3,5-Diiodo-L-thyronine prevents high-fat-diet-induced insulin resistance in rat skeletal muscle through metabolic and structural adaptations

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ABSTRACT The worldwide prevalence of obesity-associated pathologies, including type 2 diabetes, requires thorough investigation of mechanisms and interventions. Recent studies have highlighted thyroid hormone analogs and derivatives as potential agents able to counteract such pathologies. In this study, in rats receiving a high-fat diet (HFD), we analyzed the effects of a 4-wk daily administration of a naturally occurring iodothyronine, 3,5-diiodo-L-thyronine (T2), on the gastrocnemius muscle metabolic/structural phenotype and insulin signaling. The HFD-induced increases in muscle levels of fatty acid translocase (3-fold; \( P<0.05 \)) and TGs (2-fold, \( P<0.05 \)) were prevented by T2 (each; \( P<0.05 \) vs. HFD). T2 increased insulin-stimulated Akt phosphorylation levels (\( \sim 2.5\)-fold; \( P<0.05 \) vs. HFD). T2 induced these effects while sparing muscle mass and without cardiac hypertrophy. T2 increased the muscle contents of fast/glycolytic fibers (2-fold; \( P<0.05 \) vs. HFD) and sarcolemmal glucose transporter 4 (3-fold; \( P<0.05 \) vs. HFD). Adipocyte differentiation-related protein was predominantly present within the slow/oxidative fibers in HFD-T2. In T2-treated rats (vs. HFD), glycolytic enzymes and associated components were up-regulated (proteomic analysis, significance limit: 2-fold; \( P<0.05 \)), as was phosphofructokinase activity (by 1.3-fold; \( P<0.05 \)), supporting the metabolic shift toward a more glycolytic phenotype. These results highlight T2 as a potential therapeutic approach to the treatment of diet-induced metabolic dysfunctions.—Moreno, M., Silvestri, E., De Matteis, R., de Lange, P., Lombardi, A., Glinni, D., Senese, R., Cioffi, F., Salzano, A. M., Scaloni, A., Lanni, A., Goglia, F. 3,5-Diiodo-L-thyronine prevents high-fat-diet-induced insulin resistance in rat skeletal muscle through metabolic and structural adaptations. FASEB J. 25, 3312–3324 (2011). www.fasebj.org

Key Words: thyroid hormone • metabolism • proteomics

Fat accumulation within tissues has deleterious consequences for organ function and may lead to metabolic disorders such as insulin resistance (IR), type 2 diabetes, heart disease, and hypertension. Several strategies aim to combat these events by limiting the inappropriate deposition of fat within peripheral tissues. In this context, research on skeletal muscle (SKM) is attractive because of that tissue’s integral role in regulating whole-body glucose/lipid homeostasis. In fact, \( \sim 80\% \) of ingested glucose is taken up by SKM and either oxidized to provide energy or stored as glycogen (1). Moreover, fatty acid oxidation within SKM satisfies the large part of the energy requirements of this tissue in the resting state (2). Under situations of fat overload, such as during high-fat feeding, SKM is faced with increasing amounts of lipids that it is unable to oxidize, and these therefore accumulate, leading to derangements in insulin signaling and to muscle and, indeed, to systemic insulin resistance (3). This condition plays a key role in the metabolic disorders associated with obesity, thus contributing to the development of the metabolic syndrome (4). The mechanisms underlying muscle insulin resistance have not yet been fully elucidated, although they seem to involve derangements in lipid metabolism and lipotoxicity (1–11), alterations in the PI3K/Akt pathway (12–16), and skeletal muscle fiber type (17–20).

Most effort has been directed at reducing the accumulation of detrimental lipids within muscle and liver by decreasing the availability of triglycerides (TGs) and nonesterified fatty acids via the circulation. This can be done by using either or both of the classic preventive strategies (reducing caloric intake and/or increasing

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physical activity). A number of therapeutic options are currently available for the treatment of metabolic disorders, but because of the existence of some unwanted side effects, the development of safer and more effective agents is still a major priority.

Thyroid hormone (TH) is an important modulator of lipid metabolism and metabolic rate, favoring lipolysis and increasing the use of fatty acids as fuels, effects that have the desirable result of reducing fat accumulation (21). A number of investigations have demonstrated stimulatory effects of triiodothyronine (T3) on insulin-stimulated glucose transport and/or phosphorylation in muscle (22, 23), on the insulin-sensitive muscle glucose transporter, namely glucose transporter 4 (GLUT4; 22, 24), and on glycolysis in isolated muscle (23). Other, consistent data have shown decreased insulin-stimulated glucose transport and/or phosphorylation, as well as a lower rate of glycolysis in isolated muscles from hypothyroid animals (25). Because of the induction of adverse side effects (mostly at the cardiac level), the use of TH as an adiposity counteractor has been greatly limited.

