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# The saturation degree of fatty acids and their derived acylcarnitines determines the direct effect of metabolically active thyroid hormones on insulin sensitivity in skeletal muscle cells

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**ABSTRACT: Using differentiated rat L6 cells, we studied the direct effect of 3,5,3′-triiodo-L-thyronine (T3) and 3,5-diiodo-L-thyronine (T2) on the response to insulin in presence of fatty acids with a varying degree of saturation. We found that T3 and T2 both invert the response to insulin by modulating Akt Ser473 phosphorylation in the presence of palmitate and oleate. Both hormones prevented palmitate-induced insulin resistance, whereas increased insulin sensitivity in the presence of oleate was reduced, with normalization to (or, in the case of T3, even below) control levels. Both hormones effectively reduced intracellular acylcarnitine concentrations. Interestingly, insulin sensitization was lowered by incubation of the myotubes with relevant concentrations of palmitoylcarnitines (C16) and increased by oleylcarnitines and linoleylcarnitines (C18:1 and C18:2, respectively). The efficiency of mitochondrial respiration decreased in the order palmitate-oleate-linoleate; in the presence of palmitate, only T3 increased ATP synthesis–independent cellular respiration and mitochondrial respiratory complex activities. Both hormones modulated gene expression and enzyme activities related to insulin sensitivity, glucose metabolism, and lipid handling. Although T2 and T3 differentially regulated the expression of relevant genes involved in glucose metabolism, they equally stimulated related metabolic activities. T2 and T3 differentially modulated mitochondrial fatty acid uptake and oxidation in the presence of each fatty acid. The results show that T2 and T3 both invert the fatty acid–induced response to insulin but through different mechanisms, and that the outcome depends on the degree of saturation of the fatty acids and their derived acylcarnitines.—Giacco, A., delli Paoli, G., Senese, R., Cioffi, F., Silvestri, E., Moreno, M., Ruoppolo, M., Caterino, M., Costanzo, M., Lombardi, A., Goglia, F., Lanni, A., de Lange, P. The saturation degree of fatty acids and their derived acylcarnitines determines the direct effect of metabolically active thyroid hormones on insulin sensitivity in skeletal muscle cells. FASEB J. 33, 1811–1823 (2019). www.fasebj.org**

**KEY WORDS:** skeletal muscle · acylcarnitines · 3,5,3′-triiodo-L-thyronine · 3,5-diiodo-L-thyronine · insulin resistance

**ABBREVIATIONS:** BSA, bovine serum albumin; CPT, carnitine palmitoyltransferase; DMEM-F12, DMEM–Nutrient Mixture F-12; EPA, eicosapentaenoic acid; FAT/CD36, fatty acid transporter CD36; PFK, phosphofructokinase; PKM2, muscle pyruvate kinase; T2, 3,5-diiodo-L-thyronine; T3, 3,5,3′-triiodo-L-thyronine

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In rats, the effects of the classic thyroid hormone 3,5,3′-triiodo-L-thyronine (T3) and its emerging metabolically active metabolite 3,5-diiodo-L-thyronine (T2) include the improvement of skeletal muscle insulin sensitivity, which is associated with increased intramuscular PKB/Akt phosphorylation at Ser473 (1, 2). When injected intraperitoneally at a dose of 25 μg/100 g body weight into rats receiving a high-fat diet, the effect induced by T2 (2) is correlated with improved glycemia in response to insulin (3). Because skeletal muscle is the major site for insulin-dependent glucose uptake (4), reduced sensitivity to insulin in this organ, reflected by a less pronounced

phosphorylation of PKB/Akt, is an important index for hyperglycemia and thus diagnostic for type 2 diabetes mellitus.

The response to insulin in skeletal muscle is partially a consequence of cytokine signaling involving multiple organs such as liver and adipose tissue (5). Dietary fatty acids can increase (ω-6, arachidonic acid, and palmitic acid) or reduce [ω-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid] insulin resistance through their inflammatory action [as reviewed in Sears and Perry (5)]. *In vivo*, the effect of T2 and T3 on insulin sensitivity through phosphorylation of Akt in skeletal muscle (1, 2) is very likely the result of a multitude of metabolic changes induced in skeletal muscle as well as in liver (4, 6–10), and white (4, 6, 7, 10) and brown (10, 11) adipose tissue. It is unknown to what extent the *in vivo* effects of T2 and T3 described up to now in the literature are exerted by direct action of the thyroid hormones on the skeletal muscle cell itself.

Fatty acids can modulate insulin resistance by acting directly in the muscle cell: incomplete oxidation of palmitate and the resulting accumulation of palmitoylcarnitines in the cell and efflux from the cell upon lipid overload have been shown to induce insulin resistance in muscle cells (12, 13). Interestingly, using rat L6 myotubes, the direct effect of fatty acids on muscle cell insulin sensitivity has been shown to be related to the degree of saturation: palmitate, oleate, and linoleate have indeed been shown to differentially modulate Akt Ser473 phosphorylation, with palmitate being inhibitory and oleate and linoleate being significantly stimulatory (14). It is currently unknown whether and how the saturation grade of individual acylcarnitines relates to the response of insulin in the muscle cell.

In this context, mitochondria play a crucial role. Mitochondrial dysfunction is often responsible for the onset of skeletal muscle insulin resistance, as these organelles are mainly devoted to fatty acid oxidation. Because modifications in mitochondrial energetic efficiency are associated with changes in the amount of oxidized fuels, including fatty acids (15), studying respiratory parameters in the presence of fatty acids would increase our understanding as to why and how thyroid hormones modulate insulin resistance in a differential manner in various dietary contexts (4, 16). Mitochondrial dysfunction is linked to accumulation of intracellular acylcarnitines; in humans, accumulation of acylcarnitines due to incomplete long-chain fatty acid oxidation has been associated with the occurrence of type 2 diabetes mellitus (17).

In view of this information, coincubation of the fatty acids with T2 and T3 in myotubes would offer the possibility to study whether the effects of the aforementioned hormones on insulin sensitivity are directly exerted in the muscle cell and how these effects differ in the presence of each fatty acid. The goal of this study was to investigate the direct modulation of insulin signaling through Akt Ser473 phosphorylation by T2 and T3 in response to the presence of the aforementioned fatty acids in the muscle cell. We studied the association of intracellular acylcarnitine accumulation with their individual effect on insulin sensitivity, and assessed cellular respiration and mitochondrial respiratory complex activity. We furthermore investigated the expression/activity of several genes/enzymes involved in insulin signaling, glycolysis, and mitochondrial fatty acid transport and oxidation.

## MATERIALS AND METHODS

### Cell culture and treatment

Rat myoblastic L6C5 cells (a subline of the American Type Culture Collection line CRL 1458) were obtained from the Cell Bank (Interlab Cell Line Collection) of the National Institute for Cancer Research (Genoa, Italy) and cultured at early passage numbers in DMEM–Nutrient Mixture F-12 (DMEM-F12) containing 10% fetal bovine serum (HyClone, Logan, UT, USA).

Fatty acids (all from MilliporeSigma, Milan, Italy) palmitoylcarnitine (MilliporeSigma), oleylcarnitine (Santa Cruz Biotechnology, Dallas, TX, USA), and linoleylcarnitine (Chempex International, Inc., Wood Dale, IL, USA) were dissolved in ethanol to a final percentage of 0.5%, and T3 and T2 (MilliporeSigma) were dissolved in sodium hydroxide to a final concentration 600 nM. This concentration did not influence the pH of the cell culture medium. During the experiments, control cells were incubated in DMEM-F12 containing the appropriate concentrations of the aforementioned solvents.

L6 cells were grown to 75% confluence in DMEM-F12 containing 10% fetal bovine serum (HyClone) and differentiated in the same medium (differentiation medium) containing 2% fetal bovine serum for 5 d, when myotube formation became evident. Cells were then treated with fatty acids and/or thyroid hormones. To avoid the influence of the presence of endogenous fatty acids and thyroid hormones, during fatty acid/hormone treatment, the medium was changed to differentiation medium containing 2% dextran/charcoal-treated fetal bovine serum (HyClone); this treatment deprives the medium of fatty acids and hormonal residues (18). The medium was supplemented with 1% (w/v) fatty acid–free bovine serum albumin (BSA) (MilliporeSigma), with or without fatty acids [palmitate (0.5, 0.75, or 1.0 mM), oleate (0.75 mM), or linoleate (0.75 mM)] with or without T2 or T3 (100 nM), or their respective acylcarnitines: palmitoylcarnitine (C16), oleylcarnitine (C18:1), or linoleylcarnitine (C18:2, concentrations were 2.5, 5.0, or 10 μM). Control cells were incubated with the appropriate solvents used to dissolve the compounds (for fatty acids and acylcarnitines: ethanol final concentration, 0.5 and 0.05%, respectively; for thyroid hormones: sodium hydroxide final concentration, 5 nM). The used percentage of fatty acid–free BSA together with the BSA present in the charcoal-treated serum resulted in FFA/albumin molar ratios of 3.1, 4.7, or 6.3 in the presence of 0.5, 0.75, or 1.0 mM fatty acids, respectively [calculations based on those published in Oliveira *et al.* (19)]. Treated cells were incubated for 16 h. For experiments with insulin, cells were serum starved for 2 h and then treated for 15 min with 20 nM bovine insulin (MilliporeSigma).

### Western immunoblot analysis

Akt and phosphorylated Akt (Ser473), AMPK and phosphorylated AMPK (Thr172), and caspase-3 (35 and 19 kDa) protein levels were determined in cell lysates by using specific pAb (Cell Signaling Technology, Danvers, MA, USA). Sarcolemmal fatty acid transporter CD36 (FAT/CD36) levels were determined by using a pAb (Abcam, Cambridge, United Kingdom). The β-actin antibody was from MilliporeSigma.

