Impact on fatty acid metabolism and differential localization of FATP1 and FAT/CD36 proteins delivered in cultured human muscle cells

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Submitted 7 June 2004; accepted in final form 6 January 2005

Martínez, Cèlia García, Mario Marotta, Rodrigo Moore-Carrasco, Maria Guitart, Marta Camps, Silvia Busquets, Eulàlia Montell, and Anna M. Gómez-Foix. Impact on fatty acid metabolism and differential localization of FATP1 and FAT/CD36 proteins delivered in cultured human muscle cells, Am J Physiol Cell Physiol 288: C1264–C1272, 2005; doi:10.1152/ajpcell.00271.2004.—We compared the intracellular distribution and regulatory role of fatty acid transporter protein (FATP1) and fatty acid translocase (FAT/CD36) on muscle cell fatty acid metabolism. With the use of adenoviruses, FATP1 and FAT genes were delivered to primary cultured human muscle cells. FATP1 and FAT moderately enhanced palmitate and oleate transport even at concentrations of 0.05, 0.5, and 1 mM. Long-term (16 h) consumption of palmitate and oleate from the media, and particularly incorporation into triacylglyceride (TAG), was stimulated equivalently by FATP1 and FAT at all fatty acid concentrations tested. In contrast, long-term CO₂ production was reduced by FATP1 and FAT at all doses of palmitate and at the lower concentrations of oleate. Neither FATP1 nor FAT markedly altered the production of acid-soluble metabolic intermediates from palmitate or oleate. The intracellular localization of fusion constructs of FATP1 and FAT with enhanced green fluorescent protein (EGFP) was examined. Independently of fatty acid treatment, FATP1-GFP was observed throughout the cytosol in a reticular pattern and concentrated in the perinuclear region, partly overlapping with the Golgi marker GM-130. FATP1-GFP was found in the extracellular membrane and in cytosolic vesicles not coincident with GM-130. Neither FATP1 nor FAT proteins colocalized with lipid droplets in oleate-treated cells. We conclude that whereas FAT is localized on the extracellular membrane, FATP1 is active in the cytosol and imports fatty acids into myotubes. Overall, both FATP1 and FAT stimulated transport and consumption of palmitate and oleate, which they channeled away from complete oxidation and toward TAG synthesis.

palmitate; oleate; fatty acid binding proteins; skeletal muscle

MUSCLE CELLS TAKE UP FATTY ACIDS from the extracellular medium in a process facilitated by specialized fatty acid binding proteins that transport or trap the lipid moiety. Skeletal muscle cells express several types of fatty acid binding proteins: fatty acid translocase (FAT/CD36), plasma membrane-bound fatty acid binding protein (FABP-PM), the cytoplasmic heart-type fatty acid binding protein, and fatty acid transporter protein (FATP1) (5). FATP1 was identified by expression cloning based on the enhancement of intracellular accumulation of a fluorescent long chain fatty acid analog (25). It has no sequence homology with either FAT or FABP-PM, only one transmembrane domain, and multiple membrane-associated domains (17), but it displays high similarity to a family of very long chain CoA synthases and exhibits such enzyme activity in vitro (7). When overexpressed in adipocytes (26) or COS cells (7), FATP1 is predominantly found associated with a multitude of vesicles distributed throughout the cytoplasm, although in adipocytes, insulin induces accretion of endosome-like structures at the cell periphery (26). Indeed, whether FATP1 is a membrane transporter (20) or a fatty acid binding protein that facilitates import remains unclear.

In mice, FATP1 expression is highest in fat, skeletal muscle, and the heart (25). Intracellular FATP1 protein distribution has not been studied in muscle cells. Information about its physiological role in muscle comes exclusively from FATP1-knockout (FATP1-KO) mice (15). Deletion of the gene does not alter the metabolic phenotype of mice fed a starch-based diet but protects them from intramuscular accumulation of fatty acyl-CoA and triacylglyceride (TAG) induced by a fat diet without altering whole-body adiposity (15). Notably, in humans, homozygosity in a single nucleotide polymorphism within the FATP1 gene, consisting of an AG change at position 48 in intron 8, is associated with increased plasma TAG levels (9, 21), probably due to impaired peripheral postprandial uptake of fatty acids.

In contrast, genetic models have revealed much regarding the role of the very ubiquitous membrane transporter FAT (31). Overexpression of the protein in muscle from transgenic mice (13) does not stimulate oxidation of palmitate in isolated resting soleus muscle but greatly enhances this process in response to contraction. Strikingly, palmitate incorporation into TAG is unaffected or lower in both resting and contracting muscles from these transgenic mice. On the other hand, deletion of the FAT gene in mice impairs fatty acid transport and incorporation into TAG in skeletal muscle, while it increases free fatty acid and TAG levels in plasma (6). Similarly, spontaneous inactivating mutation of the gene in the insulin-resistant hypertensive rat is associated with hyperlipidemia (10). FAT/CD36 has been localized unequivocally in the plasma membrane of muscle cells. Furthermore, this localization is stimulated in myocytes by insulin or contraction at the expense of intracellularly stored protein (5). However, insulin concomitantly enhances fatty acid esterification, whereas contraction prompts oxidation, suggesting that proteins other than membrane-recruited FAT restrict fatty acid metabolism.