We showed that a natural TH derivative, namely 3,5-diiodo-L-thyronine (T2), has the potential to prevent adiposity when administered to rats receiving a high-fat diet (HFD) without the undesirable side effects attributed to THs (26), and they were accompanied at the cellular level by an increase in fatty acid oxidation in the liver, and reductions in the circulating levels of cholesterol and TGs (26). These actions may ultimately reduce the amount of lipids available to skeletal muscle, thus counteracting the fat-induced IR attributable to an HFD. To test the above idea, we designed this study to investigate the capacity of T2 to counteract HFD-linked muscle IR in rats. To that end, we directly assessed the effects of T2 on muscle insulin signaling; the muscle content of lipids and TGs; the muscle fiber type profile; the sarcolemmal levels of Akt, fatty acid translocase (FAT/CD36), and GLUT4; the muscle proteomic profile; and the fiber-specific localization of adipocyte differentiation-related protein (ADRP).

MATERIALS AND METHODS

Animals

Male Wistar rats (aged 8 wk) were purchased from Charles River (Lecco, Italy). They were housed in individual cages in a temperature-controlled room at 28°C (thermoneutral temperature for rats) with a 12-h light-dark cycle. A commercial mash (Charles River) was available ad libitum, and the animals also had free access to water.

At the beginning of this study, which was after 7–10 d of acclimatization (d 0), rats were divided into 3 groups (15 animals/group). In each group, body weight was normally distributed, and group means were similar (300 ± 5 g). The first group (N group) was fed a standard diet (Muscedola s.r.l., Milan, Italy); the total metabolizable percentage of energy was made up as follows: 60.4% carbohydrates, 29% proteins, and 10.6% fat; J/J (19.85 kJ gross energy/g; 26). The total metabolizable percentage of energy was made up as follows: 21% carbohydrates, 29% proteins, and 50% fat; J/J (19.85 kJ gross energy/g; 26). The second group (HFD group) was fed an HFD; the third group (HFD-T2 group) received the above HFD together with a daily intraperitoneal injection of T2 (25 µg/100 g body weight). N and HFD rats were sham-injected. Each group was divided into 3 subgroups of 5 rat. Ten rats were subjected to food deprivation for 5 h and were subsequently injected with either insulin (10 U/kg body weight; 5 rats) or saline (5 rats) for the determination of insulin-stimulated Akt phosphorylation. The remaining rats were used for all other measurements.

Animal care and experiments were conducted in accordance with the guidelines issued by the Italian Ministry of Health. At the end of the treatment, rats were anesthetized by an intraperitoneal injection of chloral hydrate (40 mg/100 g body weight) and were then killed by decapitation. Gastrocnemius muscles were excised, weighed, and either immediately processed or stored at -80°C for later processing.

Metabolic measurements

Total lipid and TG content of muscle were determined by means of an Infinity kit (Sigma-Aldrich Corp., St. Louis, MO). Protein levels of Akt and phosphorylated Akt (Ser-473) were determined in the supernatants of ultracentrifuged gastrocnemius lysates. Polyclonal antibodies were used for this purpose (27). Phosphofructokinase (PFK) was assayed by the method described by Opie and Newsholme (28), except that nonspecific oxidation of nicotinamide adenine dinucleotide and hydroxide was inhibited by 1 mM potassium cyanide (27). The serum levels of cholesterol and TGs were determined by following standard procedures. Thyroid hormone levels were determined using materials and protocols supplied by Byk-Sangtec Diagnostica (Hessen, Germany).

Muscle samples were fixed by overnight immersion in 4% formaldehyde and 0.1 M sodium phosphate buffer (PB), pH 7.4. After a brief wash in PB, tissues were either dehydrated and paraffin embedded or immersed in sucrose (30% w/v; with 0.1% sodium azide) and were then embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and immediately frozen in liquid N2-cooled isopentane. For immunohistochemical analysis, adjacent serial sections (4 µm) were cut, collected onto uncoated glass slides, and used to stain ADRP, GLUT4, and myosin heavy chain type I (MHC Ib) and type II (MHC IIb). The following antibodies were used: anti-ADRP (guinea pig polyclonal, RDI-PROGP40, 1:5000; Fitzgerald Industries RDI Division, Concord, MA, USA), anti-GLUT4 (rabbit polyclonal, 1:100; Abcam, Cambridge, UK), anti-fast myosin (clone MY-32, monoclonal, 1:4000; Sigma-Aldrich Corp.), and anti-slow myosin (clone NOQ7.5.4D, monoclonal, 1:6000; GeneTex, Irvine, CA, USA). Bound antibody was finally stained by the ABC peroxidase (Vector Laboratories, Burlingame, CA, USA) method. Peroxidase activity was revealed by incubation with 3,3'-diaminobenzidine tetrahydrochloride as substrate. Nuclei were counterstained with hematoxylin, and the sections were mounted in Eukitt (Kindler, Freiburg, Germany). Control sections for nonspecific staining were subjected to the same incubation protocol but with the primary antibody omitted.

