Palmitoleic acid (n-7) increases white adipocyte lipolysis and lipase content in a PPARα-dependent manner

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Bolsoni-Lopes A, Festuccia WT, Farias TS, Chimin P, Torres-Leal FL, Derogis PB, de Andrade PB, Miyamoto S, Lima FB, Curi R, Alonso-Vale MI. Palmitoleic acid (n-7) increases white adipocyte lipolysis and lipase content in a PPARα-dependent manner. Am J Physiol Endocrinol Metab 305: E1093–E1102, 2013. First published September 10, 2013; doi:10.1152/ajpendo.00082.2013.—We investigated whether palmitoleic acid, a fatty acid that enhances whole body glucose disposal and suppresses hepatic steatosis, modulates triacylglycerol (TAG) metabolism in adipocytes. For this, both differentiated 3T3-L1 cells treated with either palmitoleic acid (16:1n7, 200 μM) or palmitic acid (16:0, 200 μM) for 24 h and primary adipocytes from wild-type or PPARα-deficient mice treated with 16:1n7 (300 mg·kg−1·day−1) or oleic acid (18:1n9, 300 mg·kg−1·day−1) by gavage for 10 days were evaluated for lipolysis, TAG, and glycerol 3-phosphate synthesis and gene and protein expression profile. Treatment of differentiated 3T3-L1 cells with 16:1n7, but not 16:0, increased basal and isoproterenol-stimulated lipolysis, mRNA levels of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) and protein content of ATGL and Pser680-HSL. Such increase in lipolysis induced by 16:1n7, which can be prevented by pharmacological inhibition of PPARα, was associated with higher rates of PPARα binding to DNA. In contrast to lipolysis, both 16:1n7 and 16:0 increased fatty acid incorporation into TAG and glycerol 3-phosphate synthesis from glucose without affecting glyceroenogenesis and glycerokinase expression. Corroborating in vitro findings, treatment of wild-type but not PPARα-deficient mice with 16:1n7 increased primary adipocyte basal and stimulated lipolysis and ATGL and HSL mRNA levels. In contrast to lipolysis, however, 16:1n7 treatment increased fatty acid incorporation into TAG and glycerol 3-phosphate synthesis from glucose in both wild-type and PPARα-deficient mice. In conclusion, palmitoleic acid increases adipocyte lipolysis and lipases by a mechanism that requires a functional PPARα.

ATGL; HSL; lipogenesis; triacylglycerol/fatty acid cycle

WHITE ADIPOSE TISSUE is the major energetic reservoir in mammals storing the excess of energy from the diet as triacylglycerol (TAG), a neutral lipid composed of three fatty acids bound to the carbon backbone of a glycerol molecule (14). During periods of increased energetic demand, adipose tissue TAG stores are hydrolyzed to fatty acids and glycerol in a process denominated lipolysis. Although most lipolysis-derived fatty acids are released in the circulation to be used as energy substrates by other tissues (9), parts of these molecules are kept inside adipocytes, acting as precursors for the synthesis of other lipids, as intracellular signaling mediators, and as substrates for either oxidation or reesterification into TAG (1, 15, 46).

Lipolysis consists of the sequential hydrolysis of TAG subsequently to diacylglycerol (DAG), monoacylglycerol (MAG), and glycerol and fatty acids catalyzed in a stepwise manner by the lipases adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL), respectively (5, 43). The hydrolytic activities of these lipases are finely regulated by several mechanisms including, but not limited to, 1) covalent modifications driven by neurohormonal factors; 2) allosteric interactions with auxiliary proteins such as perilipins, comparative gene identification-58 (CGI-58), and G0/G1 switch gene 2 (G0S2) that facilitate lipase interactions with lipid droplets and act as coactivator and coinhibitor of ATGL, respectively; and 3) changes in lipase protein content due to the modulation in their gene expression (19, 39, 46).

Recent studies have demonstrated that the lipid sensors and nuclear receptors peroxisome proliferator-activated receptors (PPARs), which are well known for their hypolipidemic effects, also modulate gene expression of proteins involved in lipolytic cascade, acting as an important modulator of lipolysis in adipose tissue. Pharmacological PPARγ activation with rosiglitazone is associated with a marked increase in adipose tissue lipase expression (ATGL and MGL) and lipolysis (7, 16), such as release of lipolytic products that is counteracted by reesterification and reuptake, processes that are also exacerbated by PPARγ activation (7, 28, 41). In addition, the endogenously produced monounsaturated lipid oleoylthanolamide was demonstrated to increase epididymal adipose tissue lipolysis in a PPARα-dependent manner (12). This indicates that, in addition to synthetic compounds, naturally occurring lipids with ligand properties toward PPARs might be important modulators of lipase expression and lipolysis in adipose tissue.