In this study, we overexpressed FATP1 and FAT genes in human muscle cells, allowing us to compare their intracellular localization and role in the control of uptake and direction of palmitate and oleate toward lipid pools or oxidation.

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MATERIALS AND METHODS

Construction of recombinant adenoviruses with FATP1 or FAT cDNA. Adenovirus AdCMV-FATP was constructed as follows. The full-length cDNA of mouse FATP1 gene was amplified by RT-PCR from total RNA isolated from C57BL/6 mouse muscle and verified by sequencing. A pAdCMV-FATP plasmid was constructed after subcloning the KpnI/EcoRI PCR fragment (1.9 kb) into pACCMVpLpA downstream from the cytomegalovirus (CMV) promoter. AdCMV-FATP was generated by homologous recombination (3). Meanwhile, AdCMV-FAT was constructed as follows. A 1.3-kb DNA fragment containing the full-length cDNA of rat FAT gene was excised with BamHI (1.3 kb) from the pSG5-FAT vector (kindly provided by Dr. K. Macé, Nestlé Research Center, Switzerland) and subcloned into pACCMVpLpA downstream from the CMV promoter. Plasmid pAdCMV-FAT was used to isolate the corresponding AdCMV-FAT virus. Titers of the viral stocks were determined using plaque assay of human embryonic kidney (HEK)-293 cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Adenoviruses, including the Escherichia coli lacZ gene expressing β-galactosidase, AdCMV-β-gal, or the enhanced green fluorescent protein (EGFP) AdCMV-GFP, were used as control virus.

We constructed the plasmids pFATGPFP and pFATGFP, which include NH2-terminal fusion protein constructs with EGFP. A 0.6-kb fragment of the FATP1 cDNA was isolated from pAdCMV-FATP by performing PCR. A mutation was introduced to knock out the stop codon while inserting a BamHI restriction site. **\footnote{Restriction site at 1,582 bp}** Ligation with 5'-CATTTCCTACATGCAAGTC-3' and 5'-TGGATCCCCGGTACCTCCGAGTGAGA-3' were used as upstream and downstream oligonucleotides, respectively, in the PCR. The PCR-amplified fragment was digested with Espl (restriction site at 1,582 bp) and BamHI and thereby ligated with a 1,582-bp EcoRI-Espl fragment of the FATP1 cDNA, which was isolated from the pAdCMV-FATP, into EcoRI-BamHI-digested pEGFP-N1 vector to generate pFATGPFP. To prepare pFATGFP, a 0.35-kb fragment of the FAT cDNA was isolated from pSG5-FAT by performing PCR, in which the stop codon was replaced by an AgeI restriction site, using 5'-CATTTGTCATGCAAGTC-3' and 5'-ATTATCCGGTITTTACCTTAG-3' as upstream and downstream oligonucleotides. This fragment was cloned into pGEM-T to generate pGEM-T-FAT0.35. A second fragment of the FATP1 cDNA was isolated from pAdCMV-FATP, into EcoRI-BamHI-digested pEGFP-N1 vector to generate pFATGPFP. To prepare pFATGFP, a 0.35-kb fragment of the FAT cDNA was isolated from pSG5-FAT by performing PCR, in which the stop codon was replaced by an AgeI restriction site, using 5'-CATTTGTCATGCAAGTC-3' and 5'-ATTATCCGGTITTTACCTTAG-3' as upstream and downstream oligonucleotides. This fragment was cloned into pGEM-T to generate pGEM-T-FAT0.35. A second fragment of the FAT cDNA was prepared by digesting pSG5-FAT vector with AgeI and NsiI and ligated into pGEM-T-FAT0.35 to generate pGEM-T-FAT. Finally, pFATGPFP was constructed by cloning the AgeI-AgeI FAT cDNA from pGEM-T-FAT into pEGFP-N1 vector digested with the same restriction enzymes. pFATGPFP and pFATGFP were excised with EcoRI-AflII and BamHI-NotI, respectively. The isolated bands were inserted into pACCMVpLpA to generate the corresponding adenovirus: AdCMV-FATGPFP or AdCMV-FATGFP.