To study the fiber type-specific ADRP content, double-staining was performed. Sections processed for ADRP staining (as described above) were washed in running tap water, incubated in PBS for 5 min, and subsequently incubated with monoclonal anti-fast myosin (clone MY-32, dilution 1:4000; Sigma-Aldrich). The immunoreaction was detected by means of a different visualization system from that described above, using an ABC-AP reagent (Vectastain ABC-AP Standard Kit; Vector Laboratories) and Vector Blue Alkaline Phosphatase.
Substrate Kit III as the alkaline phosphatase substrate solution (Vector Laboratories). Gray-stained fibers were classified as type II fibers, whereas unstained fibers were classified as type I fibers. The fiber type-specific ADRP-immunoreactive lipid droplet content was validated by counting the number of ADRP-stained fibers for either slow and fast fibers. Approximately 3000 fibers were individually counted in each experimental group (n=4).

Immunohistochemical fiber type determination

Muscle fiber types were classified as fiber types I and II on the basis of myosin monoclonal antibody immunostaining. Several adjacent serial cross-sections (from each rat) were examined for fast and slow MHC expression. Entire cross-sectional areas were photographed and collected at ×20. Then, recomposed tissue sections stained with slow and fast myosin-directed antibodies were compared to confirm the position of each fiber. Muscle fiber composition was determined by counting the number of positive fibers stained exclusively for either slow or fast myosin. Muscle fibers expressing multiple MHC isoforms were taken to indicate a transitional MHC phenotype and were counted as mixed (fast/slow myosin). Approximately 4000 fibers were individually counted in each experimental group. To confirm colocalization of slow and fast MHCs within fibers, a double-labeling protocol was designed using the above antibodies against fast and slow myosin, each with a different visualization system. In brief, slow myosin was visualized as blue/gray type I fibers, using an SG Substrate Kit (Vector Laboratories) for peroxydase and alkaline phosphatase-conjugated antibody, whereas fast myosin was visualized as pink type II fibers, using a Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories). Fibers expressing multiple MHC isoforms appeared as a pale greyish-pink color (29).

Staining of lipid droplets

Muscle cryosections (6 μm) obtained using a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany) at −29°C were collected onto room-temperature glass slides; lipid droplets were stained by means of a neutral lipid dye [oil red O (ORO)]. ORO staining of muscle cross-sections, combined with immunohistochemistry (fast myosin), has been described previously for the simultaneous visualization of intramyocellular lipids (IMCLs) and identification of the muscle fiber type (30).

Western blotting

Western blot analysis was performed as described previously (27). The following antibodies were used: anti-fast myosin (clone MY-32, monoclonal, 1:4000; Sigma-Aldrich), anti-slow myosin (clone NOQ7.5.4D, monoclonal, 1:6000; Genetex), anti-GLUT4 (raised in rabbit, polyclonal, 1:50; Cell Signaling Technology, Danvers, MA, USA), and anti-FAT/CD36 (mouse monoclonal FA6-152 to CD36 AB 1744; Abcam). A β-actin antibody was purchased from Sigma-Aldrich.

Protein extraction and sample preparation for 2-dimensional gel electrophoresis (2-DE)