Among the several types of lipids produced and released by adipocytes, palmitoleic acid, a monounsaturated n-7 fatty acid (16:1n7) synthesized by the desaturation of palmitic acid (16:0) catalyzed by the stearoyl-CoA desaturase-1 (SCD-1), has been shown to act systemically in peripheral tissues modulating important metabolic processes (3). Accordingly, palmitoleic acid was demonstrated to enhance whole body glucose disposal and to attenuate hepatic steatosis in high-fat-fed mice (3, 45) and to protect pancreatic β-cells from death induced by...
palmitic acid (4, 25). Furthermore, intake of a diet enriched in palmitoleic acid was associated with improvement of circulating lipid profile in both rodents (24) and humans (10, 13). Despite of all these important metabolic effects in liver, skeletal muscle, and β-cells, whether palmitoleic acid also affects adipose tissue TAG metabolism remains to be investigated. In the present study, therefore, by combining in vitro and in vivo experiments with differentiated 3T3-L1 adipocytes and murine primary adipocytes, we tested the hypothesis that palmitoleic acid is an important modulator of TAG metabolism in adipocytes.

MATERIALS AND METHODS

Cell culture. 3T3-L1 preadipocytes were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% calf serum and penicillin-streptomycin 1% until confluence. After 2–3 days post-confluence, differentiation was induced by addition of a cocktail composed of dexamethasone (1 μM), isobutylmethylxanthine (0.5 mM), and insulin (1.67 μM). After 48 h, medium was replaced with DMEM and 10% FBS containing 0.41 μM insulin (29). Differentiated 3T3-L1 cells (6 days after cocktail) were incubated with vehicle (ethanol 0.05%) or palmitic (16:0, 200 μM) or palmitoleic acid (16:1n7, 200 μM) or a combination of palmitic (100 μM) plus palmitoleic (100 μM) acids. This dose of fatty acids was found to have no cytotoxic or deleterious effects on cell viability, as evaluated by membrane integrity and DNA fragmentation (data not shown).

Importantly, we chose for the in vitro experiments a dose of palmitoleic acid higher than that found in plasma of humans and/or rodents (8, 26, 31, 32), because, in contrast to regular adipocytes, palmitoleic acid is the most abundant fatty acid in differentiated 3T3-L1 cells (11, 35). Thus, a higher dose of palmitoleic acid is required to challenge these cells. After 18 h, cells were washed with PBS and cultured in serum and insulin-free medium containing fatty acids for an additional 6 h. PPARγ and PPARα inhibition was achieved by adding their respective antagonists, GW-6471 (10 μM) and GW-9662 (10 μM) to the medium containing fatty acids. All reagents and drugs were purchased from Sigma Chemical (St. Louis, MO).

Animals. All experimental protocols were approved by the Animal Care Committee of the Institute of Biomedical Sciences, University of São Paulo, Brazil. (#89/10/CEUA). Male 8-week-old C57BL/6 wild-type (WT) or PPARα knockout (KO) mice (from the Animal Facility of the Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil) were kept individually in cages at 23°C on a 12:12-h light-dark cycle with food (balanced chow pellet diet; Nuvilab CR1, Nuvital, Colombo, PR, Brazil) and water ad libitum. Mice were randomly assigned to one of the five groups that received 300 mg/kg·day–1 of pure palmitoleic acid (16:1n7), oleic acid (18:1n9) (Sigma), or water (45) by gavage. Instead of palmitic acid, a solid that has no cytotoxic or deleterious effects on cell viability, as evaluated by membrane integrity and DNA fragmentation (data not shown).

Animals were filtered through a plastic mesh (150 μm) and washed three times in the same buffer without collagenase. After washing, medium was thoroughly aspirated and adipocytes were harvested. A small amount of adipocytes was photographed under an optic microscope (×100 magnification) using a microscope camera (Motican 1000; Motic, Richmond, BC, Canada), and mean adipocyte diameter was determined by measuring 50 cells using Motic-Images Plus 2.0 software.

Plasma biochemical analysis. Blood glucose, TAG, and total cholesterol levels were determined using commercial kits (Labtest Diagnostica from Lagoa Santa, MG, Brazil). NEFA were measured using the HR NEFA series kit (Wako Diagnostic, Richmond, VA) according to the manufacturer’s instructions. Insulin was quantified by ELISA (Millipore kit, St. Charles, MO).

Lipolysis. Lipolysis was estimated as the rate of glycerol release in the incubation medium. For this, differentiated 3T3-L1 cells or primary epididymal adipocytes (1 × 106 cells/ml) were incubated in Krebs-Ringer-phosphate buffer (pH 7.4) containing BSA (20 mM) and glucose (5 mM) for 30 min at 37°C in the presence or absence of isoproterenol (2 × 10–6 M). The reaction was stopped on ice, and medium was carefully collected for measurement of glycerol release (Free Glycerol Determination Kit, Sigma). Results are expressed as nanomoles of glycerol per milligram of protein per hour for 3T3-L1 cells and as nanomoles of glycerol per 1 × 106 adipocytes for epididymal adipocytes.