### Gene expression analysis

Total RNA was extracted from cells treated for 16 h with 0.75 mM fatty acids with or without thyroid hormones (100 nM). RNA

extraction and qPCR analysis were performed as previously described (20). Primers used for gene expression analysis were the following: P13KP85α S:5′-ATTCAGTGATGGCCAGGAAG-3′, AS: 5′-GTCTCCCCTCTCCCCAGTAG-3′; β-ACT S: 5′-GC-TACAGCTTCACCACCACA-3′, AS: 5′-AGGGCAACATAGC-ACAGCTT-3′; carnitine palmitoyltransferase (CPT) 1b S: 5′-ATCGAACGTGCTGCTTTCTT-3′, AS: 5′-ATTTGCCGTAGA-GGCTGAGA-3′; CPT2 S: 5′-GCAATGAGGAAACCCTGAAG-3′, AS: 5′-GATCCTTCATCGGGAAGTCA-3′; phosphofructokinase (PFK) S: 5′-CTGACACAGCACTGAACACC-3′, AS: 5′-TGATAAACACTCGCCGCTTG-3′; muscle pyruvate kinase (PKM2) S: 5′-GGACATGGTGTTTGCGTCTT-3′, AS: 5′-TTCT-TGCCCTTCTCTCCCAG-3′; MYOG S: 5′-CCTGCCCTGA-GATGAGAGAG-3′, AS: 5′-GCACTGGAAGGTTCCCAATA-3′.

### Determination of cellular oxygen consumption

Cells were resuspended in differentiation medium containing 2% dextran/charcoal-treated fetal bovine serum (HyClone). Cellular respiration was measured by using an Oxygraph 2K respirometer (Oroboros Instruments, Innsbruck, Austria). Sampling intervals of 1 s were elaborated by using the DatLab 6 software designed for the respirometer (Oroboros Instruments). Zero calibration was performed by adding Na-dithionite to the medium, and instrumental background was corrected by elaborating the values obtained with Na-dithionite and open-chamber oxygen flux in the presence of the medium. After calibration, the medium was replaced by 2 ml of 0.5, 1.0, and 2.0 × $10^6$ (cells/ml) untreated cells, respectively; with closed chambers, linearity of basal respiration was verified and confirmed (data not shown). Subsequently, respiratory parameters of cell suspensions (1.0 × $10^6$ cells/ml) from each treatment were tested. After measuring basal respiration, cells were exposed sequentially to oligomycin (2.5 μM), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (0.2 μM), and rotenone/myxothiazol. Nonmitochondrial respiration, obtained after the final addition, was subtracted from the other values. Basal respiration, ATP synthesis–independent respiration, coupling efficiency, maximum respiration rate, respiratory control ratio, and spare respiratory capacity were determined as reported in Brand *et al*. (21).

### Acylcarnitine measurement

Intracellular acylcarnitine concentrations were measured both in cellular pellets and lysates. The cellular pellets were directly lysed in cold methanol by using a dounce homogenizer. The extracts were centrifuged at 12,000 rpm at 4°C for 30 min. The pellets were resuspended in resuspension buffer {7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate, Tris-hydrochloride 30 mM, and pH 7.5}, and protein abundance was measured (22). The supernatants were dried under nitrogen flow at 50°C.

To quantify acylcarnitines from cellular lysates, 15 μl of each sample was loaded on SS903 grade paper and dried. Metabolites were extracted from 3.2 mm of dried spot using methanol. Acylcarnitines were dissolved in esterifying buffer (3 N hydrochloric acid/*n*-butanol) at 65°C for 25 min. The samples were

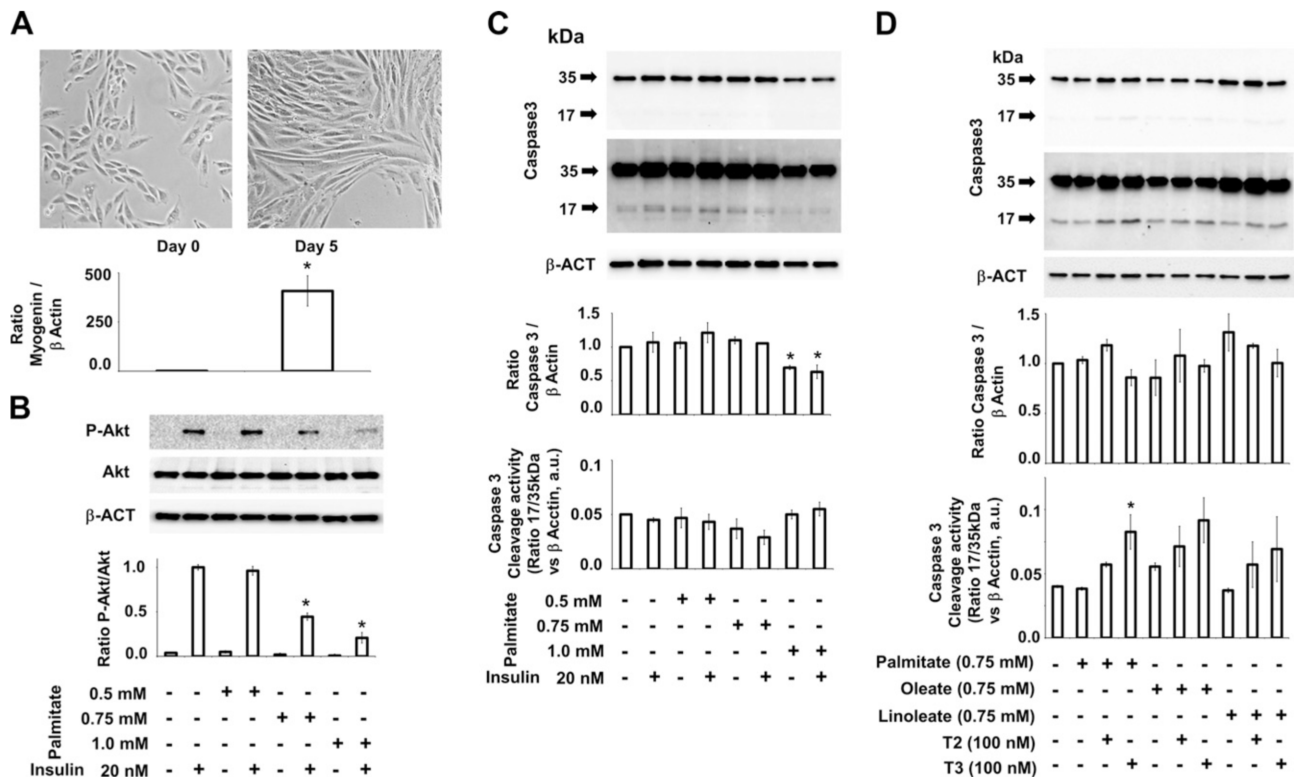



**Figure 1.** Effectiveness of myoblast differentiation and determination of the effect of fatty acids and thyroid hormones on apoptosis. *A*) Myotube formation at 5 d and myogenin mRNA levels. *B–D*) Effect of incubation with palmitate at the indicated concentrations on Akt Ser473 phosphorylation (*B*) and caspase-3 activity (*C*), effect of incubation with palmitate, oleate, and linoleate in the absence and presence of T2 or T3 at the indicated concentrations on caspase-3 cleavage (*D*). Shown are 2 different exposures of the same filter, to reveal the low signal representing the cleaved (17 kDa) caspase-3 band (*C*, *D*). Data are shown as means ± SEM. *$P < 0.05$ insulin-treated palmitate-incubated *vs.* insulin-treated control cells (*B*), *$P < 0.05$ palmitate-incubated *vs.* control cells, in absence or presence of insulin (*C*). *$P < 0.05$ T3-treated, palmitate-incubated *vs.* control cells (*D*).

dried under nitrogen at 50°C and then resuspended in 300 μl of acetonitrile/water (70:30) containing 0.05% formic acid (23). Three independent aliquots of the sample (100 μl) were injected in the API 4000 triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled with an Agilent HPLC system (Agilent Technologies, Waldbronn, Germany). The samples were run using precursor ion scan mode (24). Quantitative analyses were performed by using ChemoView v.1.2 software (Sciex, Framingham, MA, USA). The protein content of cellular lysates was measured. Final acylcarnitine concentrations, measured both in cellular lysates and pellets, were calculated based on 1 mg protein from the cellular extract.

### Measurement of mitochondrial respiratory complex activity

Separation of membrane and mitochondrial fractions, and evaluation of the electrophoretic profiles of mitochondrial oxidative phosphorylation complexes, blue-native PAGE staining, densitometric quantification, and complex activity, were performed as previously described (25, 26). The membrane fractions were used for the measurement of sarcolemmal FAT/CD36 protein levels.

### Measurement of CPT activity

Total CPT (CPT1 plus CPT2) activity was measured spectrophotometrically by following (at 450 nm) the kinetics of carnitine-dependent coenzyme A production in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) using palmitoyl–coenzyme A as substrate, as previously reported (6). For the calculation of total CPT activity, a Σ450 value of 8.26 mM was considered, as reported in Eyer *et al*. (27).

### Measurement of PFK activity

PFK was assayed by using a method adapted from that described by Opie and Newsholme (28) as previously reported (1). The assay was initiated by the addition of 3 mm fructose 6-phosphate. Controls, from which fructose 6-phosphate was omitted, were run concurrently.

### Statistical analysis

Data are expressed as means ± SEM. Statistical differences of normally distributed data between 2 treatments were determined by using an unpaired Student's *t* test or, for more treatments, by 1-way ANOVA (*post hoc* test: Student-Newman-Keuls), by using Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Differences between treatments were considered significant at a value of $P < 0.05$.

## RESULTS

### Modulation of insulin sensitivity in myotubes by T2 and T3 varies in the presence of palmitate, oleate, and linoleate

We first determined the optimal experimental conditions. Differentiation of the myoblasts after 5 d was pronounced, reflected by myotube formation and a strong increase in myogenin expression (**Fig. 1*A***). We tested the optimal concentration of palmitate to induce insulin resistance (Fig. 1*B*) without significantly inducing apoptosis (Fig. 1*C*). Because incubation with 0.75 mM palmitate caused a drop in Akt phosphorylation at Ser473, but did not cause decreased caspase-3 expression (35 kDa) or an increased formation of the cleaved 17 kDa caspase-3 product, we studied the response to insulin using this concentration with all fatty acids to be tested. Incubation with fatty acids at the concentration of 0.75 mM did not change the expression of caspase-3 (35 kDa) nor induce cleavage to the 17 kDa product, whereas T2 and T3 both induced cleavage, albeit only significantly in the case of T3 in the presence of palmitate (Fig. 1*D*). This induction caused a caspase activity below 0.1 (arbitrary units), which indicates nonsignificant induction of apoptosis.

