associated with skeletal muscle lipid handling in hypothyroidism with or without iodothyronine (T3 or T2) administration.

We also directed our attention toward changes in FAT/CD36 protein expression/localization. For an animal model we used rats in which hypothyroidism had been induced by combined treatment with propylthiouracil and iopanoic acid. This treatment induces a severe hypothyroidism and at the same time inhibits all of the three known types of deiodinase enzyme (31). This allows us to attribute the observed effects to the iodothyronines actually injected rather than to any of their deiodinated products.

In addition, in view of the occurrence of rapid translocations of FAT/CD36 from its endosomal compartment to the plasma membrane and mitochondria, we thought it would be interesting to investigate whether T3 and/or T2 might influence FAT/CD36 levels in the short term (viz., within 1 h after their injection into hypothyroid animals).

To gain further insight into the subcellular distribution of FAT/CD36, we performed immunohistochemistry on gastrocnemius skeletal muscle (GSkM), and in addition we used an in vitro model of skeletal muscle fibers (C2C12 cell line) to examine the intracellular and sarcosomal localizations of FAT/CD36, and also the T3- and T2-induced changes in its distribution, by the application of immunocytochemistry and confocal microscopy.

METHODOLOGICAL

Animals. Male Wistar rats (275–300 g) were obtained from Harlan Laboratories (Italy). They were kept one per cage in a temperature-controlled room at 28°C under a 12:12-h light-dark cycle. A commercial mash and water were available ad libitum. Six groups of rats were used throughout, with each one comprising six animals. Group N (N) consisted of euthyroid rats that received vehicle. Group H (H) consisted of hypothyroid rats [hypothyroidism being induced by the intraperitoneal (ip) administration of propylthiouracil (1 mg/100 g body wt) for 4 wk together with a weekly ip injection of iopanoic acid (6 mg/100 g body wt) (31)]. H + T3 1 wk and H + T2 1 wk contained hypothyroid rats (treated like those in group H) that in the last week of treatment received a daily injection of either T3 (15 μg/100 g body wt) or T2 (25 μg/100 g body wt).

H + T3 for 1 h and H + T2 for 1 h contained hypothyroid rats (treated like those in group H) that received T3 (25 μg/100 g body wt) or T2 (25 μg/100 g body wt) 1 h before being euthanized (see below).

At the end of the treatments, rats were anesthetized by an ip injection of chloral hydrate (40 mg/100 body wt) and euthanized by decapitation. This was done at 6 h after the end of the nocturnal periods. Blood was collected, and then gastrocnemius muscles were excised, weighed, and immediately processed for mitochondrial isolation or frozen in liquid nitrogen.

Authorization to perform these experiments on rats was given by the Italian Ministero della Sanità (decreet no. 176/2005-A).

Metabolic parameters. Oxygen consumption (V O2), carbon dioxide production (V CO2), and respiratory quotient (V CO2/V O2) measurements were made using a four-chamber, indirect, open-circuit calorimeter Oxymax system (Pan Lab, Cornell, Barcelona, Spain) with one rat per chamber. After a 1-h period of adaptation to the metabolic chamber, V O2 and V CO2 were measured in individual rats at 15-min intervals for 4 h.

Isolation of mitochondria for examination of palmitate and succinate oxidations. Skeletal muscle mitochondria were isolated as described previously, with slight modification (28). Briefly, tissue fragments were immersed in ice-cold buffer consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, and 5 mM EGTA, pH 7.4, and then homogenized in a Potter-Elvehjem homogenizer. Nuclei and cell debris were removed by centrifugation at 500 g for 10 min, with the resulting supernatant being centrifuged at 3,000 g. The mitochondrial pellet was washed twice and resuspended in a minimal volume of isolation medium and kept on ice. The protein concentration was determined by the method of Hartree (15), using bovine serum albumin (BSA) as standard.

Measurements of fatty acid and succinate oxidation rates. Mitochondrial fatty acid and succinate oxidation rates were assessed polarographically using a Clark-type electrode at 30°C in a final volume of 0.5 ml of 80 mM KCl, 50 mM HEPEs (pH 7.0), 1 mM EGTA, 5 mM K2HPO4, 1% BSA (wt/vol), and ADP (120 μg/ml). To detect succinate oxidation, mitochondria (0.25 mg) were incubated for 3 min in the respiratory medium, and the reaction was started by addition of succinate (5 mM). To detect palmitate oxidation, the respiratory medium was supplemented with 2.5 mM malate, 1 mM l-carnitine, 2 mM ATP, 0.05 mM CoA, and 1 mM DTT. Mitochondria (0.5 mg) were incubated for 3 min in the respiratory medium, and the reaction was started by addition of palmitate (120 μM).