Incorporation of [1-14C]palmitate into TAG. Differentiated 3T3-L1 (~8 × 105 cells/well) or primary epididymal adipocytes (106 cells/ml) were incubated in Krebs-Ringer-phosphate buffer (pH 7.4) containing BSA (1%), glucose (2 mM) and [1-14C]Palmitate (200 μM, 1,850 Bq/tube or well) for 2 h at 37°C in a water bath. At the end of incubation, 3T3-L1 cells were washed with PBS followed by Dole lipid extraction with 2.5 ml of isopropanol-n-heptane-H2SO4 (4:1:0.25, vol/vol/vol). For primary isolated adipocytes, the mixture was transferred to 1.5-ml tubes containing 400 μl of silicone oil and centrifuged for 30 s. The cell pellet on the top of the oil layer was transferred to polypropylene tubes containing 2.5 ml of Dole’s reagent for lipid extraction. After addition of n-heptane (1.5 ml) and distilled water (1.5 ml), tubes were vortexed, and the mixture was decanted for 5 min. An aliquot of the upper phase was collected into a scintillation vial for determination of the radioactivity trapped into TAG (1450 LSC, Coulter MicroBeta, Trilux; PerkinElmer). Results are expressed as nanomoles of palmitate incorporated into TAG per 1 × 106 cells per hour.

Incorporation of [1-14C]glucose into glycerol of TAG. Differentiated 3T3-L1 cells or primary epididymal adipocytes (1 × 105 cells/ml) were incubated in Krebs-Ringer-phosphate buffer (pH 7.4) containing BSA (1%) and [1-14C]Glucose (2 mM, 1,850 Bq/tube or well) for 2 h at 37°C in a water bath. Lipids were extracted by Dole’s method for measurement of glucose incorporation into total TAG as above described. For estimation of glucose incorporation into TAG, an aliquot of the upper phase was transferred to a tube containing 1 ml of ethanol (95%) and 250 μl of KOH (40%) and incubated in a water bath at 60°C for 1 h. After incubation, 2 ml of HCl (3 N) and 2 ml of n-heptane were added, tubes were vortexed, and the upper phase was collected (1 ml) into a scintillation vial for determination of glucose incorporation into fatty acids of TAG.

Glucose incorporation into glycerol of TAG was calculated by the difference between the incorporation of [1-14C]glucose into TAG and fatty acids. The results were expressed as nanomoles of glucose incorporated into glycerol per 1 × 106 cells per hour.

Incorporation of [1-14C]pyruvate into glycerol of TAG. Differentiated 3T3-L1 cells were incubated in Krebs-Ringer-phosphate buffer (pH 7.4) containing BSA (1%) and [1-14C]pyruvate (5 mM, 1,850 Bq/tube or well) for 2 h at 37°C in a water bath. Lipids were extracted by Dole’s method for measurement of pyruvate incorporation into glycerol of TAG as described above. The results were expressed as nanomoles of pyruvate incorporated into glycerol per 1 × 106 cells per hour.
RNA extraction and quantitative real-time polymerase chain reaction. Total RNA from 3T3-L1 cells or epididymal adipocytes were extracted with TRIzol reagent (Invitrogen Life Technologies), analyzed for quality on agarose gel and ratios 260/280 and 260/230 nm on NANO DROP (Thermo Scientific), and reverse transcribed to cDNA using the High-Capacity cDNA Kit (Applied Biosystems). Gene expression was evaluated by real-time quantitative PCR (RTqPCR) using a Rotor Gene (Qiagen) and SYBR Green as fluorescent dye with GAPDH as a housekeeping gene for 3T3-L1 cells and 36B4 for epididymal adipocytes. Primers used and annealing temperatures are presented in Table 1. Primer efficiency was evaluated with a standard curve of different cDNA concentrations. After proper setting of the baseline and threshold, the slope of the standard curve was translated into an efficiency ($R^2$) value. Primer efficiencies were around 90–100%. PCR products were run on agarose gel to confirm the size of the fragment and specificity of amplification. Some PCR products were extracted from gel with a kit (Qiagen) and submitted to sequencing for identity confirmation.

Western blot analysis. Protein aliquots (20 μg) of 3T3-L1 lysates were resolved on Nupage gradient gels (4–12%, Life Technologies) and transferred to nitrocellulose membranes. After blocking with 5% milk for 1 h, membranes were incubated overnight at 4°C with the following primary antibodies against: HSL (no. 4107), phospho(p)-Ser660-HSL (no. 4126), ATGL (no. 2138), and γ-tubulin (no. 5886 Cell Signaling, Beverly, MA), in 5% milk (1:1,000). After a 40-min washing, membranes were subsequently incubated with appropriated peroxidase-conjugated secondary antibody (1:5,000) for 1 h and developed using the ECL enhanced chemiluminescence substrate (GE Healthcare Life Sciences, Björkgatan, Uppsala, Sweden). Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

PPARα DNA binding assay. PPARα DNA binding activity was measured on nuclear extracts from 3T3-L1 cells treated with either vehicle or palmitic acid (200 μM) or palmitoleic acid (200 μM) for 12 h. Nuclear extracts were generated using the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce, Rockford, IL). Nuclear extracts were incubated on a plate containing immobilized PPARα DNA response element (Cayman Chemical, Ann Arbor, MI). The plate was then probed for antibodies specific to PPARα and read in a colorimetric plate reader. Results were normalized by nuclear protein content.