Muscle cell cultures and adenoviral transduction. Human muscle biopsies were obtained with the approval of the Research Committee of the Hospital Vall d’Hebron, Barcelona, Spain. Human muscle primary cultures were begun from satellite cells of muscle biopsies from healthy individuals as described previously (2). Myoblasts were grown in DMEM/M-199 medium (3:1) with 10% FBS, 10 μg/ml insulin, 2 mM glutamine, 25 ng/ml fibroblast growth factor, and 10 ng/ml bovine nerve growth factor. Immediately after myoblast fusion, the medium was replaced by DMEM/M-199 devoid of growth factors and glutamine and with 10% FBS. Twelve-day-old myotubes were transduced with adenoviruses at a multiplicity of infection of 50 for 2 h. In these conditions, transduction efficiency was ~90% (data not shown). In all studies, cells were used 3 days after transfection. Twenty-four hours before the metabolic experiments, cells were depleted of insulin and FBS and incubated with DMEM containing 5 mM glucose with or without fatty acids.

Fatty acid preparation and metabolic analysis. Sodium salts of palmitic and oleic acids were prepared in deionized water containing 1.2 equivalents of NaOH at 70°C until an optically clear dispersion was observed. The fatty acid salt solution was added to DMEM containing fatty acid-free bovine serum albumin (BSA) with continuous agitation. The fatty acid-to-BSA molar ratio was 5:1 or 2.5:1, within the physiological range (6, 24, 27).

To measure fatty acid uptake, cells were rinsed with phosphate-buffered saline (PBS) containing 0.1 mM CaCl2 and 0.1% BSA and incubated in glucose-deprived DMEM with [1-14C]oleate (2.8 μCi/μmol) or [1-14C]palmitate (2.8 μCi/μmol) for 2 min. To stop the reaction, cells were rinsed with ice-cold PBS solution. Finally, monolayers were extracted in PBS that contained 1% SDS, and aliquots were measured for radioactivity in 5 ml of scintillation cocktail.

To detect the incorporation of fatty acids into lipids, cells were incubated with [1-14C]fatty acid (1 μCi/μmol). Monolayers were then rinsed with PBS, and lipids were extracted twice with hexane/isopropanol (3:2). After being dried under a nitrogen stream, the lipid extract was dissolved in chloroform:methanol (2:1) and TAG and phospholipids were separated using thin-layer chromatography (TLC) with hexane-diethyl ether-acetic acid (70:30:1). The lipid spots were identified by iodine vapor, scraped, and counted in a liquid scintillation cocktail.

To measure fatty acid oxidation rate, cells in 24-well plates were incubated with [1-14C]oleate (2.8 μCi/μmol) or [1-14C]palmitate (2.8 μCi/μmol). The incubation was terminated by addition of 125 μl of 3 M HClO4 to each well. Immediately, a Whatman no. 3 paper soaked with 25 μl of β-phenylethylamine was placed over each well, which was then tightly covered and sealed with elastic film. After 1 h of incubation at room temperature, the filter paper was cut and the trapped 14CO2 was quantified in a scintillation cocktail. Cell extracts containing acid-soluble intermediates were centrifuged at 12,000 g, and the supernatant was counted for radioactivity.

To determine the TAG content, extracts were prepared by scraping cell monolayers into a buffer consisting of 50 mM Tris, 20 mM KF, 0.5 mM EDTA, and 0.05% Lubrol PX, pH 7.9, and applying three 5-s pulses of sonication. Homogenates were centrifuged at 11,000 g for 15 min, and the resulting supernatants were collected. Protein concentration was measured with Bio-Rad protein assay reagent. TAGs were measured enzymatically with a Cobas Fara II autoanalyzer and a GPO-Trinder kit using triolein resuspended in the extraction buffer as a standard.

mRNA analysis. Total RNA from myotubes was extracted using a guanidinium hydrochloride-based method. RNA (20 μg) was denatured, electrophoresed on 1.2% formaldehyde-agarose gels, and transferred to positively charged nylon membranes. Ethidium bromide (0.2 μg) was added to RNA samples to check equal loading of the gels and transfer efficiency. Prehybridization and hybridization were performed at 65°C using 0.25 M Na2HPO4 (pH 7.2), 1 mM EDTA, 7% SDS, and 1% blocking reagent solution. Blots were hybridized using as probes a 1.2-kb BglII-BamHI fragment of the mouse cDNA for FATP1, which is highly homologous to the human sequence between nucleotides 915 and 2,082 included in the probe, and the full-length cDNA (1.3 kb BamHI) of the rat FAT, which is highly homologous to the human mRNA. DNA probes were labeled with [α-32P]dCTP using the random oligonucleotide primer method.

Confocal microscopy. Cells seeded onto coverslips were fixed with 3% paraformaldehyde, rinsed, and mounted in Immuno-Fluor. Fluorescence images were obtained with a Leica TCS four-dimensional confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope and ×63 (NA ×1.4 oil immersion) Leitz Plan-Apo lens objective. The light source was an argon/krypton laser (75 mW). Green fluorescence from GFP recombinants was excited at 488 nm, and optical sections (0.1 μm) were obtained. Lipid droplets were revealed by staining with Nile Red, which was included in the mounting medium, at 1:100 dilution from a saturated stock solution in aceton. The Nile Red image was captured as described previously (23).