Gastrocnemius muscle total lysate, plasma membranes, and mitochondrial isolation for Western blotting analysis. For Western blotting analysis, gastrocnemius muscle was homogenized in RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0; all from Sigma-Aldrich, St. Louis, MO) using an Ultra-turrax. The homogenate was left on ice for 30 min, during which it was shaken every 10 min. The lysate was then ultracentrifuged at 86,000 g for 10 min at 4°C. The supernatants of the ultracentrifuged cleared lysates were used for detection of FAT/CD36.
To isolate plasma membranes and mitochondria for Western blotting analysis, gastrocnemius fragments were homogenized in isolation buffer (consisting of 10 mM NaHCO₃, 0.25 M sucrose, 5 mM NaCl, and 100 μM PMSF, pH 7.0). The homogenate was centrifuged at 5,000 x g for 10 min. The supernatant was centrifuged at 10,000 x g for 10 min. The resulting pellet was discarded and the supernatant centrifuged at 190,000 x g for 1 h. The resulting pellet, consisting of the membrane-rich fraction, was resuspended in RIPA buffer.

**Determination of skeletal muscle TG-hydrolase activity.** Dissected-out, freeze-dried GSkMs were homogenized on ice in a buffer containing 0.25 M sucrose, 1 mM EDTA, 1 mM diethiothreitol, 40 mM β-glycerophosphate, 10 mM Na pyrophosphate, 20 μg/ml leupeptin, 20 μg/ml antipain, 6.25 μg/ml pepstatin A, and 0.31 μM okadaic acid.

The homogenate was centrifuged at 18,500 g at 4°C for 45 s; the resulting supernatant was used for TG-hydrolase activity measurements. The substrate, containing triolein and tri-[9,10(n)-3H]-olein (GE Healthcare), was emulsified with phosphatidylcholine-phosphatidyllysinoisotol (3:1) in 3 ml of 0.1 M potassium phosphate buffer (pH 7.0) and 1 ml of 20% fatty acid-free BSA in 0.1 M potassium phosphate buffer (pH 7) by sonication. To detect TG-hydrolase activities, 100 μg of lysate protein was incubated in a shaking water bath for 60 min at 37°C with 100 μl of [3H]triolein substrate (167 nmol, 1.25 × 10⁶ cpm) and an enzyme dilution buffer (20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM diithiothreitol, and 0.02% fatty acid-free BSA) in a total volume of 200 μl. Incubations were each performed in triplicate. The reaction was stopped by addition of 3.25 ml of methanol-chloroform-heptane (10:9:7) and 1 ml of 0.1 M potassium carbonate and 0.1 M boric acid, pH 10.5. The mixture was vortexed for 10 s and then centrifuged at 1,100 x g for 20 min. Finally, 1 ml of the upper phase was removed, and the radioactivity of the released ³H-labeled fatty acids was determined by liquid scintillation counting (Tri-Carb; Packard Instruments).

**Gastrocnemius muscle lipolysis assay.** In vitro release of glycerol from GSKMs under basal conditions was determined as reported by Enoksson et al. (9), with slight modifications. Briefly, three to four GSKM strips deprived of visible fat and weighing ~50 mg each were incubated for 90 min in 2 ml of Krebs-Ringer bicarbonate buffer supplemented with 20 mg/ml BSA (pH 7.4, BSA fraction V; Sigma), 1 mg/ml glucose, and 0.1 mg/ml ascorbic acid in a shaking water bath (37°C) and gassed with 95% O₂-5% CO₂. Strips were incubated in duplicate, and after incubation they were removed from the medium. Then, an aliquot of the medium was used for analysis of glycerol. A commercially available absorbance-based enzyme assay (Free Glyc- erol Reagent; Sigma) for glycerol was converted to fluorescence-based detection by the inclusion of the hydrogen peroxide-sensitive dye Amplex UltraRed, as reported by Clark et al. (6).