Gas chromatographic analysis. 3T3-L1 adipocytes treated with either vehicle or palmitic (200 μM) or palmitoleic acid (200 μM) for 24 h were washed, harvested in PBS, and used for the measurement of fatty acid composition by transmethylation reaction for gas chromatography (GC) fatty acid methyl ester (FAME) analysis as previously described (23). Briefly, harvested cells were mixed with methanol, acetyl chloride, and an internal standard solution (heptadecanoic acid in methanol) and heated at 100°C for 60 min, and lipid was extracted with hexane. Individual FAMES were analyzed by GC with flame ionization detection on a Trace 1310 (Thermo Scientific) using a capillary column (DB-FFAP, 15 m × 0.1 mm ID × 0.1 μm film thickness; Agilent Technologies). FAME was identified by direct comparison with a FAME standard mix (Supelco 37 Component FAME Mix, Sigma-Aldrich), each individual peak was integrated, and the area was normalized by internal standard. The percentage of individual FAME was calculated in relation to the total area of FAME peaks. Since the fatty acid methyl esters of interest have similar carbon chain length, it was assumed that they would have the same response factor and volatility, allowing the making of a direct comparison of the peak areas to determine sample composition.

Statistical analysis. Data are expressed as means ± SE. One- or two-way ANOVA (when indicated) followed by Tukey and Bonferroni post hoc tests, respectively, were used to compare the effects of different treatments and conditions. Analysis was performed using GraphPad Prism v. 5.0 software (GraphPad Software, San Diego, CA). The level of significance was set at $P \leq 0.05$.

RESULTS

Because differentiated 3T3-L1 cells express several enzymes that can chemically modify fatty acids (e.g., elongases, desaturases), we first investigated whether treatment with palmitic or palmitoleic acid is associated with changes in 3T3-L1 fatty acid content. As evaluated by GC, palmitoleic acid is the most abundant fatty acid (~36%) found in 3T3-L1 cells, followed by palmitic acid (~23%) and oleic acid (~10%) (Table 2), and treatments did not significantly affect total 3T3-L1 fatty acid content (means ± SE, vehicle, 564 ± 23; 16:0, 615 ± 23; and 16:1n7, 629 ± 43 μg/10⁶ cells, $P = 0.11$). Treatment with palmitic acid for 24 h induced a significant increase in 3T3-L1 palmitic acid content without significantly affecting cell composition of other fatty acids such as palmitoleic, stearic, oleic, and vaccenic acids. Similarly, treatment with palmitoleic acid for 24 h induced a significant increase in 3T3-L1 palmitic acid content without significantly affecting cell levels of palmitic, stearic, oleic, and vaccenic acids. To investigate whether palmitoleic acid (16:1n7) regulates TAG metabolism in adipocytes, differentiated 3T3-L1 cells were evaluated for lipolysis after a 24-h treatment with this fatty acid. As depicted in Fig. 1, A and B, treatment with 16:1n7 at a dose of 200 μM significantly increased basal and stimulated lipolysis, as evidenced by the higher rates of glycerol release into medium from cells treated with 16:1n7 compared with those treated with vehicle (~57 and 58%, respectively) and palmitic acid (200 μM, ~53 and 56%, respectively). Interestingly, combined treatment of 16:1n7 (100 μM) plus palmitic acid (100 μM) does not interfere with the increase in basal and stimulated lipolysis induced by 16:1n7 alone. Importantly, such increase in lipolysis induced by 16:

Table 1. Pairs of primers used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’ Primer (5’-3’)</th>
<th>3’ Primer (5’-3’)</th>
<th>Annealing</th>
<th>PubMed ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CCAAGACCTCTGTGACCTAG</td>
<td>CTTGGGCTCAGTACGAGACC</td>
<td>60°C</td>
<td>NM_000804.2</td>
</tr>
<tr>
<td>36B4</td>
<td>TAAAGACGCTGGACACAGTG</td>
<td>GTTGATCTCAGTCTGCAAGA</td>
<td>63°C</td>
<td>NM_007475</td>
</tr>
<tr>
<td>ATGL</td>
<td>GTGGCGCTGCACTGCGCTC</td>
<td>CGTGGTCGAAAGAGATCTC</td>
<td>63°C</td>
<td>NM_025802</td>
</tr>
<tr>
<td>HSL</td>
<td>GCAGGACGTCGAGCCATCACA</td>
<td>ATAGACTCGAGTGGTGAGGG</td>
<td>60°C</td>
<td>NM_010715.9</td>
</tr>
<tr>
<td>MGL</td>
<td>GGTATCGCCGAGAGCTGGT</td>
<td>GGTCGACAGTGGAGTACAGA</td>
<td>60°C</td>
<td>NM_018144</td>
</tr>
<tr>
<td>Perilipin</td>
<td>AGTGGTGGAAGCTGGCTGCTG</td>
<td>TGGACCTCGGAGACTGGTG</td>
<td>60°C</td>
<td>NM_175640</td>
</tr>
<tr>
<td>G0S2</td>
<td>AGTCGACGCTGCAAGCCAC</td>
<td>GGGCTGACAGGCGCTGCTC</td>
<td>60°C</td>
<td>NM_008059.3</td>
</tr>
<tr>
<td>CGI-58</td>
<td>TGGAGGCTGAGATGGTCTG</td>
<td>GCCATATGGGTCTGATCTTCA</td>
<td>60°C</td>
<td>NM_026179</td>
</tr>
<tr>
<td>PEPCCK</td>
<td>GAATGAGATCCGTTGCATAG</td>
<td>TCCATGCTGGTCTCTTCGA</td>
<td>60°C</td>
<td>NM_011044</td>
</tr>
<tr>
<td>GyK</td>
<td>AAGGCTGAGTCCGAGCTAG</td>
<td>ACGAGATCTTTCCAAGTCAAT</td>
<td>58°C</td>
<td>NM_008194</td>
</tr>
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</table>
Table 2. Palmitic (16:0), palmitoleic (16:1n7), stearic (18:0), oleic (18:1n9), and vaccenic (18:1n7) acids contents in different 3T3-L1 treated for 24 h with vehicle (ethanol), palmitic acid (16:0, 200 μM), or palmitoleic acid (16:1n7, 200 μM)

<table>
<thead>
<tr>
<th>Fatty Acid Methyl Ester, %</th>
<th>16:0</th>
<th>16:1n7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>212 ± 0.6</td>
<td>233 ± 2.5*</td>
</tr>
<tr>
<td>16:0</td>
<td>330 ± 1.2</td>
<td>337 ± 3.3</td>
</tr>
<tr>
<td>16:1n7</td>
<td>17.1 ± 0.1</td>
<td>15.9 ± 0.4</td>
</tr>
<tr>
<td>18:0</td>
<td>92.2 ± 0.3*</td>
<td>86.8 ± 0.8</td>
</tr>
<tr>
<td>18:1n9</td>
<td>27.4 ± 0.4†</td>
<td>24.1 ± 0.2</td>
</tr>
<tr>
<td>18:1n7</td>
<td>27.6 ± 0.5†</td>
<td>26.7 ± 0.1†</td>
</tr>
</tbody>
</table>

Results are means ± SE (n = 4–5/group). *P < 0.05 vs. all other treatments; †P < 0.05 vs. 16:0.

1n7 was reproduced at lower doses of this fatty acid (50 μM but not 10 μM; data not shown). Furthermore, the increase in lipolysis induced by 16:1n7 was associated with a significant upregulation in the mRNA levels of the lipases ATGL (−64%) and HSL (−100%) but not those of MGL and other proteins involved in lipolytic cascade such as perilipin A, CGI-58, and G0S2 (Fig. 1, C–H). Furthermore, treatment with 16:1n7, but not 16:0, significantly increased 3T3-L1 protein content of ATGL and pSer600-HSL and tended to increase (P = 0.09) those of total HSL compared with vehicle treated cells (Fig. 1, I–K).

In an attempt to investigate the putative mechanisms underlying this increase in lipolysis induced by 16:1n7, we next tested whether the “lipid sensors” PPARs, previously shown to regulate lipolysis through the modulation of lipase expression, were involved in this effect. As depicted in Fig. 2, A and B, pharmacological inhibition of PPARα (GW-6471), but not PPARγ (GW-9662) (data not shown), significantly reduced rates of glycerol release from 3T3-L1 cells at basal and stimulated conditions and completely blocked the increase in these variables induced by treatment with 16:1n7. Corroborating with a likely involvement of PPARα on palmitoleic acid actions on lipolysis, treatment with 16:1n7, but not 16:0 (data not shown), for 12 h significantly increased rates of PPARα binding to its DNA consensus sequence, also known as peroxisome proliferator-responsive element (PPRE) (Fig. 2C).