We next determined to what extent T2 and T3 modulated fatty acid–induced Akt Ser473 phosphorylation. Both T2 and T3 (each hormone tested at a 100 nM

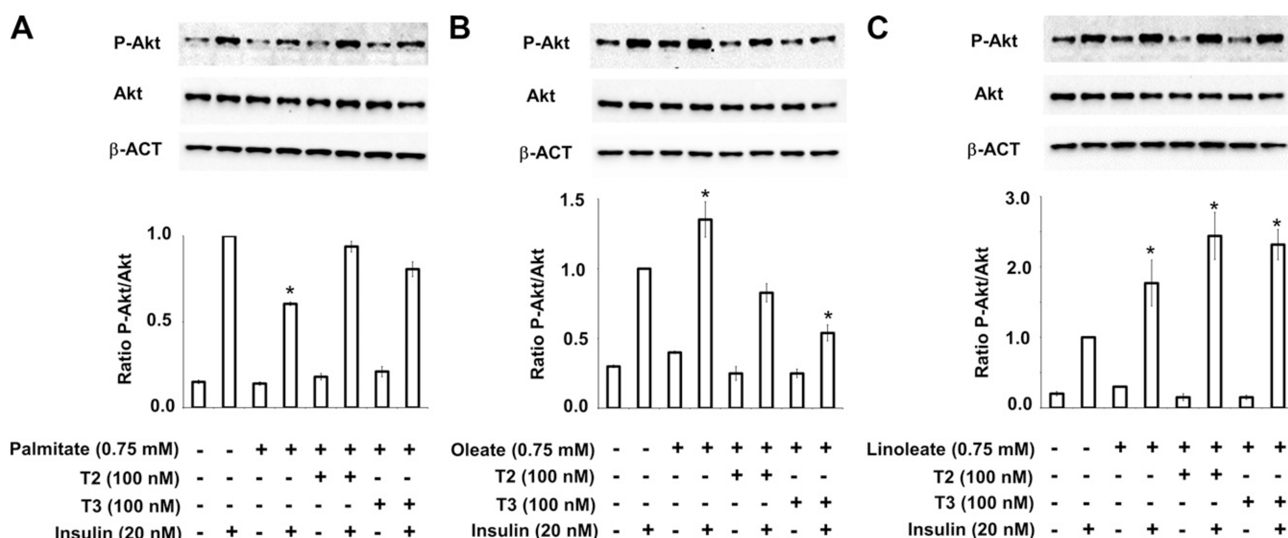



**Figure 2.** Fatty acids differentially modulate Akt Ser473 phosphorylation, being in turn differentially modulated by T2 and T3 in rat L6 myotubes. Cells were incubated with T2 or T3 in the presence of palmitate (*A*), oleate (*B*), and linoleate (*C*) at the indicated concentrations. Data are shown as means ± SEM. *$P < 0.05$, insulin-treated, hormone and/or fatty acid-incubated *vs.* insulin-treated control cells.

concentration) ameliorated palmitate-induced insulin resistance reflected by an increase in the phospho-Akt/total Akt ratio (**Fig. 2*A***); T2 was more efficient. Oleate significantly improved insulin sensitivity by increasing the phospho-Akt/total Akt ratio by 1.4-fold (Fig. 2*B*), and, interestingly, T2 normalized the insulin response (reducing the phospho-Akt/total Akt ratio to control levels), whereas T3 even reduced it. The marked (almost 2-fold) increase in insulin sensitivity caused by linoleate tended to be enhanced by T2 and T3, albeit not significantly with respect to linoleate alone (Fig. 2*C*).

## T2 and T3 both decrease intracellular acylcarnitine levels, and the degree of saturation of intracellular acylcarnitines determines the resulting effect on insulin sensitization

In the presence of palmitate, palmitoylcarnitine (C16) accumulated substantially in the cell; oleylcarnitine accumulated less so in the cells treated with oleate (C18:1). In the linoleate-treated cells, intracellular accumulation of linoleylcarnitine (C18:2) barely reached significance (**Fig. 3*A*–*C***). T2 and T3 reduced intracellular acylcarnitine levels of all tested fatty acids. To test if modulation of intracellular acylcarnitine levels could be at least partially responsible for the ability of T2 and T3 to influence insulin sensitivity, which was improved in the presence of palmitate but worsened in the presence of oleate (Fig. 2), we evaluated whether addition of the maximal physiologic concentration of the acylcarnitines themselves [5.0 μM, as obtained in humans (23)] would affect the cells' response to insulin. Indeed, we found that incubation of the cells with 5.0 μM palmitoylcarnitine (C16) resulted in a decreased Akt phosphorylation at Ser473, whereas the opposite occurred upon incubation with 5.0 μM oleylcarnitine (C18:1) and 5.0 μM linoleylcarnitine (C18:2) (**Fig. 4*A***, 5.0 μM panel). Using this concentration, the intracellular concentrations of the acylcarnitines did not significantly differ from those found

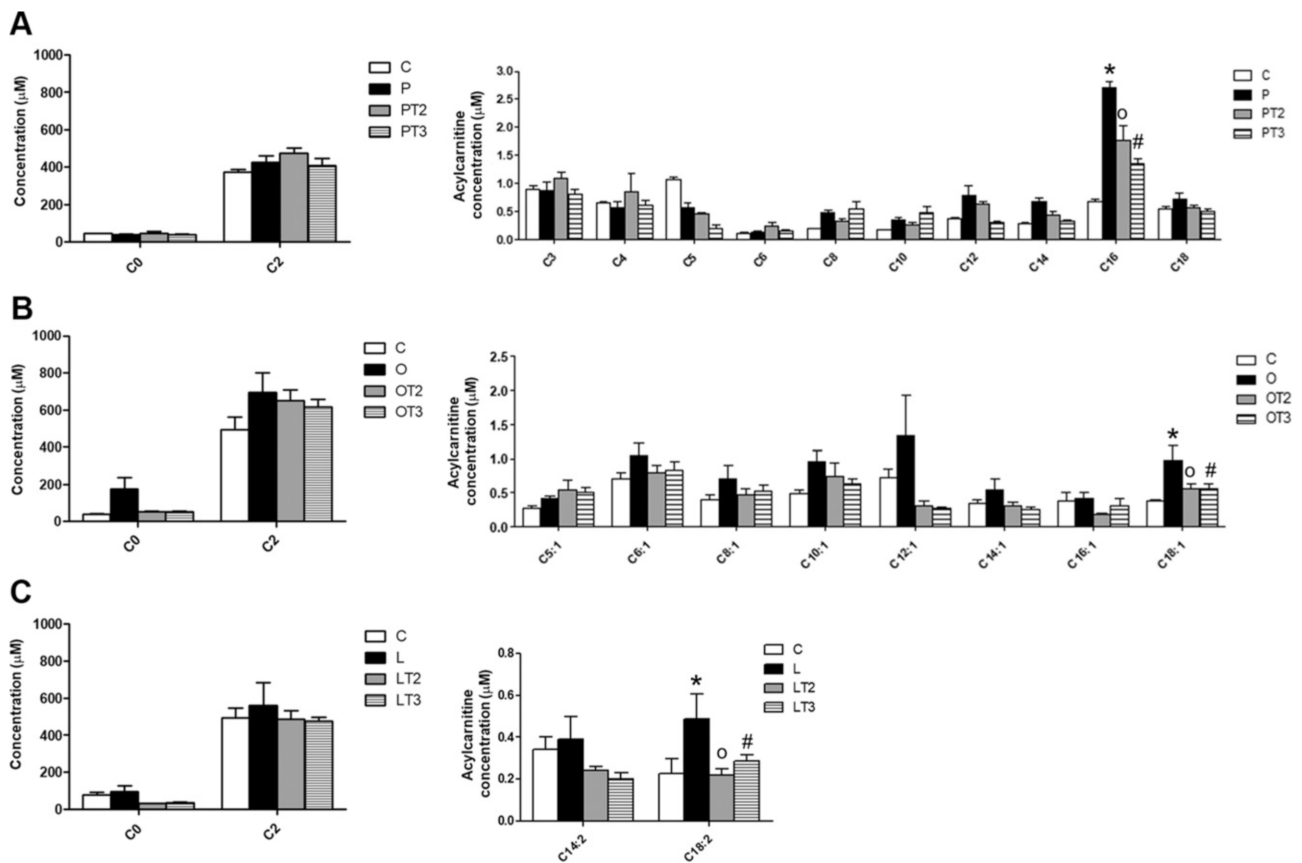



**Figure 3.** Intracellular acylcarnitine accumulation decreases in the presence of T2 and T3. Cells were incubated with T2 or T3 in the presence of palmitate (*A*), oleate (*B*), and linoleate (*C*) (all 0.75 mM). Acylcarnitine concentrations were calculated based on 1 mg protein from the cellular extract. C0, free carnitine; C2, acetylcarnitine; C3, propionylcarnitine; C4, butyrylcarnitine/isobutyrylcarnitine; C5, isovalerylcarnitine/2-methylbutyrylcarnitine; C6, hexanoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C16, palmitoylcarnitine/hexadacanoylcarnitine; C18, octadecanoylcarnitine; C5:1, tiglylcarnitine; C6:1, hexenoylcarnitine; C8:1, octenoylcarnitine; C10:1, decenoylcarnitine; C12:1, dodecenoylcarnitine; C14:1, tetradecenoylcarnitine; C14:2, tetradecadienoylcarnitine; C16:1, hexadecenoylcarnitine; C18:1, oleylcarnitines/octadecenoylcarnitine; C18:2, linoleylcarnitines/octadecadienoylcarnitine; C, control cells incubated with solvents of the various treatments. Further abbreviations indicate: cells incubated with palmitate (P), palmitate and T2 (PT2), palmitate and T3 (PT3), oleate (O), oleate and T2 (OT2), oleate and T3 (OT3), linoleate (L), linoleate and T2 (LT2), or linoleate and T3 (LT3). Data are shown as means ± SEM. *$P < 0.05$, fatty acid-incubated *vs.* control cells, $P < 0.05$, fatty acid–incubated, and °T2-incubated or #T3-incubated *vs.* fatty acid–incubated cells.