**Detection of basal and fatty acid-induced proton conductance.** Mitochondrial inner membrane (MIM) proton conductance was evaluated by measuring the ratio of the flux of protons that cross the inner membrane and are not associated with synthesis of ATP (proton leak) to the MIM potential. The respiratory rate of mitochondria measured in the presence of oligomycin is proportional to proton leak; indeed, in a steady state, proton flux can be determined by multiplying oxygen consumption by the H⁺/O ratio, which is considered to be 6 when succinate is used as substrate.

Because the relationship between proton conductance and membrane potential is not linear, the kinetic response of the proton conductance pathway to its driving force (membrane potential) was measured. To compare proton conductance values among the various mitochondrial preparations obtained from different groups of animals, proton conductance was evaluated at a fixed membrane potential (viz. the highest common membrane potential detected).

To determine respiration rate and mitochondrial membrane potential, electrodes sensitive to oxygen and to the potential-dependent probe TPMP⁺ were used.

With the aim of detecting the kinetic response of the proton conductance pathway to its driving force (membrane potential), mitochondria (0.5 mg/ml protein) were incubated in assay medium containing 80 mM KCl, 50 mM HEPES (pH 7), 1 mM EGTA, 5 mM K₂HPO₄, 5 mM MgCl₂, 1 μg/ml oligomycin, 80 ng/ml nigericin (to collapse the difference in pH across the MIM and to allow the whole proton motive force to be expressed as membrane potential), and 0.5% BSA.

The electrode was calibrated by making sequential additions of TPMP⁺ =2 μM, and then 6 mM succinate was added to start the reaction. Respiration rate and MIM potential were progressively inhibited through successive steady states by additions of malonate ≤2 mM. At the end of each run, 0.2 μM FCCP was added to dissipate the membrane potential and release all TPMP to the medium, allowing correction for any small electrode drift. The TPMP-binding correction for skeletal muscle was taken to be 0.4 μl/mg protein.

To examine the effect of FFA in inducing proton conductance, the incubation medium was supplemented with 30 μM arachidonic acid (AA). The proton conductance detected in the presence of AA represents the sum of the basal and AA-induced ones (“total” proton conductance). The proton conductance detected in the absence of AA represents “basal” proton conductance. The difference between these “total” and “basal” proton conductance values represents the AA-induced proton conductance.

**Western immunoblot analysis.** Mitochondrial membranes and sarclemma from gastrocnemius muscle were resuspended in SDS loading buffer, followed by heating for 10 min at 80°C. Mitochondrial or skeletal muscle plasma membranes containing 60 μg of protein from a single rat were loaded in each lane and electrophoresed on an 8% SDS-PAGE gel. A monoclonal antibody (mouse monoclonal ab17044; AbCam, Cambridge, UK) and anti-rabbit antibody were used as primary and secondary antibodies, respectively, in a chemiluminescence-protein detection method (NEN Life Science Products, Boston, MA). Equal loading was verified by Ponceau S staining or, where stated, by detecting β-actin levels.

To evaluate the purity of the mitochondrial and sarclemma fractions, Na/K ATPase, uncoupling protein 3 (UCP3), and stearoyl-CoA desaturase 1 (SCD1) were detected by immunoblotting with the use of the following antibodies: anti-α1 sodium potassium ATPase antibody (mouse monoclonal Ab6761 from AbCam), anti-human UCP3 antibody (rabbit polyclonal Ab3046 from Chemicon International), and anti-SCD1 (goat polyclonal sc-14720 from Santa Cruz Biotechnology).

**RNA isolation.** Total gastrocnemius muscle or liver RNA was isolated using the Trizol standard protocol (Invitrogen Life Technologies, Milan, Italy). Tissue/Trizol mixtures were homogenized using a polytron, keeping the viscosity of the solution to a minimum to ensure effective inactivation of endogenous RNAase activity.

**Quantitative real-time PCR.** PCR primers were designed using Primer Express version 2.0 (Invitrogen). The expression levels of cyclophilin F were used for normalization. The primers used were cyclophilin F sense 5'-AGGCAGATGTCGTGCCAAAGAC-3', cyclophilin F antisense 5'-GACAACCACT-3', cyclophilin F antisense 5'-GACAACCACT-3', cyclophilin F antisense 5'-GACAACCACT-3', and FAT/CD36 antisense 5'-ATGTCAGCA-CACCATACGA-3'.