Muscle cells seeded onto glass coverslips were fixed in PBS containing 3% (wt/vol) paraformaldehyde-60 mM sucrose. The cells
Overexpression of FATP1 or FAT in primary cultured human muscle. Myotubes were exposed to AdCMV-FATP or AdCMV-FAT. Three days after transduction, mRNA levels of FATP1 and FAT were analyzed using Northern blotting. While expression of the two genes was hardly detectable in control cells exposed to AdCMV-β-gal (data not shown), AdCMV-FATP- or AdCMV-FAT-treated cells showed high expression of FATP1 and FAT mRNA, respectively. These levels were too high to be compared with basal values (Fig. 1).

Palmitate and oleate transport. Fatty acid transport was measured in cells incubated with 0.05, 0.5, or 1 mM [1-14C]palmitate or [1-14C]oleate, the prevalent circulating fatty acids, and glucose (Fig. 2). In control and genetically engineered cells, uptake of palmitate and oleate increased linearly with the concentration of the substrate ($R^2 = 0.9802$ for palmitate; $R^2 = 0.9806$ for oleate). In controls, no significant differences between uptake of palmitate and oleate were detected. The fatty acid-to-BSA ratio was tested. At 0.5 mM fatty acid to 0.2 mM BSA, the palmitate uptake in control cells was $3.7 \pm 0.30$ nmol·mg of protein$^{-1}$·min$^{-1}$ and that of oleate was $4.9 \pm 0.28$ nmol·mg of protein$^{-1}$·min$^{-1}$. Therefore, no significant differences were observed by halving this ratio. Overexpression of FATP1 and FAT enhanced palmitate uptake similarly at all concentrations of fatty acids tested (increments were between 20 and 40%) (Fig. 2). The enhancement was similar when fatty acids were provided at a ratio of BSA of 2.5 (data not shown).

Palmitate and oleate long-term consumption. We measured fatty acid depletion from cell media during a 16-h incubation with 0.05, 0.5, or 1 mM [1-14C]palmitate or [1-14C]oleate (Fig. 3). In control cells, palmitate and oleate consumption were comparable at all doses tested, but slightly higher (1.2-fold) for oleate. Consumption rose linearly for palmitate ($R^2 = 0.9807$) and oleate ($R^2 = 0.9922$) with the fatty acid concentration. Overexpression of both FATP1 and FAT equivalently enhanced palmitate depletion, causing increments of >40% at 0.05 mM and of 20% at both 0.5 and 1 mM. FATP1 and FAT overexpression led to similar increments (~40%) in oleate depletion. The effect of reducing the fatty acid-to-BSA ratio from 5 to 2.5 was examined. Depletion at 0.5 mM fatty acid (expressed as nmol·mg of protein$^{-1}$·16 h$^{-1}$), were $219 \pm 23$, $262 \pm 10$, and $266 \pm 5$ (for palmitate) and $332 \pm 19$, $395 \pm 13$, and $407 \pm 16$ (for oleate) in control, FATP1-, and FAT-overexpressing cells, respectively. Therefore, the quantity of 0.5 mM palmitate or oleate depleted was reduced to about half, while the effects of FATP1 or FAT were preserved.
Palmitate and oleate incorporation into cell lipids. We examined the effects of these two fatty acid binding proteins on [1-14C]palmitate or [1-14C]oleate incorporation during a 16-h period in cell lipid fractions at 0.05, 0.5, or 1 mM (Table 1). In control cells, radioactivity incorporated in phospholipids increased linearly with palmitate (R² = 0.9979) and oleate (R² = 0.9985), whereas that in TAGs tended to saturate from 0.5 to 1 mM. Unlike palmitate, oleate was more readily incorporated in TAGs than in phospholipids as shown earlier (22). In control cells, total incorporation of oleate was higher (at least 1.4-fold) than that of palmitate in all experimental conditions. Both FATP1 and FAT enhanced the utilization of 0.05 mM palmitate or oleate for TAG (>2-fold) and phospholipid (~1.5-fold) synthesis. No significant effects on phospholipid synthesis by FATP1 and FAT were observed for either fatty acid at higher doses (0.5 or 1 mM). In contrast, TAG synthesis from 0.5 or 1 mM palmitate was enhanced, ~2- and ~1.5-fold, respectively, by either FATP1 or FAT overexpression. TAG synthesis from 0.5 or 1 mM oleate was also enhanced, but only ~1.5-fold, by FATP1 and FAT overexpression. The effect of halving the fatty acid-to-BSA ratio was tested. The amounts of fatty acids incorporated in control, FATP1-, or FAT-overexpressing cells, respectively, into TAGs at 0.5 mM fatty acid (expressed as nmol·mg of protein⁻¹·16 h⁻¹) were 19 ± 3, 46 ± 5, and 51 ± 3 (for palmitate) and 98 ± 7, 122 ± 6, and 129 ± 12 (for oleate), and in phospholipids they were 61 ± 4, 61 ± 2, and 69 ± 5 (for palmitate) and 75 ± 9, 68 ± 5, and 82 ± 6 (for oleate). Thus, whereas incorporation in phospholipids was preserved for either fatty acid, synthesis of TAG was strongly reduced in all cell types. Stimulation of TAG synthesis by FATP1 and FAT was similar.