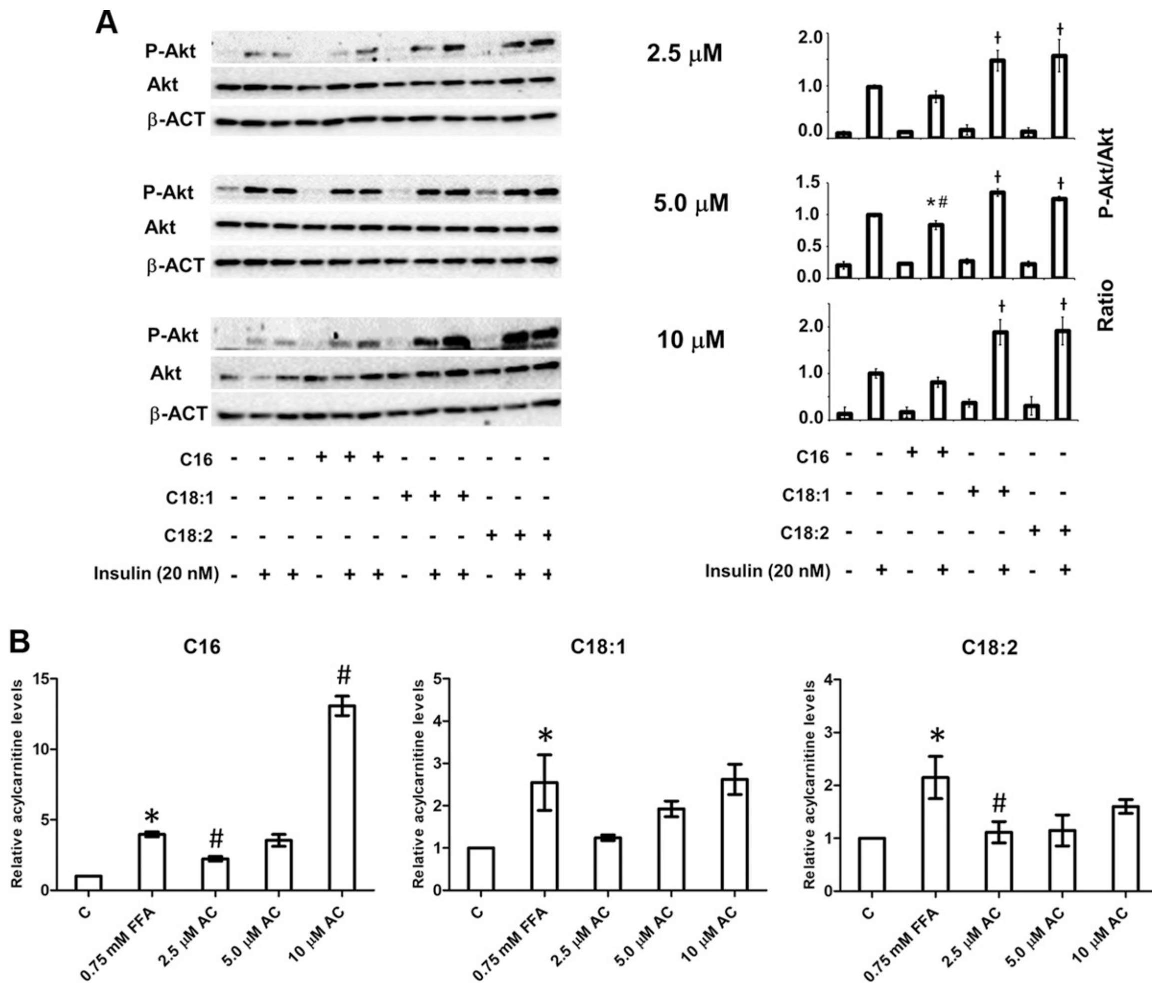



**Figure 4.** Intracellular acylcarnitines differentially affect muscle insulin resistance. *A*) Akt phosphorylation at Ser473 in cells after incubation with 2.5, 5.0, or 10 μM acylcarnitines. *B*) Comparison of relative intracellular acylcarnitine levels measured in cellular extracts used in (*A*) with those derived from incubation with 0.75 mM FFA (Fig. 3). Data are shown as means ± SEM. AC, acylcarnitine; FFA, free fatty acid. *A*) $^{*}P < 0.05$ C16-incubated *vs.* control cells, $^{\#}P < 0.05$ *vs.* all other acylcarnitines, $^{\dagger}P < 0.05$ *vs.* control cells and palmitoylcarnitine-incubated cells. *B*) $^{*}P < 0.05$ *vs.* C; $^{\#}P < 0.05$ *vs.* 0.75 mM FFA.

when cells were loaded with the functional concentration of the respective fatty acids (0.75 mM) (Fig. 4*B*). We next studied the effect of halving the acylcarnitine concentration to 2.5 μM on the modulation of Akt phosphorylation at Ser473. At this concentration, the palmitoylcarnitines failed to suppress Akt phosphorylation, whereas both oleylcarnitines and linoleylcarnitines at this concentration retained their stimulatory effect (Fig. 4*A*, 2.5 μM panel). Loading the cells with 10 μM of the unsaturated acylcarnitines doubled Akt phosphorylation, whereas significant suppression of Akt phosphorylation by palmitoylcarnitines failed to be achieved at this concentration (Fig. 4*A*, 10 μM panel). Intracellular acylcarnitine concentrations after incubation with 2.5 and 10 μM palmitoylcarnitines were halved and tripled, respectively, relative to the intracellular concentrations measured after incubation with 5.0 μM palmitoylcarnitines or 0.75 mM palmitate (Fig. 4*B*, panel C16). Linearity was maintained with oleylcarnitines, although at 2.5 μM and 10 μM, their levels were not significantly lower, or higher, respectively, compared with those derived from 0.75 mM oleate (Fig. 4*B*, panel C18:1). No linearity was observed with linoleylcarnitines, although their levels were significantly reduced at 2.5 μM with respect to those derived from incubation with 0.75 mM linoleate (Fig. 4*B*, panel C18:2).

## Palmitate, oleate, and linoleate increasingly stimulate AMPK phosphorylation, being unaltered by T2 and enhanced by T3 in the presence of oleate

To investigate how T2 and T3 brought about their modulating effect on insulin sensitivity, we next determined how AMPK phosphorylation varied under the different experimental conditions. AMPK phosphorylation was progressively enhanced by palmitate, oleate, and linoleate (by ~1.5, 2.0, and 5.0-fold, respectively) (**Fig. 5*A*–*C***). Neither T2 and T3 nor insulin significantly altered AMPK phosphorylation; although in the presence of oleate, T3 enhanced the P-AMPK/AMPK ratio due to a slight decrease in total AMPK protein levels (Fig. 5*B*). In the presence of linoleate, insulin reduced AMPK phosphorylation throughout, but this change did not reach statistical significance.

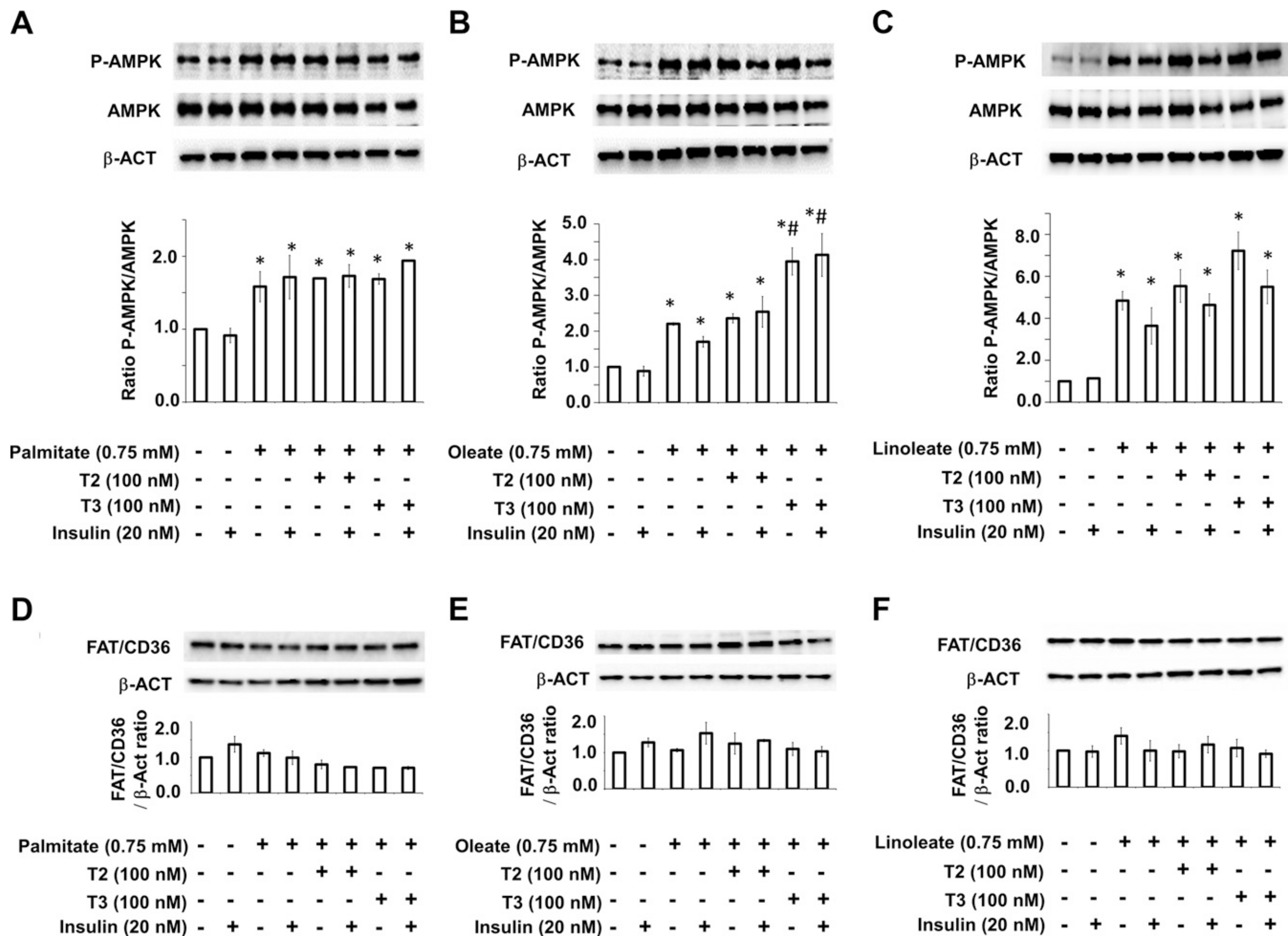
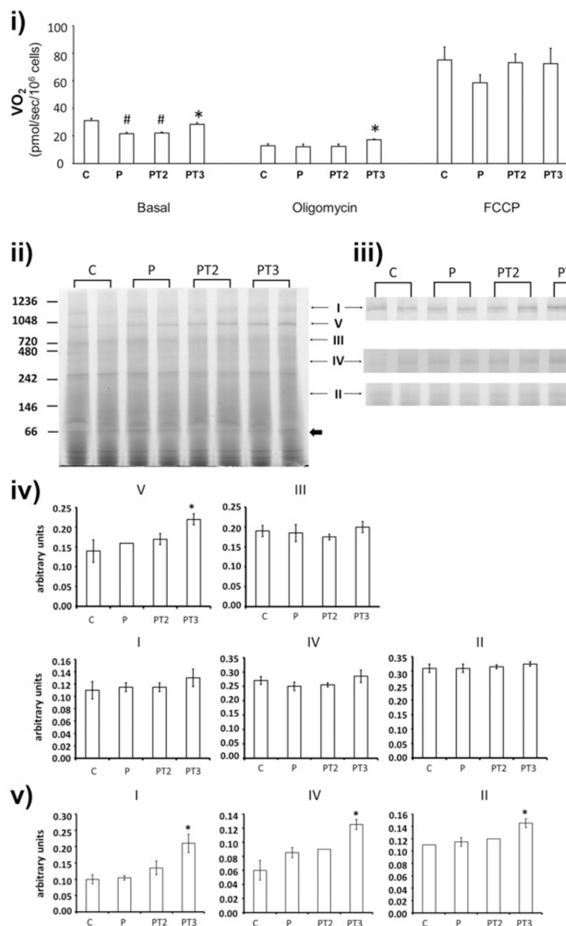