A possible modulation of the anabolic branch of TAG metabolism, namely, TAG synthesis by palmitoleic acid, was also investigated. As depicted in Fig. 3A, in contrast to lipolysis, treatment of 3T3-L1 with either 16:0 or 16:1n7 significantly increased TAG synthesis (−50%) as evidenced by the higher rates of fatty acid incorporation into TAG. Noteworthy, since palmitoleic acid increased TAG lipolysis and extracellular levels of fatty acids, this may have led to a dilution of the radiolabeled fatty acid and a likely underestimation of true values of fatty acid incorporation into TAG in palmitoleic-treated cells. This higher TAG synthesis induced by 16:0 or 16:1n7 was associated with a significant increase in the generation of glycerol 3-phosphate via glycolysis (~29%), as evidenced by the higher rates of glucose incorporation in the glycerol fraction of TAG (Fig. 3B). Importantly, such increase in glucose incorporation into TAG-glycerol was not affected by treatment with the pharmacological PPARα antagonist GW-6471 (Fig. 3B). In contrast to glycolysis, treatment with 16:0 or 16:1n7 did not affect the other two possible pathways of glycerol 3-phosphate generation in adipocytes, namely glyceroneogenesis, estimated by pyruvate incorporation in the glycerol fraction of TAG and PEPCK expression (Fig. 3, C and D), and glycerokinase, estimated by its mRNA levels (Fig. 3E).

To extend the significance and relevance of these in vitro findings, we also investigated whether palmitoleic acid also affects TAG metabolism in vivo. As shown in Table 3, treatment of wild-type and PPARα KO mice with pure 16:1n7 or oleic acid (18:1n9) by gavage for 10 days did not affect body weight, food intake, masses of white and brown adipose depots and liver, and plasma levels of cholesterol, free fatty acids, insulin, and glucose. In contrast to these variables, treatment with 18:1n9, but not 16:1n7, significantly increased plasma TAG levels in wild-type mice. As depicted in Fig. 4, A and B, similarly to its in vitro actions, treatment of wild-type mice by gavage with 16:1n7, but not with 18:1n9, significantly increased basal and stimulated lipolysis (~21%), as evidenced by the higher rates of glycerol release into medium from isolated epididymal adipocytes of mice treated with 16:1n7 compared with those treated with water.

To confirm the involvement of the PPARα pathway in the increase in lipolysis induced by 16:1n7, epididymal adipocytes from wild-type and PPARα KO mice treated with vehicle or 16:1n7 for 10 days were evaluated for glycerol release and lipase expression. Supporting our in vitro findings, treatment of wild-type mice with 16:1n7 significantly increased basal and isoproterenol-stimulated lipolysis as estimated by glycerol release (~21%) and adipose tissue mRNA levels of ATGL (~48%) and HSL (~62%), such effects that were completely blunted in epididymal adipocytes of PPARα KO mice (Fig. 5, A and B). In fact, epididymal adipocytes of PPARα KO mice had reduced rates of lipolysis at basal conditions and lower mRNA levels of ATGL than wild-type mice.

Regarding TAG synthesis, similarly to the in vitro findings, treatment of wild-type mice with either 16:1n7 or 18:1n9 significantly increased the incorporation of fatty acids into TAG, as well as 16:1n7 increased glycerol 3-phosphate generation from glycolysis, as estimated by glucose incorporation into the glycerol fraction of TAG (Fig. 6, A and B). Interestingly, in contrast to lipolysis, PPARα deficiency did not affect the increase induced by 16:1n7 on these parameters.

**DISCUSSION**

In the present study, we have unveiled important actions of palmitoleic acid (16:1n7) in the regulation of adipose tissue TAG metabolism and possible mechanisms underlying these effects. Our main findings indicate that palmitoleic, but not
palmitic (in vitro) or oleic (in vivo), acid increases adipose tissue lipolysis under both basal and stimulated conditions as well as the mRNA expression and protein content of the major TAG lipases ATGL and HSL. Here, we present strong evidence suggesting that the positive effects of palmitoleic acid on lipolysis and lipase expression require the proper functioning of the nuclear receptor and lipid sensor PPARα.

Several studies have found compelling evidence indicating that palmitoleic acid is an important signaling molecule, produced mainly by white adipose tissue, that regulates major metabolic processes such as skeletal muscle glucose disposal and insulin sensitivity and hepatic lipid deposition (3, 45). Here, we extend this notion by showing that palmitoleic acid also acts in white adipose tissue, where it modulates both
catabolic and anabolic branches of TAG metabolism. Accordingly, treatment of differentiated adipocytes in vitro or of mice in vivo with palmitoleic acid was associated with a significant increase in adipocyte lipolysis, under both basal and stimulated conditions. Such upregulation of lipolysis by palmitoleic acid was not affected by the presence of an equimolar concentration of palmitic acid. Interestingly, palmitoleic acid does increase lipolysis in vitro at dose of 50 $\mu$M, such a concentration of palmitoleic acid that can be attained in human plasma upon overnight fasting (8). Conversely, adipocyte treatment with palmitic acid, which can be intracellularly converted to palmitoleic acid by the adipocyte-expressed SCD1 had no effects on lipolysis. In fact, adipocyte treatment with palmitic acid did not translate into a higher generation of palmitoleic acid, as evidenced by the absence of changes in 3T3-L1 palmitoleic acid content estimated by GC analysis upon 24-h treatment with palmitic acid. These findings strongly indicate that SCD1 may preferentially use palmitic acid from de novo fatty acid syn-