A total of 1 μg of total RNA was used to generate cDNA strands in a 20-μl reaction volume using a Cloned AMV First Strand Synthesis Kit (Invitrogen). The equivalent of 25 ng of total RNA was used subsequently in the amplification step, with 50 nM gene-specific primers and 4 ml of SYBR green mix (Applied Biosystems) in a total volume of 8 μl, using standard cycle parameters in an Applied Biosystems Model 7500.
Detection of serum levels of FFA and glycerol. FFA and glycerol levels were determined in serum samples by the use of kits from Wako Chemicals (Richmond, VA) and Sigma, respectively.

Determination of skeletal muscle FFA and triglyceride levels. Lipid was extracted from frozen tissues in chloroform-methanol using the method of Folch et al. (11). The levels of FFA and triglyceride in the skeletal muscle lipid extract were determined using kits from Wako and Sigma, respectively.

Determination of carnitine palmitoyl transferase system activity. Mitochondrial carnitine palmitoyl transferase (CPT) system (CPT1 plus CPT2) activity was measured spectrophotometrically by following (at 412 m) the kinetics of carnitine-dependent CoASH production in the presence of 5,5'-dithio-bis(2-nitrobenzoic acid) using palmitoyl-CoA as substrate, as described by Alexson and Nedergaard (1). An 412 value of 13.6 nM/cm was applied for the calculation of total CPT activity.

Immunohistochemistry. Paraffin-embedded gastrocnemius muscle sections (5 μm) were incubated with anti-FAT/CD36 polyclonal antibody (ab 78054 diluted 1:200; Abcam). The immunoreaction was detected by the avidin-biotin-peroxidase complex (ABC) method using a Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA), as described previously (32). The muscle sections were counterstained with hematoxylin to reveal nuclei and then mounted in Eukitt (Kindler, Freiburg, Germany). Omission of the primary antibody served as the negative control.

Cell culture. C57BL/6 mouse myoblasts were routinely cultured in growth medium consisting of DMEM supplemented with heat-inactivated 10% vol/vol fetal bovine serum, 2 mM glutamine, and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin). Medium of the same composition was used to promote C57BL/6 cell differentiation, when 70–80% confluence was obtained, with the only exception that heat-inactivated fetal bovine serum was reduced to 1% vol/vol. The cells were maintained in a humidified 5% CO2 atmosphere at 37°C. For the immunofluorescence staining study, all of the cells were maintained in a humidified 5% CO2 atmosphere at 37°C. Concerning the respiratory quotient, no significant change was expected. Resting metabolic rate was reduced significantly (−25%) by hypothyroidism. Long-term administration of TT3 values. Resting metabolic rate was reduced significantly (−25%) by hypothyroidism. Long-term administration of either TT3 or TT4 to H rats increased resting metabolic rate significantly to values higher than those observed in N rats. Concerning the respiratory quotient, no significant change was induced by hypothyroidism, on the other hand, long-term administration of either TT3 or TT4 to H rats significantly increased NO3 uptake rate, indicating an increase in the use of lipids as a substrate for oxidation in H + T3 1 wk rats and also in H + T3 1 wk rats. The rats’ body weight gain with time was reduced significantly by hypothyroidism (see Table 1). When we looked at the whole treatment period (4 wk of treatment with propylthiouracil and iopanoic acid with or without either T2 or T2 administration during the last week of treatment), the administration of those iodothyronines did not affect body weight gain.  

Table 1. Effects of thyroid state and of long-term administration of iodothyronines on serum levels of T3 and T4, metabolic parameters (RMR and RQ), body weight gain, food intake, adiposity, and serum levels of glycerol and free fatty acids