Accumulation of TAG in cells overexpressing FATP1 or FAT. TAG was measured in extracts from cells incubated with glucose with or without fatty acids (Fig. 4). TAG concentration increased 2.6-fold 16 h after addition of 0.5 mM palmitate and 17.6-fold with 0.5 mM oleate compared with cells incubated without fatty acids. In cells transduced with either AdCMV-FATP or AdCMV-FAT, minor differences in TAG levels were detected in a medium devoid of fatty acids. FATP1 increased TAG levels (57%) after addition of palmitate, while the FATP1 and FAT enhanced the utilization of 0.05 mM palmitate or oleate for TAG (>2-fold) and phospholipid (~1.5-fold) synthesis. No significant effects on phospholipid synthesis by FATP1 and FAT were observed for either fatty acid at higher doses (0.5 or 1 mM). In contrast, TAG synthesis from 0.5 or 1 mM palmitate was enhanced, ~2- and ~1.5-fold, respectively, by either FATP1 or FAT overexpression. TAG synthesis from 0.5 or 1 mM oleate was also enhanced, but only ~1.5-fold, by FATP1 and FAT overexpression. The effect of halving the fatty acid-to-BSA ratio was tested. The amounts of fatty acids incorporated in control, FATP1-, or FAT-overexpressing cells, respectively, into TAGs at 0.5 mM fatty acid (expressed as nmol·mg of protein⁻¹·16 h⁻¹) were 19 ± 3, 46 ± 5, and 51 ± 3 (for palmitate) and 98 ± 7, 122 ± 6, and 129 ± 12 (for oleate), and in phospholipids they were 61 ± 4, 61 ± 2, and 69 ± 5 (for palmitate) and 75 ± 9, 68 ± 5, and 82 ± 6 (for oleate). Thus, whereas incorporation in phospholipids was preserved for either fatty acid, synthesis of TAG was strongly reduced in all cell types. Stimulation of TAG synthesis by FATP1 and FAT was similar.

Table 1. Effects of FATP1 and FAT on fatty acid incorporation into cell lipids

<table>
<thead>
<tr>
<th></th>
<th>AdCMV-β-Gal</th>
<th>AdCMV-FATP</th>
<th>AdCMV-FAT</th>
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<tr>
<td></td>
<td>TAG, nmol</td>
<td>PL, nmol</td>
<td>TAG, nmol</td>
</tr>
<tr>
<td>Palmitate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 mM</td>
<td>14 ± 0.8</td>
<td>14 ± 0.4</td>
<td>34 ± 2‡</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>55 ± 7</td>
<td>47 ± 6</td>
<td>118 ± 8‡</td>
</tr>
<tr>
<td>1 mM</td>
<td>67 ± 6</td>
<td>90 ± 7</td>
<td>90 ± 5*</td>
</tr>
<tr>
<td>Oleate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 mM</td>
<td>24 ± 1</td>
<td>15 ± 0.8</td>
<td>59 ± 5‡</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>150 ± 8</td>
<td>51 ± 10</td>
<td>217 ± 9*</td>
</tr>
<tr>
<td>1 mM</td>
<td>170 ± 9</td>
<td>86 ± 7</td>
<td>207 ± 21</td>
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Cells transduced with adenovirus (Ad) cytomegalovirus (CMV)-β-galactosidase (AdCMV-β-gal; control), AdCMV-fatty acid transporter protein (FATP), or AdCMV-FAT were incubated for 16 h with 0.05, 0.5, or 1 mM [1-14C]palmitate or [1-14C]oleate in medium containing 5 mM glucose with neither fetal bovine serum (FBS) nor insulin. Cell lipids were extracted, separated using thin-layer chromatography (TLC), and radioactivity associated with triacylglycerols (TAGs) and phospholipids (PL) was quantified. Data are means ± SE from 3 experiments performed in triplicate and are expressed as nmol fatty acid incorporated·mg of protein⁻¹·16 h⁻¹. Significance of differences vs. cells treated with control virus: *P < 0.05. †P < 0.01. ‡P < 0.001.
production levels were consistently higher from palmitate than from oleate; they were >2-fold at 0.5 and 1 mM and ~1.5-fold at 0.05 mM. FATP1 and FAT overexpression lowered the production of [14CO2] from palmitate (between 20% and 30%) at all doses tested. [14CO2] production from oleate was also inhibited by both FATP1 and FAT at 0.05 mM (~40% reduction) and 0.5 mM (20%), whereas at 1 mM, they had no significant effect. Neither FATP1 nor FAT had a clear effect on ASI production. The reduction of the fatty acid-to-BSA ratio to half did not alter the amount of 0.5 mM palmitate or oleate incorporated into either [14CO2] or ASI compared with the ratio of 5. The effects of FATP1 or FAT were unchanged (data not shown).