2-DE was performed essentially as reported previously (31). In brief, frozen muscle tissue (40 mg) was homogenized in 0.25–1 ml of 8.3 M urea, 2 M thiourea, 2% 3-[3-cholamidopropyl] diethylammonio]-1 propane sulfonate, 1% dithiothreitol, and 2% immobilized pH gradient buffer (pH 3–10). The extracts were shaked vigorously for 30 min at 4°C, followed by a 30-min centrifugation at 10,000 g. Protein concentration was determined using the detergent-compatible method (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA, USA). Protein extracts were prepared for each animal, and each individual animal was assessed separately. Protein samples (650 μg) were applied to immobilized pH 3–10 nonlinear gradient strips (17 cm; Bio-Rad Laboratories). For each sample, triplicate runs were performed as independent experiments. Focusing started at 200 V; the voltage was gradually increased to 3500 V and then kept constant for a further 66,500 V h (Protein IEF System; Bio-Rad Laboratories). The second-dimensional separation was performed using 12% SDS-polyacrylamide gels. After protein fixation, the gels were stained with colloidal Coomassie blue (Sigma-Aldrich). Electronic images of the gels were acquired by means of a calibrated GS-800 densitometer (Bio-Rad Laboratories) and analyzed using PDQuest software (Bio-Rad Laboratories). For all spot-intensity calculations, normalized values were used to calculate the relative intensity (RI) for each spot: RI = vi/vt, where vi is the volume of the individual spot and vt the sum of the volumes of all matched spots. Spots with values of P < 0.05 and ≥1.5-fold variation in pairwise comparisons were considered to display a significant difference between experimental groups.

Protein digestion and mass spectrometry analysis

Spots from 2-DE were excised from gels, Salkylated, and digested with trypsin as reported previously (32). Digests were desalted on µZipTipC18 (Millipore Corp., Billerica, MA, USA) before mass spectrometry analysis. During matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) peptide mass fingerprinting (PMF) experiments, peptide mixtures were loaded on the instrument target with α-cyano-4-hydroxycinnamic acid as matrix, using the dried droplet technique. Samples were analyzed on a Voyager-DE PRO mass spectrometer (Applied Biosystems, Foster City, CA, USA). Spectra were acquired in reflectron mode; internal mass calibration was performed using peptides from trypsin autoproteolysis. Data were elaborated using DataExplorer software (Applied Biosystems). Digests were eventually analyzed by nano-liquid chromatography-electrospray ionization-linear ion trap-tandem mass spectrometry (nLC-ESI-LIT-MS/MS) using a LTQ XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Proxeon nanospray source connected to an Easy-nLC system (Thermo Fisher Scientific; ref. 33). Peptide mixtures were separated on an Easy C18 column (10×0.075 mm, 3 μm; Thermo Fisher Scientific) using a gradient of acetonitrile containing 0.1% formic acid in aqueous 0.1% formic acid; acetonitrile was ramped from 5 to 35% over 10 min and from 35 to 95% over 2 min and remained at 95% for 12 min, at a flow rate of 300 nl/min. Spectra were acquired in the range of m/z 400–2000. Acquisition was controlled by a data-dependent product ion-scanning procedure over the three most abundant ions, enabling dynamic exclusion function.

Protein identification

MASCOT 2.2.06 software (Matrix Science Ltd., London, UK; ref. 34) was used to identify spots from a Rattus norvegicus sequence database (National Center for Biotechnology Information no. 2010/08/07). MALDI-TOF PMF data were searched using a mass tolerance value of 50 ppm, with trypsin as the proteolytic enzyme, a missed-cleavage maximum value of 2, and Cys carbamidomethylation and Met oxidation as fixed and variable modifications, respectively. nLC-ESI-LIT-MS/MS data were analyzed on a Voyager-DE PRO mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Proxeon nanospray source connected to an Easy-nLC system (Thermo Fisher Scientific; ref. 33). Peptide mixtures were separated on an Easy C18 column (10×0.075 mm, 3 μm; Thermo Fisher Scientific) using a gradient of acetonitrile containing 0.1% formic acid in aqueous 0.1% formic acid; acetonitrile was ramped from 5 to 35% over 10 min and from 35 to 95% over 2 min and remained at 95% for 12 min, at a flow rate of 300 nl/min. Spectra were acquired in the range of m/z 400–2000. Acquisition was controlled by a data-dependent product ion-scanning procedure over the three most abundant ions, enabling dynamic exclusion function.
MS/MS data were searched using the same criteria but with a mass tolerance value of 2 Da for the precursor ion and 0.8 Da for MS/MS fragments. MALDI-TOF PMF candidates with a cumulative MASCOT score or nLC-ESI-LIT-MS/MS candidates with assigned peptides with an individual MASCOT score both corresponding to P=0.05 for a significant identification, were further evaluated for their experimental mass and pI values obtained from 2-DE. Protein identification was checked with a reverted sequence database to provide a false-positive rate of 1%.

Statistical analysis

Results are expressed as means ± se. The statistical significance of differences between groups was determined using 1-way ANOVA followed by a Student-Newman-Keuls test. Differences were considered significant at P<0.05.