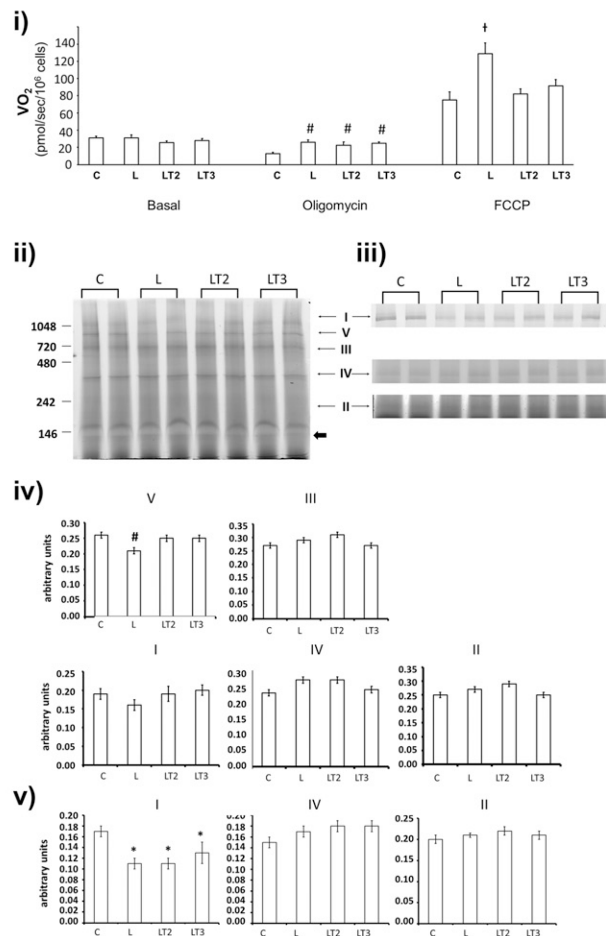

**Figure 5.** Phosphorylation of AMPK at Thr172 in rat L6 myotubes is increasingly stimulated by fatty acids with a lower saturation grade and is modulated by T3 only in the presence of oleate, whereas FAT/CD36 accumulation in the sarcolemma is not altered in any of the tested conditions. Cells were incubated with T2 or T3 in the presence of palmitate (*A*, *D*), oleate (*B*, *E*), and linoleate (*C*, *F*) at the indicated concentrations. Data are shown as means ± SEM. $^*P < 0.05$, fatty acid and fatty acid with hormone-incubated cells, in absence or presence of insulin, *vs.* control cells; $^\#P < 0.05$, oleate and T3-incubated *vs.* oleate-incubated cells, in the absence or presence of insulin.

The treatments of the cells (absence or presence of fatty acids, hormones, and/or insulin) did not significantly alter sarcolemmal FAT/CD36 accumulation (Fig. 5*D–F*).

## Cellular respiration and mitochondrial respiratory complex activity is differentially modulated by the fatty acids, T2, and T3

Basal cellular respiration was inhibited by palmitate but not by oleate and linoleate (**Fig. 6*Ai, Bi, Ci***). The reduced cellular respiration in the presence of palmitate is attributable to a lower mitochondrial ATP synthesis, as can be estimated from the decrease in respiration in the presence of oligomycin. Mitochondrial respiration was only normalized by T3, plausibly through increased activity of mitochondrial respiratory complexes I, II, and IV (Fig. 6*Av, Bv*, palmitate panel) and increased ATP synthesis–independent respiration, revealed in the presence of oligomycin (Fig. 6*Ai, Bi, Ci*, palmitate panel). In the presence of palmitate, only T3 significantly reduced coupling efficiency and the respiratory control ratio with respect to controls (**Table 1**). Although the action of T2 did not include significant ameliorations in mitochondrial activity, T2 did tend to normalize the spare respiratory capacity (representing the ability of substrate supply and electron transport to respond to an increase in energy demand) with respect to palmitate (Table 1). Oleate moderately increased ATP synthesis–independent respiration (Fig. 6*Ci*), accounting for ~50% of the basal respiration rate. This parameter was unaltered in the presence of T2 and T3, as were the activities of mitochondrial complexes (histograms not shown). In the presence of oleate, either alone or in combination with T3, the respiratory control ratio was decreased with respect to the controls (Table 1). In the presence of linoleate, ATP synthesis–independent respiration was increased to account for ~75% of the basal respiration rate, not being altered in presence of T2 and T3 (Fig. 6*Bi*). Indeed, linoleate alone or in the presence of either T2 or T3 decreased the respiratory control ratio with respect to controls. Complex I activity was reduced by linoleate, and T2 and T3 did not influence this action (Fig. 6*Bv*). The highly reduced mitochondrial energetic efficiency in the myotubes induced by linoleate compensates



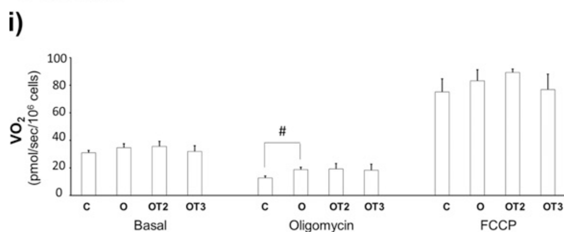




**Figure 6.** Fatty acids and T2 and T3 differentially modulate mitochondrial respiratory complex activity and ATP-independent respiration. Cells were incubated with T2 or T3 in the presence of palmitate, oleate, and linoleate (indicated near the panels) at the concentrations as indicated in Figs. 2 and 4. *Ai, Bi, Ci*) Respiration measured in intact cells. Data are shown as means ± SEM. $*P < 0.05$, palmitate and T3-incubated *vs.* palmitate cells; $^{\#}P < 0.05$, fatty acid or fatty acid and hormone-treated cells *vs.* control cells for each panel; $^{\dagger}P < 0.05$, linoleate-incubated *vs.* all other conditions. In presence of oligomycin, ATP synthesis-independent respiration is measured, whereas in presence of FCCP maximal uncoupled respiration is measured (*Aii, iii, Bii, iii, Cii, iii*). Measurement of intra-mitochondrial respiratory complex abundance levels *Aii, Bii, Cii* and activity *Aiii, Biii, Ciii* in isolated mitochondria. Capital Roman numerals indicate the various mitochondrial complex numbers. *Aiv, Biv*) Histograms of the complex abundance. *Av, Bv*) Histograms of complex activity. Palmitate panel: $*P < 0.05$, palmitate and T3-incubated cells *vs.* all other conditions. Linoleate panel: $^{\#}P < 0.05$, linoleate-incubated cells *vs.* all other experimental conditions. $*P < 0.05$, all experimental conditions *vs.* control cells.

impairment of respiratory chain activity reflected by a decrease in complex I presence and activity. This compensatory effect guarantees maintenance of high mitochondrial oxidation levels. The induction of maximal uncoupled respiration by linoleate was restored to control levels by T2 and T3 (Fig. 6*Bi*); as a result, spare respiratory capacity was significantly reduced with respect to that observed in the presence of linoleate alone (Table 1).

Expression of the p85 subunit of PI3K, PFK, PKM2, PFK enzyme activity, CPT1b/CPT2 ratios, and CPT enzyme activity are differentially influenced by the fatty acids and by T2 and T3.

We studied how T2 and T3 influenced the expression of genes and the activity of enzymes involved in cellular metabolism related to insulin sensitivity. Palmitate increased the expression of the p85 subunit of PI3K but T2

TABLE 1. *Effect of coincubation of cells with various fatty acids in the presence and absence of T2 and T3 on respiratory parameters in L6 myotubes*

| Variable | C | P | PT2 | PT3 | O | OT2 | OT3 | L | LT2 | LT3 |
|---|---|---|---|---|---|---|---|---|---|---|
| Coupling efficiency | 0.63 ± 0.04 | 0.43 ± 0.08 | 0.44 ± 0.09 | 0.39* ± 0.04 | 0.50 ± 0.04 | 0.53 ± 0.07 | 0.44 ± 0.03 | 0.24† ± 0.04 | 0.13† ± 0.05 | 0.13† ± 0.03 |
| Respiratory control ratio | 6.39 ± 0.31 | 5.2 ± 1.39 | 6.33 ± 1.32 | 4.23* ± 0.70 | 4.37* ± 0.54 | 4.97 ± 1.07 | 4.14* ± 0.24 | 4.58† ± 0.02 | 3.66† ± 0.17 | 3.74† ± 0.23 |
| Spare respiratory capacity | 43.53 ± 11.88 | 37.44 ± 5.77 | 50.11 ± 7.13 | 44.16 ± 9.80 | 45.18 ± 5.97 | 48.90 ± 1.85 | 43.20 ± 5.08 | 91.26 ± 13.86 | 57.01‡ ± 3.84 | 62.69‡ ± 5.02 |

C, carnitine; L, linoleate; O, oleate; P, palmitate. Values are based on absolute respiratory values measured as depicted in Fig. 6*Ai*, *Bi*, *Ci* (pmol/s/$10^6$ cells). *$P < 0.05$ *vs.* C, †$P < 0.05$ *vs.* C, ‡$P < 0.05$ *vs.* L.

and T3 reduced it; in all other treatments, the expression of the p85 subunit of PI3K was consistently reduced with respect to untreated controls (**Fig. 7*A***). We also determined the expression of 2 key genes in glucose metabolism, namely PFK and PKM2. The presence of palmitate reduced the expression of PFK, and T2 and T3 did not affect this. In the presence of oleate, expression of both genes was stimulated only by T2 (Fig. 7*B*). PFK enzyme activity doubled in the presence of all fatty acids, being further stimulated by T2 and T3; although, despite significantly increasing PFK expression (Fig. 7*C*), T2 only tended to increase PFK activity. CPT1b expression was reduced in the presence of palmitate and linoleate and remained unaltered while being increased by T2 and T3 in the presence of oleate (Fig. 7*D*). CPT2 expression was decreased by all fatty acids. Interestingly, the CPT1b/CPT2 ratio (inversely correlating with fatty acid transport and oxidation efficiency) was increased in the presence of palmitate and oleate but not linoleate. In the presence of palmitate, the CPT1b/CPT2 ratio was normalized only by T2. CPT enzyme activity significantly decreased by ~20% in the presence of palmitate, and only T2 prevented this drop (Fig. 7*E*). Oleate and linoleate did not affect CPT activity, and this outcome was not altered by coincubation with T2 or T3.