**Localization of FATP1 and FAT in transduced myotubes.** To determine the intracellular localization of overexpressed FATP1 and FAT (Fig. 5), NH2-terminal fusion constructs with EGFP were prepared and inserted into AdCMV-FATPGFP or AdCMV-FATGFP, respectively. The stability of the fusion proteins was confirmed using Western blot analysis of muscle proteins. EGFP was localized intracellularly in a reticular pattern throughout the cytoplasm and at a higher density in the perinuclear region. In cells devoid of fatty acids, in which the FATGFP gene was transferred (Fig. 5, C–E), the GFP protein was observed along the cell membrane and inside the cell, where it was present in vesicles of various sizes. These two distribution patterns clearly differed from the pattern of GFP alone, which was present uniformly throughout the cytoplasm and concentrated inside the nuclei (Fig. 5F). Therefore, despite overloading myotubes with FATPGFP or FATGFP proteins, the former did not target the plasma membrane, whereas the latter was evidently localized in this compartment. We further examined FATPGFP localization in rat C2C12 myotubes and found a homologous pattern of distribution (data not shown). To analyze the potential overlap of lipid droplets with FATP1 and FAT proteins, human muscle cells were incubated with 0.5 mM oleate to induce the accumulation of TAG stores and lipid vesicles were labeled with Nile Red stain, which specifically binds to neutral lipids (Fig. 6). Control cells showed abundant lipid vesicles in the cytoplasm, which were either aligned or concentrated in the perinuclear region (Fig. 6, B and E). No significant alteration in the lipid droplet pattern was detected in cells overexpressing either of the fatty acid binding proteins as deduced by comparison between nontransfected and transected cells (Fig. 6, B and E). In addition, the localization of FATP1 or FAT was not affected in oleate-treated cells [compare Fig. 6A (FATP1) and Fig. 6D (FAT) with Fig. 5B (FATP1) and Fig. 5C (FAT)]. The profile of lipid vesicles differed markedly from the intracellular distribution of FATPGFP or FATGFP as evidenced by overlapping the corresponding images (Fig. 6, C and F, respectively). To examine the nature of the vesicles in which fatty acid binding proteins were associated, a specific marker of the Golgi system, GM-130, was coanalyzed using immunofluorescence (Fig. 7, B and E). The merging of FATPGFP and the Golgi marker images revealed widespread, yet partial, overlap of these two proteins (Fig. 7C). In contrast, no overlap was detected between the Golgi resident protein GM-130 and FATGFP (Fig. 7F).

**DISCUSSION**

In this study, we have demonstrated, first, that overexpression of FATP1 in cultured myotubes evenly enhances palmitate and oleate transport at a range of concentrations from 0.05 to 1 mM. The equivalent specificity of FATP1 for these two long chain fatty acids, shown in cultured muscle, is consistent with

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**Table 2. Oxidation of fatty acids in FATP1- and FAT-overexpressing cells**