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**Fig. 2.** Basal and isoproterenol-stimulated rates of glycerol release (µmol/mg protein; A and B, respectively) by 3T3-L1 cells treated for 24 h with vehicle or palmitoleic acid (16:1n7, 200 $\mu$M) in association or not with a PPAR$\alpha$ antagonist (GW-6471, 10 $\mu$M) and PPAR$\alpha$ DNA binding activity (absorbance/mg of protein; C) by nuclear extracts from 3T3-L1 cells treated with vehicle or palmitoleic acid (16:1n7, 200 $\mu$M) for 12 h. Results are means ± SE (n = 6–7/group). *$P \leq 0.05$, 16:1n7 vs. vehicle; #$P < 0.05$ GW-6471 and GW-6471 + 16:1n7 vs. vehicle and 16:1n7; &$P < 0.05$ GW-6471 + 16:1n7 vs. 16:1n7.

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**Fig. 3.** Incorporation of fatty acids (A), glucose (B), or pyruvate (C) into triacylglycerol (TAG, nmol/10$^6$ cells and pmol/10$^6$ cells, respectively) and into the glycerol fraction of TAG (nmol/10$^6$ cells), respectively, and mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK; D) and glycerokinase (GyK; E) in 3T3-L1 cells treated for 24 h with vehicle or palmitic acid (16:0, 200 $\mu$M) or palmitoleic acid (16:1n7, 200 $\mu$M) in the presence or not of DMSO or GW-6471 (GW, 10 $\mu$M), a PPAR$\alpha$ antagonist. GAPDH was used as a housekeeping gene. Results are means ± SE (n = 6–8/group). *$P < 0.05$ 16:0 and 16:1n7 vs. vehicle; +$P \leq 0.05$ GW + 16:0 and GW + 16:1n7 vs. GW; &$P < 0.05$ 16:1n7 vs. 16:0.
Table 3. Body and organ weights, food intake, and plasma biochemical profile in WT or PPARα KO mice daily treated for 10 days by gavage with H2O or palmitoleic acid (16:1 n7, 300 mg/kg) or palmitic acid (18:1 n9, 300 mg/kg)

<table>
<thead>
<tr>
<th></th>
<th>WT  + H2O</th>
<th>WT + 16:1n7</th>
<th>WT + 18:1n9</th>
<th>KO  + H2O</th>
<th>KO + 16:1n7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight, g</td>
<td>25.6 ± 0.6</td>
<td>25.4 ± 0.6</td>
<td>26.6 ± 1.3</td>
<td>24.8 ± 0.4</td>
<td>24.7 ± 0.4</td>
</tr>
<tr>
<td>Final body weight, g</td>
<td>24.4 ± 0.5</td>
<td>24.6 ± 0.4</td>
<td>25.7 ± 1.04</td>
<td>25.4 ± 0.4</td>
<td>25.2 ± 0.5</td>
</tr>
<tr>
<td>Food intake, g/day</td>
<td>3.6 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>Epididymal WAT, mg/g BW</td>
<td>13.3 ± 1.3</td>
<td>12.5 ± 1.2</td>
<td>13.1 ± 1</td>
<td>11.1 ± 0.8</td>
<td>10 ± 0.7</td>
</tr>
<tr>
<td>Inguinal WAT, mg/g BW</td>
<td>8.3 ± 1</td>
<td>9.3 ± 1.4</td>
<td>7.4 ± 0.9</td>
<td>6.8 ± 0.6</td>
<td>6.4 ± 0.1</td>
</tr>
<tr>
<td>Retropitoneal WAT, mg/g BW</td>
<td>2.3 ± 0.5</td>
<td>2.3 ± 0.6</td>
<td>2.0 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>BAT, mg/g BW</td>
<td>2.1 ± 0.1</td>
<td>2.3 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>1.9 ± 0.09</td>
<td>2 ± 0.06</td>
</tr>
<tr>
<td>Liver, mg/g BW</td>
<td>44.7 ± 1.2</td>
<td>46.1 ± 2.1</td>
<td>44 ± 0.6</td>
<td>44.3 ± 1.3</td>
<td>46.5 ± 1.2</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>79.6 ± 18.2</td>
<td>79.3 ± 8.7</td>
<td>139.2 ± 17.7</td>
<td>58.9 ± 5.9</td>
<td>83.7 ± 22.1</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>87.2 ± 8.6</td>
<td>100 ± 8</td>
<td>107.3 ± 12</td>
<td>109 ± 1.8</td>
<td>102 ± 10</td>
</tr>
<tr>
<td>Free fatty acid, mEq/l</td>
<td>0.72 ± 0.07</td>
<td>0.60 ± 0.08</td>
<td>0.90 ± 0.15</td>
<td>0.81 ± 0.12</td>
<td>0.70 ± 0.11</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.75 ± 0.21</td>
<td>0.89 ± 0.25</td>
<td>0.66 ± 0.04</td>
<td>0.51 ± 0.4</td>
<td>0.75 ± 0.21</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>101 ± 10.6</td>
<td>107 ± 14.1</td>
<td>108.3 ± 16.3</td>
<td>103 ± 11.4</td>
<td>109 ± 12.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–7. PPAR, peroxisome proliferator-activated receptor; WT, wild type; KO, knockout; 16:1n7, palmitoleic acid; 18:1n9, oleic acid; BW, body weight; WAT, white adipose tissue; BAT, brown adipose tissue. *P < 0.05 WT + 18:1n9 vs. WT + H2O and WT + 16:1n7.