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>H</th>
<th>H + T3 1 wk</th>
<th>H + T4 1 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT3 serum level, nM</td>
<td>58 ± 2.0a</td>
<td>7.1 ± 0.9b</td>
<td>6.2 ± 0.6a</td>
<td>6.9 ± 0.5a</td>
</tr>
<tr>
<td>TT4 serum level, nM</td>
<td>0.83 ± 0.01a</td>
<td>0.19 ± 0.05b</td>
<td>1.5 ± 0.17a</td>
<td>0.18 ± 0.07b</td>
</tr>
<tr>
<td>RMR, ml O2/min kg-0.75</td>
<td>10.2 ± 0.8a</td>
<td>7.6 ± 0.8b</td>
<td>14.4 ± 0.6c</td>
<td>12.8 ± 0.2a</td>
</tr>
<tr>
<td>RQ</td>
<td>0.92 ± 0.02a</td>
<td>0.96 ± 0.02a</td>
<td>0.82 ± 0.03b</td>
<td>0.85 ± 0.04a</td>
</tr>
<tr>
<td>Serum glycerol level, mg/dl</td>
<td>0.33 ± 0.07a</td>
<td>0.17 ± 0.01b</td>
<td>0.68 ± 0.01c</td>
<td>0.18 ± 0.04a</td>
</tr>
<tr>
<td>Free fatty acid level, mg/dl</td>
<td>17.9 ± 0.70a</td>
<td>17.9 ± 0.70a</td>
<td>45.5 ± 4.5b</td>
<td>7.0 ± 0.5a</td>
</tr>
<tr>
<td>Body weight gain, g</td>
<td>110 ± 4e</td>
<td>47.3 ± 5.0b</td>
<td>46.11 ± 4.9b</td>
<td>55.1 ± 4.3b</td>
</tr>
<tr>
<td>Body weight gain during last week of treatment, g</td>
<td>19.25 ± 1.00a</td>
<td>0.63 ± 0.68b</td>
<td>−5.8 ± 2.9b</td>
<td>10.2 ± 2.5a</td>
</tr>
<tr>
<td>Food intake, g</td>
<td>501 ± 15a</td>
<td>368 ± 10b</td>
<td>416 ± 15b</td>
<td>413 ± 16b</td>
</tr>
<tr>
<td>Food intake during last week of treatment, g</td>
<td>122 ± 8g</td>
<td>70 ± 4b</td>
<td>80 ± 2b</td>
<td>85 ± 4a</td>
</tr>
<tr>
<td>Visceral adipose tissue/weight × 100</td>
<td>4.5 ± 0.3a</td>
<td>3.9 ± 0.2ab</td>
<td>3.0 ± 0.2c</td>
<td>3.4 ± 0.2bc</td>
</tr>
</tbody>
</table>

Values represent means ± SE from 6–10 different experiments. T3, 3,5,3′-triiodothyronine; T4, thyroxine; RMR, resting metabolism; RQ respiratory quotient; N, group N (consisting of euthyroid rats that received vehicle); H, group H (consisting of hypothyroid rats); TT3 and TT4, total T3 and T4, respectively. Values labeled with different letters are significantly different (P < 0.05) from each other.
iodothyronines to oxidize succinate as substrate in experimental conditions in which the synthesis and export of ATP are at maximal rates (Table 2). The succinate oxidation rate was reduced significantly by hypothyroidism (−25% vs. N). Long-term administration of T3 to H rats increased it significantly, restoring it to the levels observed in N animals. However, T2 was ineffective at 1 h after its acute administration. Long-term administration of T2 to H rats increased the succinate oxidation rate significantly (+30%), and its effect was very rapid since it was already evident at 1 h after a single T2 administration.

Lipolysis in gastrocnemius skeletal muscle and triglyceride hydrolase activities. To assess the ability of one week’s iodothyronine administration to H rats to affect skeletal muscle lipolysis and lipase activities, we evaluated glycerol release by muscle strips and triglyceride hydrolase activity in whole homogenate. As shown in Fig. 1, hypothyroidism did not affect GSKM’s ability to release glycerol. One week’s administration of T3 to H animals increased glycerol release from GSKM significantly (+70% vs. H and N), whereas administration of T2 tended to increase glycerol release by only ~25% (not significant).

Triglyceride hydrolase activities displayed the same trend as that observed for glycerol release; viz. hypothyroidism caused no significant change, and administration of T3 to H rats induced a significant increase (+70% vs. H), whereas administration of T2 led to only a nonsignificant tendency to increase (+12%).

Effect of hypothyroidism and iodothyronine administration on mitochondrial oxidative phosphorylation efficiency. To examine whether an alteration in the efficiency of mitochondrial oxidative phosphorylation might contribute to the effects of iodothyronines on the fatty acid oxidation rate, we measured mitochondrial proton conductance in mitochondria from N, H, H + T3 1 wk, and H + T2 1 wk rats, and we discriminated between the contributions of basal and fatty acid-induced proton conductance (Fig. 2).

An inhibition of proton conductance (−40%) was observed in mitochondria from H rats compared with N ones, with basal proton conductance being reduced by ~20% and fatty acid-induced proton conductance being reduced by 60%.

One week’s administration of T3 to H rats led to an increase in proton conductance (+33%), which did not reach the values observed in N rats. Actually, T3 increased both basal and fatty acid-induced proton conductance (+33% in each case), and although basal proton conductance reached levels not significantly different from the N ones, fatty acid-induced proton conductance remained significantly lower (~45% vs. N).