RESULTS

T2 prevents HFD-induced hyperlipidemia without inducing a thyrotoxic state

Serum levels of cholesterol and TGs were higher in HFD rats than in N rats, whereas in HFD-T2 rats, they were significantly reduced (vs. HFD; Table 1). In addition, as a control for biological effect, heart weight/body weight and gastrocnemius weight/body weight ratios were determined. T2 treatment, while preventing body weight gain (Table 1), did not change either of these ratios (Table 1), and no change in heart rate was observed on T2 administration (actual beats/min of 300±32, 312±40, and 315±30 in N, HFD, and

![Figure 1](image_url)

**Figure 1.** T2 prevents HFD-induced accumulation of muscle TGs and IMCLs and increase in FAT/CD36 levels and improves insulin signaling. A) Representative cross-section of gastrocnemius muscle from HFD rats. Tissues were stained for neutral lipids with ORO and for MHC Ib immunolocalization. Large lipid droplets (arrows) in the HFD condition were localized in fast fibers (f; no MHC Ib immunoreactivity) and only rarely in slow fibers (s). Scale bar = 8 µm. B) TG content in muscles from N, HFD, and HFD-T2 rats. C, D) Sarcolemmal FAT/CD36 protein expression (C) and phospho (p)-Akt/Akt ratio (D) in muscles from N, HFD, and HFD-T2 rats. Data represent means ± se; n = 5. Bars labeled with dissimilar letters are significantly different (P<0.05).
T2 prevents HFD-induced increases in gastrocnemius muscle TG, IMCL, and FAT/CD36 levels and significantly improves insulin signaling

Skeletal muscles adapt to a given stimulus with structural, biochemical, and functional modifications in line with the fuel used and the energy demand (35). This is particularly true for a mixed-fiber-type muscle, such as gastrocnemius, which contains regions of slow- and fast-twitch fibers. As expected, HFD feeding raised the

Figure 2. T2 induces a structural shift toward fast/glycolytic myofibers in gastrocnemius muscle. A–E) Western blot analyses of MHC Ib (A) and MHC IIb (B) protein levels, together with representative images of gastrocnemius muscle sections, double-stained by immunohistochemistry for type I (blue-gray) and type IIb (pink) MHC isoforms, from N (C), HFD (D), and HFD-T2 (E) rats. Fibers coexpressing type I and type IIb MHC isoforms (arrows) were identified by their greyish-pink staining. F, G) Immunohistochemistry on serial sections of gastrocnemius muscles from HFD-T2 rats for type IIb (F) and type I (G) MHC showed that most fibers were “pure,” containing only the IIb (F) or only the I (G) MHC isoform. Fibers 1–4 were “hybrids,” containing both I and IIb MHC isoforms. Some showed a weaker staining for one type, e.g., type I (fiber 1) or type IIb (fibers 3 and 4) MHC isoforms. H) Quantitative analysis of gastrocnemius fiber composition (shown as percentage of total fiber number). Data represent means ± se; n = 5. Bars labeled with dissimilar letters are significantly different (P<0.05). Scale bars = 100 μm (C, D, E); = 85 μm (F, G).
gastrocnemius content of IMCLs and TGs (Fig. 1A, B). Large lipid droplets were noted within muscle fibers in the HFD condition (Fig. 1A). Combined ORO staining and MHC Ib immunolocalization showed that those large lipid droplets (Fig. 1A, arrows) were localized to fast fibers. This augmented IMCL accumulation was associated with an elevated basal sarcolemmal-associated FAT/CD36 level (~3-fold vs. N; Fig. 1C). T2 treatment normalized muscle TG levels (Fig. 1B) and prevented the increase in the sarcolemmal FAT/CD36 protein level (Fig. 1C), when compared with HFD rats, in the absence of any increase in fatty acid oxidation (data not shown). Therefore, it appears that under HFD conditions, gastrocnemius responds by maintaining a higher concentration of FAT/CD36 on the cell membrane; as a consequence, uptake of fatty acids is increased, and they are stored as IMCLs. By reducing the fatty acid uptake, T2 evidently leads to decreases in IMCL and TG levels in muscle. This result suggests a protective role for T2 in preventing IMCL accumulation and consequently ameliorating the condition of IR induced by the HFD. Indeed, under in vivo insulin-stimulated conditions, T2 treatment enhanced the concentration of the phosphorylated form of Akt (~2.5-fold; P<0.05 vs. HFD), demonstrating that it preserves insulin signaling against the deleterious effect of HFD feeding (Fig. 1D).