## DISCUSSION

To our knowledge, this study is the first to show that the direct effects of T2 and T3 on the insulin sensitivity of muscle cells are related to the degree of saturation of fatty acids and intracellular fatty acylcarnitines, and that both hormones exert these effects in a differential manner. In the presence of palmitate and oleate, both T2 and T3 substantially completed fatty acid oxidation, judged by a decrease in intracellular acylcarnitine accumulation, which in both cases was associated with an inversion of the response to insulin. In particular, palmitate-induced insulin resistance was prevented and oleate-induced insulin sensitivity was abolished. This scenario is in line with the recent observation that patients with type 2 diabetes exhibit relatively high fasting serum C16:0 levels (29), whereas a separate study linked a decline in serum oleylcarnitines (C18:1) to type 2 diabetes (30).

In this context, the question that has arisen in the literature is whether intracellular acylcarnitines are merely markers of insulin resistance or directly involved in the modulation of insulin sensitivity [reviewed in Schooneman *et al.* (31)]. It has previously been shown that physiologic concentrations of intracellular palmitoylcarnitines are directly involved in the modulation of insulin sensitivity in mouse C2C12 cells (13). Our results obtained in rat L6 cells confirm this observation: loading the cells with physiologic concentrations of palmitoylcarnitine [5.0 μM, representing the maximal concentration observed in humans (23)] reduces muscle cell insulin sensitivity. Because we observed that at this concentration the intracellular palmitoylcarnitine levels were comparable to those observed after loading the cells with the functional dose (0.75 mM) of the corresponding fatty acids, as expected,

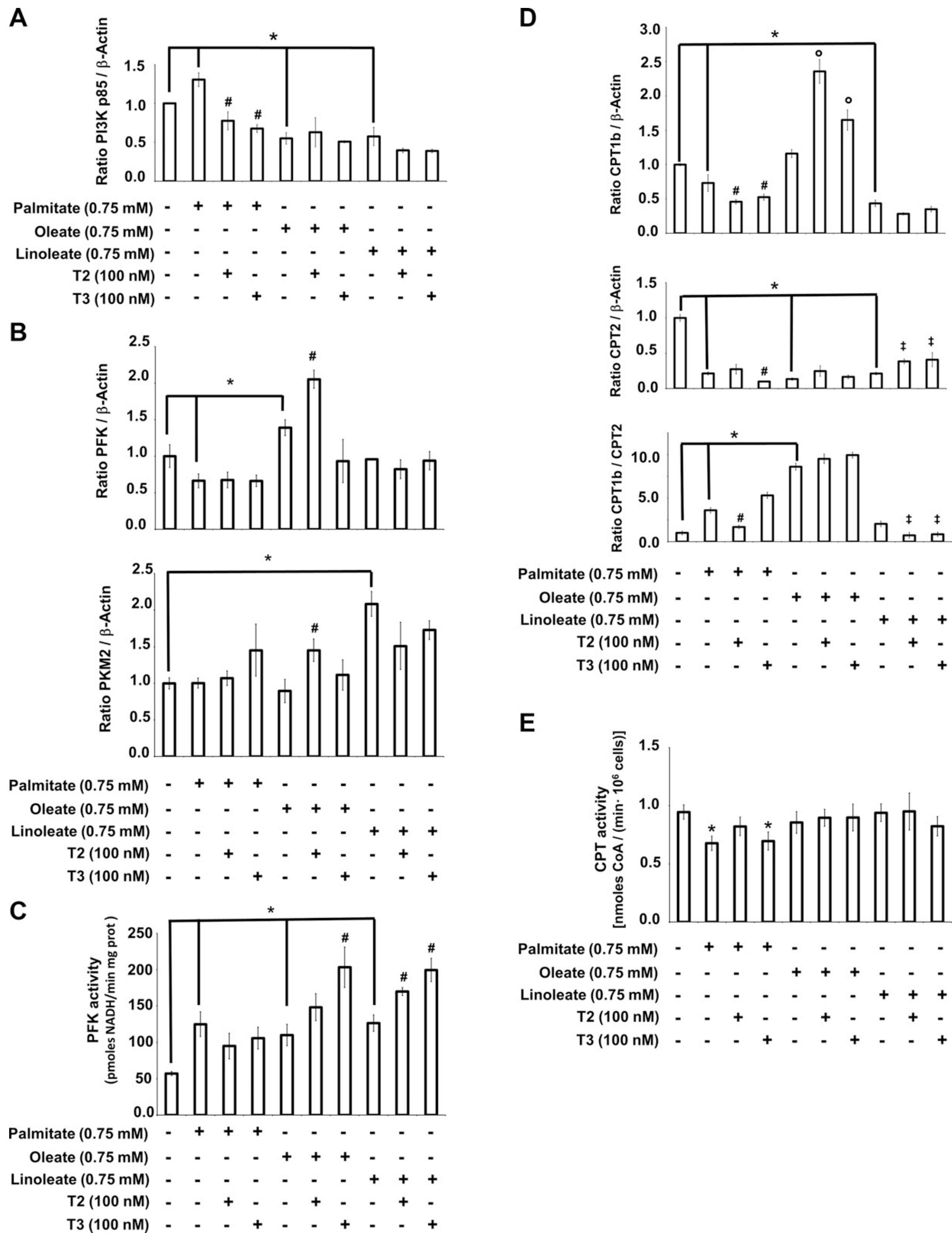



**Figure 7.** Both T2 and T3 normalize expression of the p85 subunit of P13K; T2 normalizes the CPT1b/CPT2 ratio and CPT activity in the presence of palmitate; only T2 increases the expression of PFK and PKM2 in the presence of oleate in L6 myotubes, whereas in the presence of each fatty acid, PFK activity is increased by both hormones. Cells were incubated with T2 or T3 in the presence of palmitate, oleate, and linoleate at the indicated concentrations. Data are shown as means ± SEM. $^{*}P < 0.05$, fatty acid–incubated *vs.* control cells; $^{\#}P < 0.05$, fatty acid–incubated and T2- or T3-incubated *vs.* fatty acid–incubated cells (*A, C*); $^{*}P < 0.05$, fatty acid–incubated *vs.* control cells; $^{\#}P < 0.05$, oleate and T2-incubated *vs.* oleate-incubated cells (*B*). $^{*}P < 0.05$, fatty acid–incubated *vs.* control cells; $^{\#,\bigcirc,‡}P < 0.05$, fatty acid–incubated and T2- or T3-incubated *vs.* fatty acid–incubated cells, palmitate (#), oleate (○), and linoleate (‡) (*D*). $^{*}P < 0.05$, palmitate and palmitate/T3-incubated cells *vs.* control cells (*E*).

loading the cells with one half this concentration (mimicking, as demonstrated here, the intracellular palmitoylcarnitine concentrations found in the presence of T2 and T3) abolished the effect of the palmitoylcarnitines on Akt phosphorylation. In the previously cited study, it was also shown that the significant suppression of Akt phosphorylation was not achieved when supraphysiological concentrations of the palmitoylcarnitine were used (13). Our results confirm this observation, and because incubation with 1.0 mM palmitate resulted in apoptosis, it is unlikely that intracellular palmitoylcarnitine concentrations higher than those that derive from 0.75 mM palmitate can be reached, at least in rat L6 cells. In addition, we have shown here that incubation with physiologic concentrations (5.0 μM) of oleylcarnitine and linoleylcarnitine increase muscle cell insulin sensitivity, being further increased at the 10 μM concentration. At lowered concentrations (2.5 μM), oleylcarnitines and linoleylcarnitines continued to be insulin sensitizing. In agreement, upon coincubation with 0.75 mM of the related fatty acids with T2 and T3, resulting in a significant reduction of the intracellular acylcarnitine levels, linoleate continued to be insulin sensitizing, but, seemingly in contrast, the significantly reduced oleylcarnitine levels caused a loss of insulin sensitivity. Of note, upon addition of 2.5 μM oleylcarnitines, intracellular oleylcarnitine levels were not significantly different compared with those derived from the addition of 0.75 mM oleate, although they tended to be reduced. This suggests that only a substantial lowering of intracellular oleylcarnitine levels (as observed after incubation with T2 and T3) provokes decreased insulin sensitivity.