Administration of T2 to H rats increased mitochondrial proton conductance by 27%, but it did not reach the values observed in N rats. T2 affected only fatty acid-induced proton...
conductance (+67% vs. H), with basal proton conductance being unaffected.

FFA and triglyceride levels in gastrocnemius muscle. We next investigated whether the alterations in GSkM oxidative capacity and ability to hydrolize triglycerides induced by 1) the hypothyroid state and 2) long-term administration of either T3 or T2 to hypothyroid rats might alter FFA and triglyceride levels in GSkM.

GSkM FFA levels were not affected by either hypothyroidism or long-term administration of iodothyronines to H rats (Fig. 3A). In contrast, the triglyceride content of GSkM was significantly higher (by 26%) in H rats than in N ones. Long-term T2 treatment reduced the triglyceride level in GSkM by 40% (vs. H) so that it reached a value lower than that seen in N (Fig. 3A).

Tissue and subcellular FAT/CD36 levels in GSkM. To evaluate whether FAT/CD36 might be affected by the thyroid state of the animal, the GSkM content was assessed at both tissue and subcellular levels. Hypothyroidism greatly increased the GSkM FAT/CD36 mRNA level (+200%; Fig. 4A), and this enhancement was accompanied by increases in the total lysate (Fig. 4B) and sarcolemmal and mitochondrial (Fig. 5) FAT/CD36 content.
CD36 protein contents, with the increases detected being +50, +60, and +213%, respectively, vs. N (Fig. 5). Long-term administration of T3 to H rats did not alter the GSkM FAT/CD36 mRNA level, but T2 administration reduced it slightly (−30%; Fig. 4A). Long-term administration of either T3 or T2 reduced the FAT/CD36 GSkM protein lysate level, with the effect of T2 being the greater of the two (−60 and −25% for T2 and T3, respectively; Fig. 4B).

Long-term administration of T3 to H rats increased both the sarcolemmal and mitochondrial levels of FAT/CD36 (+70 and +184%, respectively, vs. H; Fig. 6A), with the effects being very rapid since they were already evident at 1 h after T3 administration (Fig. 6A). Long-term administration of T2 to H rats had effects similar to those elicited by T3, with the increases in the FAT/CD36 sarcolemmal and mitochondrial contents being −90 and 165%, respectively (Fig. 6B). Again, the effects were rapid, being evident at 1 h after T2 administration.

We could exclude the possibility of the FAT/CD36 detected in the sarcolemmal and mitochondrial fractions having origins different from the fraction under consideration, since they did not cross-contaminate each other. To establish this, we evaluated the presence of Na-K-ATPase (a protein present in the sarcolemma but not in mitochondria), UCP3 (a protein present in the inner mitochondrial membrane), and SCD1 (a protein present at the endoplasmic reticulum level) in each of the above fractions. The data shown in Fig. 7 indicate that neither Na/K-ATPase nor SCD1 was present in mitochondrial membrane-rich fractions, whereas neither UCP3 nor SCD1 was present in the sarcolemma-rich fraction.

**Immunohistochemical analysis.** Immunohistochemical detection of FAT/CD36 in the GSkM of rats subjected to the experimental conditions already described (N, H, H + T3 1 h, and H + T2 1 h) revealed that T2 and T3 each rapidly induced an increased sarcolemmal-associated FAT/CD36 immunoreactivity and also that there was intense staining of subsarcolemmal regions (Fig. 8).

The intracellular distribution of FAT/CD36 was examined by immunofluorescence detection using an in vitro model of skeletal muscle. Such fluorescence immunocytochemistry showed that within 1 h after addition to C2C12 cells in vitro, T2 and T3 enhanced the sarcolemmal FAT/CD36 staining (Fig. 9), but such staining was also within the cytoplasm, mainly in the perinuclear region (Fig. 10). To determine whether the cytoplasmic FAT/CD36 might be localized to mitochondria, FAT/CD36 antibody staining and the mitochondrial tracking dye Mitotracker Red were colocalized by confocal microscopy. In T2-treated and T3-treated cells, but not in control cells, FAT/CD36 was partially colocalized with Mitotracker Red (Fig. 10).
DISCUSSION

Despite the metabolic effects of T3 having first been demonstrated more than a century ago (29), the mechanisms by which it causes metabolic changes are still not completely understood. The present study sought to provide detailed insight into the effects of both hypothyroidism and treatment with T3 or T2 on GSkM fatty acid uptake and utilization, with particular focus on the role played by FAT/CD36.