T2 induces in gastrocnemius muscle a structural and biochemical shift toward glycolytic myofibers

Because the skeletal muscle fiber-type profile might play a role in the IR observed in HFD rats, we measured the relative protein expression levels of the MHC isoforms Ib and IIb in gastrocnemius. Western blot analysis revealed that the MHC Ib level was

**Figure 3.** T2 induces an increase in sarcolemmal GLUT4 levels in gastrocnemius muscle. A) Western blot analysis of sarcolemmal GLUT4 protein levels. C–D) Cell type-specific GLUT4 localization in muscles from N (B), HFD (C), and HFD-T2 (D) rats. GLUT4 immunoreactivity was localized as a scattered granular surface-associated reaction (arrows). s, type I fibers; f, type II fibers. Data represent means ± se; n = 5. Bars labeled with dissimilar letters are significantly different (P<0.05). Scale bars = 10 μm.
significantly increased in gastrocnemius from HFD rats, whereas T2 treatment significantly reduced it to the level observed in N rats (Fig. 2A). The MHC IIb level, on the other hand, was significantly reduced in gastrocnemius muscles from HFD rats, but it, too, was restored by T2 treatment (Fig. 2B). Quantitative analysis of fiber types revealed that slow/oxidative fibers were replaced to a significant extent by fast/glycolytic fibers after T2 treatment (Fig. 2C–H). Of note, although some 4% of fibers were joint fast/slow immunoreactive in both HFD and HFD-T2 rats, the fast component prevailed after T2 treatment (Fig. 2E, F), indicating an ongoing structural shift toward the glycolytic phenotype. This shift was supported by the observation that many of the slow/fast immunoreactive fibers were only weakly stained for slow myosin in muscles from HFD-T2 rats (Fig. 2G). The sarcomembrane-associated GLUT4 protein content was up-regulated by T2 treatment, in accordance with the observed structural shift (Fig. 3A). The plasma membrane-associated labeling in fast/glycolytic fibers was more intense in the HFD-T2 gastrocnemius.

Figure 4. T2 induces a structural and biochemical shift toward the glycolytic phenotype in gastrocnemius muscle. A) Gastrocnemius muscle protein profiling by 2-DE. The 38 circled and numbered spots mainly correspond to deregulated protein components after T2 treatment. B) Histogram of protein abundance ratio HFD-T2/HFD. C) Representative subsections of 2-DE images are shown as examples of differential expression among experimental groups. Data (relative intensity) represent means ± se; n = 5. P < 0.05 in pairwise comparisons: HFD vs. N; HFD-T2 vs. HFD.
(Fig. 3D) than in the N (Fig. 3B) or HFD (Fig. 3C) rats.

To delve more deeply into the effects of long-term T2 treatment on the muscle phenotype, proteomic analysis was performed (Fig. 4). The number of proteins identified as significantly differing between HFD-T2 and HFD muscle samples was 38 (Fig. 4A, B and Table 2). Furthermore, several proteins were identified at multiple spot positions, putatively reflecting the occurrence of post-translational modifications. In these cases, however, on average, the changes in the spots were quite similar (Fig. 4B and Table 2).

Proteomic analysis revealed that T2 treatment significantly altered the protein expression profile of muscle in the HFD condition. In particular, the protein expression levels of the fast isotypes of myosin light chains (MLC1f, spot2; MLC2f, spot 4) and of the tropomyosin α chain fast (spot 6) increased significantly after T2 treatment, whereas the content of the slow isotypes MLC1s (spot 1) and MLC2s (spot 3) and the tropomyosin α chain slow (spot 5) decreased, in agreement with a shift toward the fast phenotype (Fig. 4B, C). Coherently, other proteins, identified as glycolytic enzymes, were all up-regulated in muscles from HFD-T2 rats (vs. HFD; Fig. 4B, C). These proteins included α- and β-enolase (spots 32 and 30, respectively), which participate in the glycolytic conversion of glucose to pyruvate; phosphoglucomutase-1 (spot 17), an enzyme regulating an important step in both glycolysis and gluconeogenesis; triosephosphate isomerase (spots 14–16), which promotes the formation of glyceraldehyde 3-phosphate molecules to be metabolized further down the glycolytic pathway; glyceraldehyde-3-phosphate dehydrogenase (spot 38), an enzyme yielding NADH from glyceraldehyde 3-phosphate; lactate dehydrogenase (A chain, M type; spot 37), which provides for the interconversion of glycolysis end-products (i.e., pyruvate and lactate) and may regulate the turnover of lactate within the muscle cell; creatine kinase (spots 20 and 21), which, by phosphorylating creatine, plays a crucial energy transduction role; isocitrate dehydrogenase (spot 28); and adenylate kinase isoenzyme 1 (spot 19), which catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP and is involved in energy metabolism and nucleotide synthesis (Fig. 4A, B).