In the absence of hormones, the decrease of insulin sensitivity by palmitate and the increase by oleate and linoleate correlated with the following: *1*) decreased

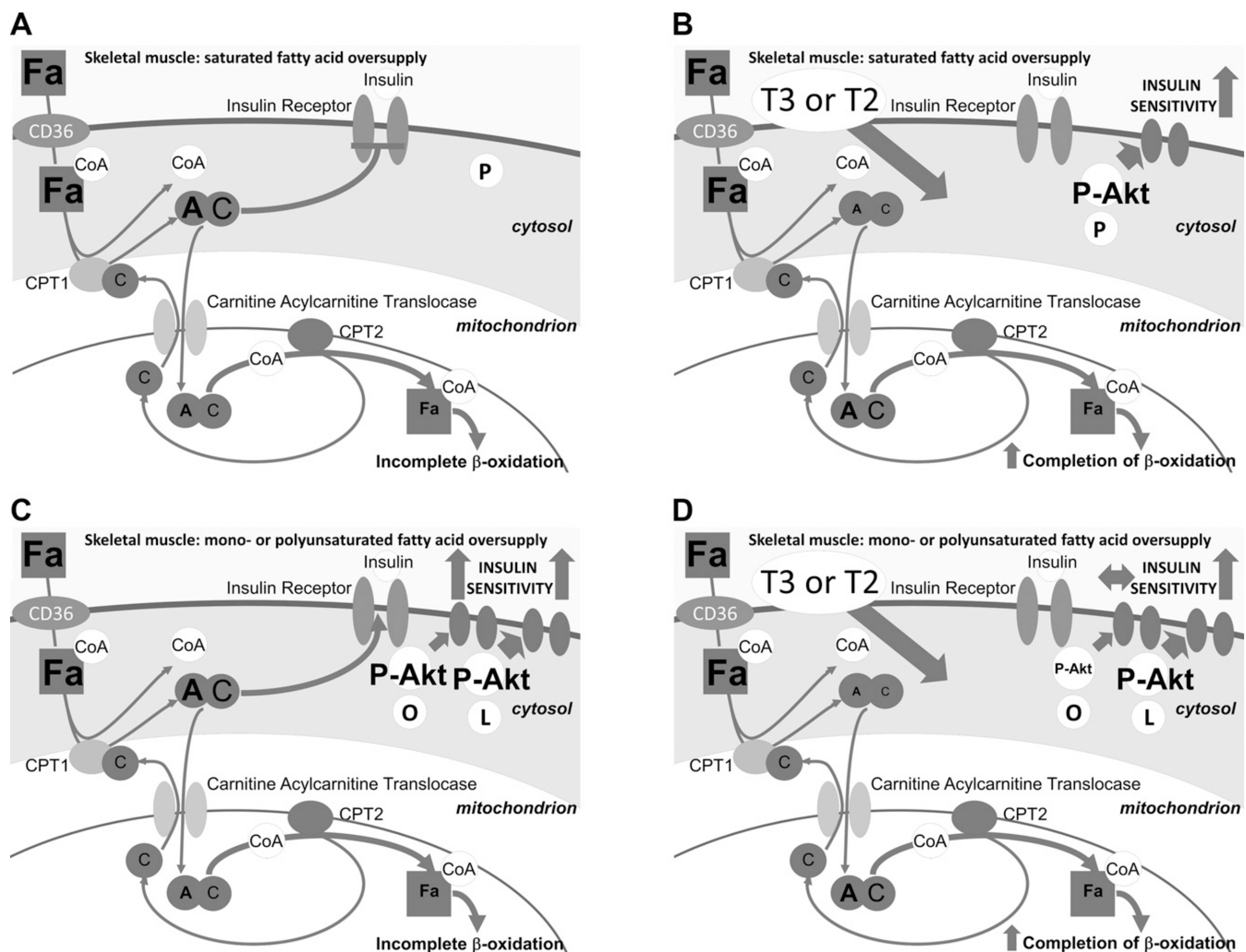



**Figure 8.** Schematic overview depicting the differential effects of thyroid hormones on Akt phosphorylation in muscle cells incubated with fatty acids with different degrees of saturation. *A*, *B*) An oversupply of saturated fatty acids results in intracellular accumulation of saturated acylcarnitines with concomitant induction of insulin resistance (*A*) that is overcome in the presence of T2 or T3, concomitant with a reduction in intracellular acylcarnitine levels (*B*). *C*, *D*) Oversupply of monounsaturated fatty acids, although leading to intracellular accumulation of acylcarnitines, does not induce insulin resistance but increases it (*C*), and thyroid hormones normalize insulin sensitivity in presence of oleylcarnitines but do not alter insulin sensitivity in presence of linoleylcarnitines, concomitant with a reduction of the intracellular acylcarnitine to control levels (*D*). The perpendicular line (*A*) indicates an inhibitory effect of the acylcarnitines on insulin signaling. A, acyl chain; AC, acylcarnitine; C, carnitine; CD36, fatty acid transporter FAT/CD36; CoA, coenzyme A; Fa, fatty acid; L, linoleylcarnitine; P, palmitoylcarnitine; O, oleylcarnitine.

respiration in the presence of palmitate *vs.* increased ATP synthesis–independent mitochondrial respiration and lowered respiratory control ratios in the presence of oleate, both parameters being additionally stimulated in presence of linoleate; and *2*) increased AMPK phosphorylation together with decreased respiratory control ratios in the order palmitate-oleate-linoleate. Indeed, increased mitochondrial inefficiency leads to a progressive decline in ATP that is known to give rise to a concomitant increase in AMP levels through adenylate kinase activity, resulting in activation of AMPK (32), which has been shown to induce fatty acid oxidation in L6 cells (33). Because the observed progressive induction of ATP synthesis–independent respiration in the order palmitate-oleate-linoleate, leading to a consequent enhancement in substrate oxidation, maintains a low NADH/NAD+ ratio and facilitates fatty acid oxidation, the obtained results explain the gradual decrease of accumulation of acylcarnitines of the fatty acids in the same order, and indeed, linoleylcarnitines barely accumulated in the cell. These results are in line with the observation that in rat skeletal muscle, increased mitochondrial efficiency with age leads to reduced substrate use that is associated with increased insulin resistance (34). Regarding the effect of thyroid hormones on AMPK phosphorylation, T3 further increased the P-AMPK/AMPK ratio only in the presence of oleate.

The palmitate-induced reduction insulin sensitivity was normalized by both T2 and T3, and both hormones reduced intracellular palmitoylcarnitine accumulation, whereas only T3 reduced mitochondrial respiratory efficiency. Palmitate-induced insulin resistance was associated with increased expression of the marker gene encoding the p85 subunit of PI3K, known to cause reduced Akt phosphorylation at Ser473 (35). Palmitate-induced down-regulation of Akt phosphorylation was normalized both by T2 and T3, and this action was indeed associated with a normalization of PI3K p85 expression to (or even below) control levels in the presence of both hormones. Although the differential modulation by T2 and T3 of the expression of relevant genes involved in lipid/glucose metabolism and insulin sensitivity in the presence of each fatty acid underlines their diversity of action, both hormones increased the activity of PFK under all conditions. Thus, despite nonsyntonized transcriptional regulation, both hormones equally stimulated glucose metabolism.

Insulin sensitivity can also be altered by modulation of cellular or mitochondrial fatty acid uptake and handling. Whereas thyroid hormones have been shown to influence sarcolemmal FAT/CD36 accumulation *in vivo* (2, 36), this effect was not confirmed in our experimental conditions. Instead, fatty acid uptake and processing in the mitochondria were differentially modulated by T2 and T3. Both the increased CPT1b/CPT2 ratio (indicating a decreased efficiency of mitochondrial free fatty acid uptake) and decreased CPT activity induced by palmitate were only normalized by T2. Because mitochondrial respiratory efficiency was reduced in the presence of oleate and especially linoleate, this is likely the reason why in presence of the unsaturated fatty acids, CPT activity did not differ from that of the controls; the CPT1b/CPT2 ratio was increased in the presence of oleate, however, and CPT1b expression was decreased in the presence of linoleate. Support for our observation comes from a study performed on rats in which dietary polyunsaturated fatty acids were shown to increase CPT activity and decrease mitochondrial efficiency in skeletal muscle, accompanied by increased insulin sensitivity (37).

Taken together, the outcome of this study, schematically depicted in **Fig. 8**, reveals several important aspects: *1*) both T2 and T3 invert the muscle cell's response to insulin; *2*) the degree of saturation of the fatty acids determines the muscle cell's insulin sensitivity, at least in part due to accumulation of their derived intracellular acylcarnitines; and *3*) as a consequence, increasing the completeness of fatty acid oxidation by thyroid hormones (or other stimulators of fat oxidation) may not always continue to be insulin sensitizing. FJ

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## AUTHOR CONTRIBUTIONS

A. Giacco, G. delli Paoli, R. Senese, F. Cioffi, E. Silvestri, M. Moreno, M. Ruoppolo, M. Caterino, M. Costanzo, A. Lombardi, and P. de Lange contributed to conception and design, acquisition of data, and analysis and interpretation of data; A. Giacco, G. delli Paoli, E. Silvestri, M. Moreno, M. Ruoppolo, A. Lombardi, F. Goglia, A. Lanni, and P. de Lange drafted the article or revised it critically for important intellectual content; F. Goglia, A. Lanni, and P. de Lange conducted the final editing of the manuscript; and all authors gave final approval of the version to be published.

## REFERENCES

1. De Lange, P., Senese, R., Cioffi, F., Moreno, M., Lombardi, A., Silvestri, E., Goglia, F., and Lanni, A. (2008) Rapid activation by 3,5,3′-L-triiodothyronine of adenosine 5′-monophosphate-activated protein kinase/acetyl-coenzyme a carboxylase and akt/protein kinase B signaling pathways: relation to changes in fuel metabolism and myosin heavy-chain protein content in rat gastrocnemius muscle in vivo. *Endocrinology* **149**, 6462–6470
2. 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., and Goglia, F. (2011) 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
3. De Lange, P., Cioffi, F., Senese, R., Moreno, M., Lombardi, A., Silvestri, E., De Matteis, R., Lionetti, L., Mollica, M. P., Goglia, F., and Lanni, A. (2011) Nonthyrotoxic prevention of diet-induced insulin resistance by 3,5-diiodo-L-thyronine in rats. *Diabetes* **60**, 2730–2739
4. DeFronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J., and Felber, J. P. (1981) The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* **30**, 1000–1007
5. Sears, B., and Perry, M. (2015) The role of fatty acids in insulin resistance. *Lipids Health Dis.* **14**, 121
6. Lanni, A., Moreno, M., Lombardi, A., de Lange, P., Silvestri, E., Ragni, M., Farina, P., Baccari, G. C., Fallahi, P., Antonelli, A., and Goglia, F. (2005) 3,5-diiodo-L-thyronine powerfully reduces adiposity in rats by increasing the burning of fats. *FASEB J.* **19**, 1552–1554