<table>
<thead>
<tr>
<th>AdCMV-β-gal</th>
<th>AdCMV-FATP</th>
<th>AdCMV-FAT</th>
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<tr>
<td><strong>Palmitate</strong></td>
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<tr>
<td>0.05 mM</td>
<td>219±11</td>
<td>25±0.8</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>836±31</td>
<td>69±1.2</td>
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<td>1 mM</td>
<td>1322±91</td>
<td>108±0.7</td>
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<tr>
<td><strong>Oleate</strong></td>
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<td></td>
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<tr>
<td>0.05 mM</td>
<td>143±2</td>
<td>20±0.4</td>
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<tr>
<td>0.5 mM</td>
<td>308±11</td>
<td>24±0.8</td>
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<td>1 mM</td>
<td>442±28</td>
<td>54±0.6</td>
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<tr>
<th>14CO2, pmol</th>
<th>ASI, nmol</th>
<th>14CO2, pmol</th>
<th>ASI, nmol</th>
<th>14CO2, pmol</th>
<th>ASI, nmol</th>
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<tbody>
<tr>
<td>Palmitate</td>
<td></td>
<td></td>
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<tr>
<td>0.05 mM</td>
<td>156±15‡</td>
<td>22±0.5†</td>
<td>172±7†</td>
<td>22±0.3†</td>
<td></td>
</tr>
<tr>
<td>0.5 mM</td>
<td>638±15‡</td>
<td>71±2.2</td>
<td>643±26‡</td>
<td>67±1.1</td>
<td></td>
</tr>
<tr>
<td>1 mM</td>
<td>1031±12§</td>
<td>96±0.4§</td>
<td>883±23*</td>
<td>91±1.4§</td>
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<tr>
<td>Oleate</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0.05 mM</td>
<td>88±3‡</td>
<td>18±0.7*</td>
<td>90±2‡</td>
<td>19±0.5</td>
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</tr>
<tr>
<td>0.5 mM</td>
<td>235±8†</td>
<td>23±0.2</td>
<td>243±11‡</td>
<td>22±0.7</td>
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<tr>
<td>1 mM</td>
<td>500±34</td>
<td>64±1.9†</td>
<td>420±23</td>
<td>49±1.7</td>
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Cells exposed to AdCMV-β-gal (control), AdCMV-FATP, or AdCMV-FAT were incubated for 16 h with 0.05, 0.5, or 1 mM [1-14C]palmitate or [1-14C]oleate as indicated in medium containing 5 mM glucose without FBS or insulin. Radioactivity in the acid-soluble intermediates (ASI) or 14CO2 fractions was quantified and expressed as pmol/mg of protein of -1 h-1 (14CO2) and nmol/mg of protein of -1 h-1 (ASI). Data are means ± SE from 3 experiments performed in triplicate. Significance of differences versus cells treated with control virus: *P < 0.05, †P < 0.01, §§P < 0.001.
earlier studies in adipocytes (25). FAT stimulates palmitate and oleate transport to a similar extent. In most previous studies of FAT activity, stimulation of palmitate use has been observed (6, 13), while oleate has generated inconsistent results (14).

We also report that FATP1 and FAT moderately stimulate long-term consumption of palmitate and oleate in the range of concentrations tested, with higher enhancement at 0.05 mM than at 0.5 or 1 mM. Notably, FATP1 strongly stimulated the utilization of palmitate and oleate for TAG synthesis. Stronger effects were exerted at the lower doses of fatty acids, and the stimulation was higher for palmitate than oleate, which is more efficiently used than palmitate for TAG synthesis (22). In contrast, incorporation of fatty acids in phospholipids was stimulated by FATP1 only at the lowest dose (0.05 mM). FATP1 data are consistent with the observation that in FATP1-KO mice, intramuscular accumulation of TAGs and acyl-CoA is severely impaired in animals fed fat diets, while no differences are observed in animals fed a standard chow diet (15). There are no other studies of FATP1 overexpression in muscle cells, but in growing epithelial HEK-293 cells it has been shown to also enhance oleic acid uptake and channel its incorporation into TAGs (11). The effects of FAT on the channeling of palmitate and oleate to TAGs and phospholipids synthesis were similar to those of FATP1. In transgenic mice (13), which show FAT overexpression in muscle, palmitate incorporation into TAGs was not enhanced, suggesting that this pathway is saturated in vivo. Nevertheless, in FAT/CD36-KO mice, incorporation of a fatty acid analog to muscle TAG is restricted (6), which indicates the involvement of FAT.

Fig. 5. Green fluorescent protein (GFP) confocal microscopic images showing localization of AdCMV-FATPGFP and AdCMV-FATGFP. Cells were exposed to AdCMV-GFP (F), AdCMV-FATPGFP (A and B), or AdCMV-FATGFP (C–E). Images were obtained using a confocal microscope at ×63 magnification with diverse zoom. Bars, 10 μm. Asterisks mark the subcellular localization: perinuclear (B), plasma membrane (C–E), and vesicular (C–E).
The effects of FATP1 and FAT on fatty acid oxidation in skeletal muscle cells were studied. In all cell types and at all concentrations of fatty acid tested, palmitate was more readily oxidized than oleate to CO2 and to acid-soluble metabolites. ASI accounted for a higher proportion of the oxidation products than CO2 (1, 28). FATP1 and FAT overexpression reduced the proportion of palmitate that was completely oxidized, while the production of intermediate metabolites was not markedly altered. In transgenic mice, FAT did not stimulate palmitate oxidation in resting muscle (13), although after contraction, a marked enhancement was detected. CO2 production from oleate was also inhibited by FATP1 and FAT but only at concentrations <1 mM, while the levels of intermediate metabolites were unchanged as well. The finding that FATP1 and FAT reduce CO2 production without markedly changing acid-soluble metabolites suggests that β-oxidation is not specifically inhibited, while disposal of acetyl-CoA to oxidation is affected. Further work is necessary to elucidate the mechanism of action of FATP1 and FAT.

Moreover, we have demonstrated that reducing the ratio of 0.5 mM palmitate or oleate to BSA did not modify the metabolic effects of FATP1 or FAT. Neither transport nor fatty acid oxidation or ASI or CO2 production was affected by the BSA ratio. However, palmitate and oleate depletion were markedly reduced, as was the incorporation of either fatty acid to TAGs, but not in phospholipids. These data suggest that whereas transport, incorporation into phospholipids, and oxidation are saturated by unbound fatty acid at 0.5 mM fatty acid-0.2 mM BSA, consumption and channeling toward TAG synthesis are not, as shown for other fatty acid actions (29).