On the other hand, enzymes involved in oxidative metabolism, such as carbonic anhydrase III (spots 10 and 11) and myoglobin (spot 36), were down-regulated in muscles from HFD-T2 rats compared with those from HFD rats (Fig. 4). The increased levels of glycolytic enzymes in the muscle of HFD-T2 rats were reflected in an increased activity of phosphofructokinase, a key regulatory enzyme for glycolysis (51 ± 3 and 66 ± 4 nmol NADH/min/mg protein for HFD and HFD-T2, respectively). Taken together, these results strongly support biochemical and structural shifts toward the fast glycolytic phenotype in the HFD-T2 gastrocnemius muscle in rats. These shifts parallel the reductions in fatty acid, TG, and cholesterol levels in the serum and the reduced steatosis in the liver reported previously in HFD-T2 rats (26).

**T2 increases the localization of ADRP within slow/oxidative fibers**

We next investigated another aspect related to intramuscular TG storage, namely ADRP fiber-specific localization. Double-staining MHC IIb and ADRP allowed us to localize fiber type-specific ADRP labeling surrounding IMCLs. The data reported here, in agreement with previous studies (36, 37), showed that the observed ADRP immunoreactivity overlapped well with the lipid staining, demonstrating that this protein is associated with lipid droplets in rat gastrocnemius muscle (Fig. 5). In muscle from N rats, ADRP immunoreactivity was poorly evident (Fig. 5A) and mainly present around very small lipid droplets located predominantly within slow/oxidative fibers (65.98±5.93% of slow fibers) but also in some fast/glycolytic fibers (24.43±2.38% of fast fibers). In muscle from HFD rats (Fig. 5B), the ADRP staining was mainly located around the large lipid droplets present within the fast/glycolytic fibers (41.36±4.6% of fast fibers); most of them were very intensely ADRP immunoreactive, whereas the few lipid vacuoles located within the slow/oxidative fibers (35.74±5.73% of slow fibers), displayed only very weak ADRP labeling. After T2 treatment, ADRP labeling coated all the IMCLs (Fig. 5C); on the whole, slow/oxidative fibers exhibited increased accumulation of ADRP (86.11±4.53 of slow fibers), whereas the fast/glycolytic fibers had a reduced ADRP content (32.83±3.98 of fast fibers).

**DISCUSSION**

Increased body fat, especially the intramyocellular fat content, has been related to the development of IR (10), a maladaptive response that is currently attributed to the generation of intracellular events that antagonize insulin signaling (38). High-fat feeding has been demonstrated to trigger IR in animal models (39, 40), and the worldwide spread of fat-enriched diets may be the largest contributor to the growing incidence of the metabolic syndrome. Recent interest has focused on therapeutic agents that might be able to improve insulin sensitivity and/or ameliorate the features of metabolic disease by limiting the inappropriate deposition of fat in certain peripheral tissues not suited for lipid storage, such as skeletal muscle. We showed that 4 wk of T2 treatment of HFD rats resulted in a marked prevention of the elevation of plasma free fatty acid levels by increasing hepatic fatty acid oxidation (26). Notably, in contrast to what happens when THs are used as antiobesity agents, T2 treatment did not decrease the skeletal muscle mass and did not affect either heart mass or heart rate, indicating a lack of undesirable side effects associ-
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The T2-induced increase in hepatic fatty acid oxidation, and its lowering effects on the levels of plasma lipids, could decrease the supply of fatty acids to muscle cells, relieving lipid overload and leading to increased glucose uptake, thus improving insulin sensitivity (40). The present results show that T2 treatment effectively prevented the HFD-dependent increase in the expression of an important determinant of muscle lipid accumulation and insulin responsiveness, namely FAT/CD36 (5), without significant enhancement of fatty acid oxidation. T2 administration to HFD rats resulted in significant reductions in both IMCL and muscle TG levels, with a concomitant significant stimulation of insulin-induced Akt activation, which is implicated in glucose transport and glycogen synthesis in skeletal muscle cells (15, 41) as well as being implicated in muscle hypertrophy in vivo (17), and has been shown to be deregulated in conditions of IR (19, 42). Notably, in the gastrocnemius muscle T2 also caused a slow-to-fast fiber shift, with a concomitant increase in the total Akt protein level (vs. HFD rats). This phenomenon is consistent with results published by Izumiya et al. (20), who created obese Akt1 transgenic mice that displayed hypertrophy of fast/glycolytic muscle fibers. The growth of fast/glycolytic muscle in these obese mice normalized their responses to exogenous glucose and insulin and reversed their hepatic steatosis (20). These data indicated that increasing the fast muscle size (e.g., via strength training) could be an important intervention for the at-risk populations. Indeed, fast muscle fibers are more responsive to resistance training (e.g., weight training) than their HFI equivalents. Our proteomic analysis confirmed the T2-induced structural shift within the gastrocnemius muscle toward a more glycolytic phenotype in a situation in which the serum levels of TGs, free fatty acids, and cholesterol are lowered by increased hepatic fatty acid oxidation. Indeed, fast muscle fibers are more responsive to resistance training, which in humans has been observed to reduce adiposity and improve insulin sensitivity and is a recommended mode of exercise for patients with type 2 diabetes (23, 43). It is consistent with all this that the gastrocnemius muscles of HFD-T2 rats were more insulin-sensitive than their HFD equivalents.