7. Padron, A. S., Neto, R. A., Pantaleão, T. U., de Souza dos Santos, M. C., Araujo, R. L., de Andrade, B. M., da Silva Leandro, M., de Castro, J. P., Ferreira, A. C., and de Carvalho, D. P. (2014) Administration of 3,5-diiodothyronine (3,5-T2) causes central hypothyroidism and stimulates thyroid-sensitive tissues. *J. Endocrinol.* **221**, 415–427
8. Jonas, W., Lietzow, J., Wohlgemuth, F., Hoefig, C. S., Wiedmer, P., Schweizer, U., Köhrle, J., and Schürmann, A. (2015) 3,5-Diiodo-L-thyronine (3,5-t2) exerts thyromimetic effects on hypothalamus-pituitary-thyroid axis, body composition, and energy metabolism in male diet-induced obese mice. *Endocrinology* **156**, 389–399
9. Cavallo, A., Taurino, F., Damiano, F., Siculella, L., Sardanelli, A. M., and Gnoni, A. (2016) Acute administration of 3,5-diiodo-L-thyronine to hypothyroid rats stimulates bioenergetic parameters in liver mitochondria. *J. Bioenerg. Biomembr.* **48**, 521–529
10. Martínez-Sánchez, N., Seoane-Collazo, P., Contreras, C., Varela, L., Villarroya, J., Rial-Pensado, E., Buqué, X., Aurrekoetxea, I., Delgado, T. C., Vázquez-Martínez, R., González-García, I., Roa, J., Whittle, A. J., Gomez-Santos, B., Velagapudi, V., Tung, Y. C. L., Morgan, D. A., Voshol, P. J., Martínez de Morentin, P. B., López-González, T., Liñares-Pose, L., Gonzalez, F., Chatterjee, K., Sobrino, T., Medina-Gómez, G., Davis, R. J., Casals, N., Orešič, M., Coll, A. P., Vidal-Puig, A., Mittag, J., Tena-Sempere, M., Malagón, M. M., Diéguez, C., Martínez-Chantar, M. L., Aspichueta, P., Rahmouni, K., Nogueiras, R., Sabio, G., Villarroya, F., and López, M. (2017) Hypothalamic AMPK-ER stress-JNK1 Axis mediates the central actions of thyroid hormones on energy balance. *Cell Metab.* **26**, 212–229.e12
11. Lombardi, A., Senese, R., De Matteis, R., Busiello, R. A., Cioffi, F., Goglia, F., and Lanni, A. (2015) 3,5-Diiodo-L-thyronine activates brown adipose tissue thermogenesis in hypothyroid rats. *PLoS One* **10**, e0116498
12. Koves, T. R., Ussher, J. R., Noland, R. C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J. R., Newgard, C. B., Lopaschuk, G. D., and Muoio, D. M. (2008) Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab.* **7**, 45–56
13. Aguer, C., McCoin, C. S., Knotts, T. A., Thrush, A. B., Ono-Moore, K., McPherson, R., Dent, R., Hwang, D. H., Adams, S. H., and Harper, M. E. (2015) Acylcarnitines: potential implications for skeletal muscle insulin resistance. *FASEB J.* **29**, 336–345
14. Nardi, F., Lipina, C., Magill, D., Hage Hassan, R., Hajduch, E., Gray, A., and Hundal, H. S. (2014) Enhanced insulin sensitivity associated with provision of mono and polyunsaturated fatty acids in skeletal muscle cells involves counter modulation of PP2A. *PLoS One* **9**, e92255
15. Crescenzo, R., Bianco, F., Mazzoli, A., Giacco, A., Liverini, G., and Iossa, S. (2015) Mitochondrial efficiency and insulin resistance. *Front. Physiol.* **5**, 512
16. Vatner, D. F., Snikeris, J., Popov, V., Perry, R. J., Rahimi, Y., and Samuel, V. T. (2015) 3,5 diiodo-l-thyronine (T2) does not prevent hepatic steatosis or insulin resistance in fat-fed sprague dawley rats. *PLoS One* **10**, e0140837
17. Adams, S. H., Hoppel, C. L., Lok, K. H., Zhao, L., Wong, S. W., Minkler, P. E., Hwang, D. H., Newman, J. W., and Garvey, W. T. (2009) Plasma acylcarnitine profiles suggest incomplete long-chain fatty acid beta-oxidation and altered tricarboxylic acid cycle activity in type 2 diabetic African-American women. *J. Nutr.* **139**, 1073–1081
18. Cao, Z., West, C., Norton-Wenzel, C. S., Rej, R., Davis, F. B., Davis, P. J., and Rej, R. (2009) Effects of resin or charcoal treatment on fetal bovine serum and bovine calf serum. *Endocr. Res.* **34**, 101–108
19. Oliveira, A. F., Cunha, D. A., Ladriere, L., Igoillo-Esteve, M., Bugliani, M., Marchetti, P., and Cnop, M. (2015) In vitro use of free fatty acids bound to albumin: a comparison of protocols. *Biotechniques* **58**, 228–233
20. Senese, R., Valli, V., Moreno, M., Lombardi, A., Busiello, R. A., Cioffi, F., Silvestri, E., Goglia, F., Lanni, A., and de Lange, P. (2011) Uncoupling protein 3 expression levels influence insulin sensitivity, fatty acid oxidation, and related signaling pathways. *Pflugers Arch.* **461**, 153–164
21. Brand, M. D., and Nicholls, D. G. (2011) Assessing mitochondrial dysfunction in cells. *Biochem. J.* **435**, 297–312
22. Caterino, M., Chandler, R. J., Sloan, J. L., Dorko, K., Cusmano-Ozog, K., Ingenito, L., Strom, S. C., Imperlini, E., Scolamiero, E., Venditti, C. P., and Ruoppolo, M. (2016) The proteome of methylmalonic acidemia (MMA): the elucidation of altered pathways in patient livers. *Mol. Biosyst.* **12**, 566–574
23. Ruoppolo, M., Campesi, I., Scolamiero, E., Pecce, R., Caterino, M., Cherchi, S., Mercuro, G., Tonolo, G., and Franconi, F. (2014) Serum metabolomic profiles suggest influence of sex and oral contraceptive use. *Am. J. Transl. Res.* **6**, 614–624
24. Scolamiero, E., Cozzolino, C., Albano, L., Ansalone, A., Caterino, M., Corbo, G., di Girolamo, M. G., Di Stefano, C., Durante, A., Franzese, G., Franzese, I., Gallo, G., Giliberti, P., Ingenito, L., Ippolito, G., Malamisura, B., Mazzeo, P., Norma, A., Ombrone, D., Parenti, G., Pellecchia, S., Pecce, R., Pierucci, I., Romanelli, R., Rossi, A., Siano, M., Stoduto, T., Villani, G. R., Andria, G., Salvatore, F., Frisso, G., and Ruoppolo, M. (2015) Targeted metabolomics in the expanded newborn screening for inborn errors of metabolism. *Mol. Biosyst.* **11**, 1525–1535
25. Silvestri, E., Cioffi, F., Glinni, D., Ceccarelli, M., Lombardi, A., de Lange, P., Chambery, A., Severino, V., Lanni, A., Goglia, F., and Moreno, M. (2010) Pathways affected by 3,5-diiodo-l-thyronine in liver of high fat-fed rats: evidence from two-dimensional electrophoresis, blue-native PAGE, and mass spectrometry. *Mol. Biosyst.* **6**, 2256–2271
26. Grasselli, E., Voci, A., Demori, I., Vecchione, G., Compalati, A. D., Gallo, G., Goglia, F., De Matteis, R., Silvestri, E., and Vergani, L. (2016) Triglyceride mobilization from lipid droplets sustains the anti-steatotic action of iodothyronines in cultured rat hepatocytes. *Front. Physiol.* **6**, 418
27. Eyer, P., Worek, F., Kiderlen, D., Sinko, G., Stuglin, A., Simeon-Rudolf, V., and Reiner, E. (2003) Molar absorption coefficients for the reduced Ellman reagent: reassessment. *Anal. Biochem.* **312**, 224–227
28. Opie, L. H., and Newsholme, E. A. (1967) The activities of fructose 1,6-diphosphatase, phosphofructokinase and phosphoenolpyruvate carboxykinase in white muscle and red muscle. *Biochem. J.* **103**, 391–399
29. Bouchouirab, F.Z., Fortin, M., Noll, C., Dubé, J., and Carpentier, A.C. (2018) Plasma palmitoyl-carnitine (AC16:0) is a marker of increased postprandial nonesterified incomplete fatty acid oxidation rate in Individuals with type 2 diabetes. *Can. J. Diabetes* **42**, 382–388
30. Abu Bakar, M. H., and Sarmidi, M. R. (2017) Association of cultured myotubes and fasting plasma metabolite profiles with mitochondrial dysfunction in type 2 diabetes subjects. *Mol. Biosyst.* **13**, 1838–1853
31. Schooneman, M. G., Vaz, F. M., Houten, S. M., and Soeters, M. R. (2013) Acylcarnitines: reflecting or inflicting insulin resistance? *Diabetes* **62**, 1–8
32. Hardie, D. G., Carling, D., and Carlson, M. (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu. Rev. Biochem.* **67**, 821–855
33. Watt, M. J., Steinberg, G. R., Chen, Z. P., Kemp, B. E., and Febbraio, M. A. (2006) Fatty acids stimulate AMP-activated protein kinase and enhance fatty acid oxidation in L6 myotubes. *J. Physiol.* **574**, 139–147
34. Iossa, S., Mollica, M. P., Lionetti, L., Crescenzo, R., Tasso, R., and Liverini, G. (2004) A possible link between skeletal muscle mitochondrial efficiency and age-induced insulin resistance. *Diabetes* **53**, 2861–2866
35. Mauvais-Jarvis, F., Ueki, K., Fruman, D. A., Hirshman, M. F., Sakamoto, K., Goodyear, L. J., Iannacone, M., Accili, D., Cantley, L. C., and Kahn, C. R. (2002) Reduced expression of the murine p85alpha subunit of phosphoinositide 3-kinase improves insulin signaling and ameliorates diabetes. *J. Clin. Invest.* **109**, 141–149
36. Lombardi, A., De Matteis, R., Moreno, M., Napolitano, L., Busiello, R. A., Senese, R., de Lange, P., Lanni, A., and Goglia, F. (2012) Responses of skeletal muscle lipid metabolism in rat gastrocnemius to hypothyroidism and iodothyronine administration: a putative role for FAT/CD36. *Am. J. Physiol. Endocrinol. Metab.* **303**, E1222–E1233
37. Cavaliere, G., Trinchese, G., Bergamo, P., De Filippo, C., Mattace Raso, G., Gifuni, G., Putti, R., Moni, B. H., Canani, R. B., Meli, R., and Mollica, M. P. (2016) Polyunsaturated fatty acids attenuate diet induced obesity and insulin resistance, modulating mitochondrial respiratory uncoupling in rat skeletal muscle. *PLoS One* **11**, e0149033



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