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Table 4. Biochemical profile of epididymal adipocytes

<table>
<thead>
<tr>
<th></th>
<th>H2O</th>
<th>18:1n9</th>
<th>16:1n7</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAG lipases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal Lipolysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol, mmol/10^6 cells</td>
<td>3 ± 0.2</td>
<td>2 ± 0.2</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>Stimulated Lipolysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol, mmol/10^6 cells</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Fig. 4. Basal and isoproterenol-stimulated rates of glycerol release (nmol/10^6 cells; A and B, respectively) by isolated epididymal adipocytes from wild-type (WT) mice treated for 10 days by gavage with water or oleic acid (18:1n9, 300 mg·kg⁻¹·day⁻¹) or palmitoleic acid (16:1n7, 300 mg·kg⁻¹·day⁻¹). Results are means ± SE (n = 5–12/group). *P < 0.05 16:1n7 vs. water and 18:1n9; #P < 0.05 16:1n7 vs. water.
It is well established that ~30–40% of the fatty acids produced by lipolysis in adipocytes are reesterified back into TAG through a process that depends on intracellular glycerol 3-phosphate levels (15, 33, 42). Here, we have shown that lipolysis activation by palmitoleic acid is associated with an increase in the rates of fatty acid incorporation into TAG. This increase in fatty acid esterification is also associated with an elevation in glucose incorporation into the glycerol fraction of TAG, indicative of a higher glycerol 3-phosphate synthesis via glycolysis. Taken together, these data suggest an elevation in the recycling of the lipolytic product fatty acid back to TAG; however, this hypothesis remains to be investigated. A similar increase in fatty acid incorporation into TAG and glycerol 3-phosphate synthesis from glycolysis was also seen upon in vitro treatment with palmitic acid and in vivo treatment with oleic acid, suggesting that, in contrast to lipolysis, which is affected specifically by palmitoleic acid, an elevation into TAG synthesis is a rather common phenotype shared by all fatty acids investigated here. Furthermore, in contrast to lipolysis, TAG synthesis activation by palmitoleic acid does not depend on PPARα activity, as evidenced by the similar increase in TAG and glycerol 3-phosphate synthesis induced by palmitoleic acid in 3T3-L1 cells treated with PPARα antagonist and adipocytes of both wild-type and PPARα KO mice. All together, these findings indicate that palmitoleic acid enhances fatty acid esterification into TAG and glycerol 3-phosphate synthesis independently of lipolysis through a distinct mechanism that does not involve PPARα.

Nevertheless, the concomitant increase in antagonistic processes such as lipolysis and TAG synthesis, defined as TAG-
fatty acid cycling, by palmitoleic acid is not without precedent, resembling, with minor differences, that induced by pharmacological PPARα activation with rosiglitazone (6, 7, 20). Whereas palmitoleic acid stimulation of lipolysis is associated with increased expression of ATGL and HSL, activation of TAG hydrolysis by rosiglitazone is associated with an elevation in both ATGL and MGL mRNA levels. Furthermore, palmitoleic acid activation of TAG synthesis is linked to an increase in glycerol 3-phosphate synthesis from glycolysis, whereas PPARα activation enhances all three possible pathways of glycerol 3-phosphate synthesis, namely glycolysis, glyceroconogenesis, and glycerokinase (6, 7, 20). It is important to note that such an increase in TAG-fatty acid cycling has been shown not only to enhance cell energy expenditure but also to increase the sensitivity of both TAG synthesis and lipolysis to neuro/hormonal control in such a manner that small changes in the concentration of the neuro/hormonal factors regulating these pathways are able to induce dramatic changes in the direction of substrate flux in this cycle (2, 27, 44).

In conclusion, we have presented herein strong evidence indicating that palmitoleic acid, in addition to liver and skeletal muscle, controls important metabolic processes in adipose tissue. Our data indicate that palmitoleic acid is an important positive modulator of adipocyte lipolysis and the content of the major lipases ATGL and HSL through a PPARα-dependent mechanism. Further studies are required, however, to test whether this activation of lipolysis and lipases by palmitoleic acid would confer protective effects against excessive fat deposition found in obesity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.-B.-L., W.T.F., F.B.L., R.C., and M.I.C.A.-V.

REFERENCES


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