Growing evidence supports the idea that the metabolic adaptations that occur in the hypothyroid state are not a mirror image of those that occur in the transition from the euthyroid to the hyperthyroid state (37). The effects of hypothyroidism and T3 administration on the FAT/CD36 mRNA and protein levels (the latter at tissue, sarcolemmal, and mitochondrial levels), these effects were not reversed by long-term administration of T3. The rapidity of the effect induced by T3, being evident at 1 h after its administration to hypothyroid rats (Fig. 4A), led us to speculate that it is most likely a non-nuclear-mediated effect. However, further detailed studies are needed to examine this possibility.

The utilization of FFA by GSkM involves many mechanisms, including 1) lipolysis and release/mobilization of FFA from adipose tissue, 2) delivery of FFA to GSkM and transport across the sarcolemma, 3) lipoprotein lipase activity, 4) lipolysis of intramuscular triacylglycerol, and 5) FFA transport into mitochondria and their subsequent oxidation. (18). Another important factor involved in the ability of mitochondria to oxidize fatty acid is the mitochondrial proton conductance, which is known to modulate the efficiency of oxidative phosphorylation. Indeed, a rise in the NADH/NAD+ ratio or acetyl-CoA/CoA ratio results in inhibition of fatty acid β-oxidation, whereas an increase in mitochondrial proton conductance, by promoting the oxidation of substrates, maintains a low NADH/NAD+ or acetyl-CoA/CoA ratio, thus enhancing fatty acid oxidation. Apart from lipoprotein lipase activity that in striated muscle has been reported to not be affected by the animal’s thyroid state (23), the data presented here indicate that most of above mechanisms are affected by both hypothyroidism and T3 treatment.

Hypothyroidism did not change FFA availability to the tissues (as indicated by the serum levels of FFAs) but did result in an increased sarcolemmal FAT/CD36 level, giving rise to an enhanced ability of GSkM to import FFA for utilization. Despite the increase in the uptake of FFA by GSkM, hypothyroidism decreased the tissue’s fatty acid oxidation, leading to an imbalance between FFA supply and oxidation (with the latter also being more efficient) and hence, an accumulation of intramyocellular triglyceride.

In addition, although the presence of FAT/CD36 at the mitochondrial level has been said to be related to the ability of mitochondria to use fatty acid as a fuel substrate (17, 39), our data clearly indicate that in hypothyroidism the reduced ability of mitochondria to oxidize fatty acids (Table 1) is associated with an upregulation of the mitochondrial FAT/CD36 content (Fig. 3). This discrepancy seems to be more apparent than real since, in the hypothyroid state, a structural shift occurs in GSkM toward a slow-twitch phenotype (19, 38), which makes predominant use of lipid as an energy source. Thus, the
upregulation of FAT/CD36, which would increase fatty acid supply to the mitochondria (39), could be interpreted as a form of compensation for the impaired capacity of mitochondria to import fatty acids and to oxidize substrates.

One week’s administration of T3 to hypothyroid rats led to an increase in the availability of FFAs to the tissues. Within 1 h of its administration to hypothyroid rats, T3 induced both an increase in the import of FFA into GSkM and an increase in their oxidation therein. Although the molecular mechanism underlying the modulation by T3 of the redistribution of FAT/CD36 within the gastrocnemius muscle cells remains to be unraveled, we speculate that there is involvement of kinases such as AMPK and Akt, each of which has been shown to be involved in FAT/CD36 redistribution (12). In support of this notion, we showed recently that a single administration of T3 to hypothyroid rats rapidly activated both AMPK and Akt (8). One week’s T3 administration to hypothyroid rats did not affect the mRNA level of FAT/CD36 in skeletal muscle, a finding in apparent contrast to the observation made by Flores-Morales et al. (10) in mouse liver. They noted a significant decrease in FAT/CD36 following a 1-wk T3 administration to hypothyroid mice. This may suggest a tissue- or species-specific regulation of FAT/CD36 transcription by T3.

Following the present long-term T3 treatment, the induced increases in fatty acid uptake and oxidation were associated with an unchanged level of GSkM FFA and a reduced level of GSkM triglyceride. These data may indicate that following such treatment, the increase in FFA oxidation, which also became less efficient, exceeds the increase in the import of FFA into the tissue, with the consequence that FFAs are not deposited as triglycerides. In addition, lipolysis of triglycerides takes place in GSkM.