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ATP from ADP via the use of phosphocreatine and thus increased dependence of energy metabolism on the creatine kinase shuttle in glycolytic muscle.

Such structural and biochemical shifts seem to mimic the reported ability of T3 to convert skeletal muscle fiber type I to type II (45). Notably, however, the effects of T2, but not those of T3, are associated with a sparing of muscle mass. This finding indicates that T2 does not drive sarcopenia and argues strongly in favor of the potential utilization of T2, rather than T3, as a therapeutic option to counteract overweight and the associated metabolic derangements.

Another factor that appears to be affected by T2 treatment is fiber type-specific IMCL deposition. In particular, the ability of T2 to prevent HFD-induced lipid accumulation within glycolytic fibers, which has been shown to be an important factor contributing to IR (14), together with its ability to induce a shift toward a more glycolytic phenotype, strongly underlines the positive metabolic effects of this iodothyronine. Taken together, these results lead to its ability to limit or prevent the IR induced by HFD while lowering the serum levels of lipids. Further studies are needed to investigate whether chronic T2 treatment may affect insulin or glucagon secretion to fully address the mechanism by which T2 interferes with glucose homeostasis.

Very recently, the localization and organization of the lipid droplets within the cell have emerged as important qualitative aspects of intramuscular TG storage, together with the identity and properties of the associated proteins that can indeed account for the fate of lipids. ADRP is the predominant lipid droplet-associated protein in skeletal muscle, and a positive relationship between ADRP protein expression and insulin sensitivity has been reported in human skeletal muscle (36). This has led to the hypothesis that up-regulation of ADRP may serve to sequester fatty acids (TGs) within discrete lipid droplets, thus protecting muscle from the detrimental effects of fatty acids on the action of insulin and on glucose tolerance. However, a recent study by Minnaard et al. (37) reported a negative correlation between ADRP content and insulin-stimulated glucose uptake in gastrocnemius muscle from ZDF rats as well as in muscle from type 2 diabetic patients. Interestingly, our data extended ADRP localization to the fiber type, and indeed we demonstrated a specific presence of ADRP within fast/glycolytic fibers in the gastrocnemius muscle of HFD rats but predominantly within the slow/oxidative fibers in HFD-T2 rats. Because extrapolating from the relationship between muscle fiber type and ADRP protein expression in whole-muscle homogenates from rats to the situation in humans is an indirect approach, the discrepancies between the above-cited studies may be more apparent than real. Indeed, our results may indicate that the correlation between ADRP and insulin sensitivity relies on the fiber type localization of ADRP and at the same time on the muscle fiber type composition and enrichment. Because extensive data on ADRP fiber type localization and fiber type expression in all the conditions used in the above-mentioned studies have not yet been published (36, 37), the precise role played by ADRP in muscle lipid accumulation in situations of IR remains to be elucidated.

In summary, the work described here provides new insights into the effects of T2 on skeletal muscle metabolism in a situation in which T2 prevents systemic fat accumulation and liver steatosis by stimulating hepatic fatty acid oxidation without harmful effects on either lean muscle or heart mass. In so doing, the present study advances the case for a promotion of muscle glycolytic capacity having metabolic benefits.

This work was partially supported by the following grants: Ministero dell’Istruzione, dell’Università e della Ricerca (MIUR) Cofinanziamento (COFIN) 2008 Protocol 200808RS2X, MIUR Fondo Investimento Ricerca di Base (FIRB) Medical Research in Italy (MERIT) RBNE08YFN3_003, Regione Campania 2008, and Regione Campania Rete di Spettrometria di Massa.
REFERENCES


and SEQUEST analysis: the human proteome. *J. Proteome Res.* 4, 53–62

Received for publication February 14, 2011.
Accepted for publication June 2, 2011.