The cellular localization of FATP1 and FAT was analyzed by means of fusion constructs with GFP. FATPGFP was dispersed mainly through the cytosol in a reticular pattern and concentrated in the perinuclear region and was partially colocalized with a specific marker of the Golgi system, GM-130. Although FATP1 was originally considered an integral plasma membrane protein (25), its single transmembrane domain and very short amino-terminal stretch exposed to the exterior of the cell provide an unusual structure for facilitating transport across the membrane (17). FATP1 has been localized on the plasma membrane of HEK-293 cells (11, 25), but immunofluorescence studies of endogenous FATP1 in 3T3-L1 adipocytes (25, 26) and transfected GFP fusion constructs in COS-7 cells (17) show a distribution consistent with an internal membrane-associated protein, with endoplasmic reticulum and reticular pattern, and with great overlap with the Golgi marker GM-130 (26), in striking similarity to the muscle cell. In separate experiments, we incubated cells with oleate to induce maximal TAG accumulation, and lipid droplets were revealed by staining with Nile Red. Lipid droplets were found throughout the cytoplasm in linear arrangements or concentrated in masses around the nuclei. FATP1 localization was not affected by oleate treatment, nor was the lipid droplet pattern modified by FATP1 overexpression. Indeed, distribution of lipid droplets

![Image](http://ajpcell.physiology.org/)

**Fig. 6.** Colocalization analysis of lipid droplets and FATPGFP and FATGFP. Cells were exposed to AdCMV-FATPGFP (A–C) or AdCMV-FATGFP (D–F) and then stained with Nile Red. Confocal microscopic images of lipid droplets (B and E) and GFP (A and D) are shown. No colocalization (yellow) of lipids and FATPGFP or FATGFP was observed as shown in C and F, respectively. Bars, 10 μm.
did not overlap with that of FATP1. Overall, our data indicate that the translocation of FATP1 to the extracellular membrane is not required for the promotion of fatty acid uptake. Nevertheless, we cannot rule out the possibility that if FATP1 had been translocated in an endosome-like structure to the cell periphery as described in adipocytes (26), a greater effect might have been observed. We suggest that FATP1 may instead function within an intracellular protein complex that may include acyl-CoA synthase, which colocalizes with FATP1 in adipocytes (8) and other currently undefined proteins. FATP1 may enhance fatty acid import to this protein complex, trapping fatty acids and in that way facilitating their access to the specific acyl-CoA synthase or other enzymes in the synthetic pathway of TAG. It is also possible that FATP1 accounts for part of the synthesis of acyl-CoA by means of its reported acyl-CoA synthase activity in vitro (7, 8). The suggestion that FATP1 may not function as a fatty acid transporter has been made previously after expression of the functional FATP1-Myc/His fusion protein in COS1 cells (7).

FATGFP, independently of fatty acid availability, was predominantly localized in the extracellular membrane of muscle cells and intracellularly in the periphery of vesicles, which did not overlap with lipid droplets or the Golgi marker. Other studies, in which cell fractionation and subsequent immunodetection techniques were applied, reported a similar distribution profile in skeletal (4, 19) and cardiac (18) myotubes. FAT contains multiple lipid modification sites (16) and has been found in Chinese hamster ovary and C32 cells in association with lipid rafts and membrane domains rich in cholesterol and sphingolipids and absent from caveolae and clathrin-coated pits (30). Indeed, in myocytes, FAT, like other lipid raft-associated proteins, translocates to the plasma membrane upon stimulation by insulin (19) or contraction (18).

In summary, we have demonstrated that in cultured human myotubes FATP1 is found in the cytosol, while FAT is overtly present in the extracellular membrane. Nevertheless, both FATP1 and FAT enhance palmitate and oleate transport and consumption and direct these fatty acids toward TAG synthesis while inhibiting their aerobic oxidation. Our findings indicate that FATP1 may trap and draw fatty acid toward their accumulation rather than restrict their transport.

ACKNOWLEDGMENTS

We thank Susana Castell, Raquel García, and Nieves Hernandez at the Serveis Científicote cènics of the Universitat de Barcelona and Ana Orozco and Josep Parnau for technical assistance.

GRANTS

A. M. Gómez-Foix is the recipient of a fellowship from the Generalitat de Catalunya. C. García-Martínez is the recipient of a Ramon y Cajal fellowship from the Ministerio de Educación y Ciencia, Spain. This work was supported by Grant SAF-2003-04223 from the Ministerio de Ciencia y Tecnología (to M. Camps) and C03/08 Red de Centros de Metabolismo y Nutrición del Instituto de Salud Carlos III, Spain.
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