The raised sarcolemmal FAT/CD36 levels seen upon long-term administration of T3 and the elevated FFA serum level are in accord with data published by Klieverik et al. (23), who reported that a significant rise in the uptake of albumin-bound fatty acids by striated muscle was induced by an excess of thyroid hormone. The different time scales of the effects of T3 on the mitochondrial fatty acid oxidation rate (viz. recruitment of FAT/CD36 vs. effect on the CPT system) supports FAT/CD36 being primarily responsible for the early increase in lipid handling (vs. that in the hypothyroid condition).

![Fig. 8. Immunolocalization of FAT/CD36 protein in skeletal muscle. Gastrocnemius muscle taken from euthyroid control rats (C), hypothyroid rats (H), and hypothyroid rats at 1 h after administration of iodothyronines to hypothyroid rats (H + T2 1 h and H + T3 1 h, respectively). After T2 and T3 administration, fine punctate staining was seen around the membrane (arrows) of some skeletal fibers but also in subsarcolemmal regions (arrowheads). FAT/CD36 is stained brown by the avidin-biotin-peroxidase complex method. Nuclei were counterstained with hematoxylin (blue). Bar, 20 μm.](image1)

![Fig. 9. Localization of FAT/CD36 protein in control and in T2- and T3-treated (1 h) C2C12 cells. Confocal representative images of immunostaining of nonpermeabilized cells. Fine staining of the cell surface was seen (arrowheads) in both T2- and T3-treated cells, but not in control cells, when an anti-FAT/CD36 antibody and a polyclonal secondary antibody conjugated to Alexa 488 (green) were employed. Nuclei were counterstained with TO-PRO-3 iodide (pink). Bars, 10 μm.](image2)
It should also be mentioned that, when expressed in *Xenopus laevis* oocytes, FAT/CD36 mediates the import of T3 into the cell as well as FFA import (41). Thus, we speculate that T3, by promoting a rapid translocation of such protein to the sarcolemma, could promote its rapid uptake into muscle cells, thus facilitating its action at the cellular level and favoring skeletal muscle metabolic adaptation. However, in vivo or ex vivo functional data on such FAT/CD36-mediated import of T3 are lacking.

Unlike that of T3, administration of T2 to hypothyroid rats induces a significant reduction in the serum FFA level that could be a consequence of the T2-induced increase in lipid utilization by GSkm, which is reported here. However, the involvement of tissues other than GSkm cannot be excluded.

Concerning the effect of T2 on FAT/CD36, T2 had effects similar to those induced by T3 in promoting the translocation of the protein from cellular depots to the sarcolemma and to mitochondria, each in a very rapid fashion, thus favoring both the import of FFAs into the tissue and their subsequent oxidation at the mitochondrial level. Although T2 and T3 each increased the mitochondrial fatty acid oxidation rate, the mechanisms underlying the short-term effects of these two iodothyronines seem to differ, as indicated by the differing onsets of CPT activation and mitochondrial respiratory pathway activation. Indeed, FAT/CD36 is evidently not the main factor responsible for the rapid T2-induced activation of fatty acid oxidation, although it appears to be for T3. Rather, such activation by T2 depends also on the CPT system and the substrate oxidation rate (thus confirming the data reported in Ref. 27). Both processes, in fact, were already activated at 1 h after T2 administration, whereas T3 was ineffective at that time point. FAT/CD36, CPT activity, and the substrate oxidation rate all displayed increases as elements in the long-term effect of T2 on fatty acid oxidation rate.

Different mechanisms also seem to underlie the long-term effects of T2 and T3 on the efficiency of oxidative phosphor-
ylation since T₃ affected both basal and fatty acid-induced proton conductance, whereas T₂ affected only fatty acid-induced proton conductance. Finally, as was the case with T₃, long-term administration of T₂ to hypothyroid rats led to a reduction in triglyceride deposition. However, unlike with T₃, this was due principally to fatty acid oxidation, not to lipolysis of GSKM triglyceride.

As a whole, these data indicate that in hypothyroid rats T₃ and T₂ induced similar increases in muscle fatty acid uptake and utilization and an associated decrease in tissue triglycerides. This would result in a protection of skeletal muscle and tissue triglycerides and reutilization of free fatty acids during activated lipolysis in human skeletal muscle. J Clin Endocrinol Metab 90: 1189–1195, 2005.


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