Eicosapentaenoic acid (20:5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells

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Abstract This study was conducted to evaluate the chronic effects of eicosapentaenoic acid (EPA) on fatty acid and glucose metabolism in human skeletal muscle cells. Uptake of [^{14}C]oleate was increased >2-fold after preincubation of myotubes with 0.6 mM EPA for 24 h, and incorporation into various lipid classes showed that cellular triacylgycerol (TAG) and phospholipids were increased 2- to 3-fold compared with control cells. After exposure to oleic acid (OA), TAG was increased 2-fold. Insulin (100 nM) further increased the incorporation of [^{14}C]oleate into all lipid classes for EPA-treated myotubes. Fatty acid β-oxidation was unchanged, and complete oxidation (CO_2) decreased in EPA-treated cells. Basal glucose transport and oxidation (CO_2) were increased 2-fold after EPA, and insulin (100 nM) stimulated glucose transport and oxidation similarly in control and EPA-treated myotubes, whereas these responses to insulin were abolished after OA treatment. Lower concentrations of EPA (0.1 mM) also increased fatty acid and glucose uptake. CD36/FAT (fatty acid transporter) mRNA expression was increased after EPA and OA treatment compared with control cells. Moreover, GLUT1 expression was increased 2.5-fold by EPA, whereas GLUT4 expression was unchanged, and activities of the mitogen-activated protein kinase p38 and extracellular signal-regulated kinase were decreased after treatment with OA compared with EPA.▮▮ Together, our data show that chronic exposure of myotubes to EPA promotes increased uptake and oxidation of glucose despite a markedly increased fatty acid uptake and synthesis of complex lipids.—Aas, V., M. H. Rokling-Andersen, E. T. Kase, G. H. Thoresen, and A. C. Rustan. Eicosapentaenoic acid (20:5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells. *J. Lipid Res.* 2006. **47:** 366–374.

Supplementary key words human skeletal myotubes • lipid metabolism • glucose metabolism

Skeletal muscle is the major site of insulin-stimulated glucose disposal, and insulin resistance of skeletal muscle is strongly linked to the development of type 2 diabetes.

Increased lipid availability is thought to play a role for insulin resistance in skeletal muscle (1, 2). It has long been recognized that increased plasma FFAs as well as triacylglycerol (TAG) are associated with insulin resistance in vivo in humans, and several studies show that the intramyocellular content of lipids (e.g., TAG) is increased (1–5). Both the amount and type of fatty acids are important. Diets high in saturated fats or rich in linoleic acid and n-6 fatty acids lead to insulin resistance mainly as a result of effects in oxidative skeletal muscle (6, 7). The physiological alterations in metabolic flux induced by high-saturated-fat feeding mimic those reported in patients with type 2 diabetes (8). However, replacement of some of the fat with long-chain n-3 PUFAs prevents the development of insulin resistance caused by high-fat feeding (9–11). The mechanism by which fish oil improves peripheral insulin resistance is unclear, but it may involve changes in glucose (12) as well as fatty acid metabolism in skeletal muscle. Mechanisms may be linked to membrane incorporation (13), changes in the level of muscle lipid species such as acyl-CoA (14), diacylglycerol, and lipid storage (TAG) (2, 5), altered lipid oxidation (15, 16), and/or interference with insulin signaling (17). Reduced plasma concentrations of TAG and FFA observed after dietary intake of n-3 fatty acids may also contribute to less lipid flux to skeletal muscle, decreased muscular fatty acid oxidation, and lipid storage (9).

This study was conducted to determine the effects and explore the mechanisms of long-term exposure to eicosapentaenoic acid (EPA; an n-3 PUFA from fish oil) on glucose and lipid metabolism in human myotubes. The actions of EPA were compared with those of oleic acid (OA; a common monounsaturated fatty acid) and a fatty acid-free control (containing BSA).

EXPERIMENTAL PROCEDURES

Materials

Bovine serum albumin (essentially fatty acid-free), cytochalasin B, 2-deoxy-D-glucose, extracellular matrix gel, glycogen (rabbit liver), L-carnitine, and OA were purchased from Sigma-Aldrich (St. Louis, MO). EPA was purchased from NU-Chek-Prep (Elysian, MN). 2-[^{3}H(G)]deoxy-D-glucose (10 Ci/mmol), D-[^{14}C(U)]glucose (12.1 mCi/mmol), D-[1-^{14}C]glucose (55 mCi/mmol), and [1-^{14}C]OA (53 mCi/mmol) were provided by Du Pont NEN™ Life Sciences Products (Boston, MA). 2-[^{3}H(G)]deoxy-D-glucose (10 Ci/mmol), D-[^{14}C(U)]glucose (315 mCi/mmol), and 1-[^{14}C]glucose (55 mCi/mmol) were purchased from American Radiolabeled Chemicals. L-Glutamine, 0.05% trypsin/EDTA, penicillin/streptomycin, fetal calf serum (FCS), and minimum essential medium α (αMEM) were purchased from Gibco (Paisley, UK). Skeletal muscle cell growth medium with bullet kit was obtained from Clonetics, BioWhittaker (Verviers, Belgium). Insulin Actrapid® was obtained from Novo Nordisk (Bagsvaerd, Denmark). Cell culture equipment (Costar®; tissue culture-treated) was purchased from Corning Life Sciences (Schiphol-Rijk, The Netherlands). RNeasy Mini kit and RNase-free DNase were purchased from Qiagen Nordic (Crawley, UK), whereas the Dynabeads mRNA DIRECT kit was from Dynal (Oslo, Norway). The primers for GLUT1, GLUT4, CD36/FAT (fatty acid transporter), diacylglycerol acyltransferase-1, and β-actin, SYBR® Green, and the TaqMan reverse transcription reagents kit were from Applied Biosystems (Warrington, UK) (**Table 1**). TLC plates (silica gel) were from Merck (Darmstadt, Germany). The scintillation liquids Instagel and Hionic were from Packard Bioscience Co. (Groningen, The Netherlands). All other chemicals used were standard commercial, high-purity quality.

Human skeletal muscle cell cultures

A cell bank of satellite cells was established from muscle biopsy samples of the musculus vastus lateralis of six healthy volunteers, age 24.7 ± 0.7 years, body mass index 23.6 ± 1.1 kg/m^2, fasting glucose and insulin within normal range, and with no family history of diabetes. The biopsies were obtained with informed consent and approval by the National Committee for Research Ethics (Oslo, Norway). Muscle cell cultures free of fibroblasts were established by the method of Henry et al. (18) with minor modifications. Briefly, muscle tissue was dissected in Ham's F-10 medium at 4°C and dissociated by three successive treatments with 0.05% trypsin/EDTA, and satellite cells were resuspended in skeletal muscle cell growth medium with 2% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml amphotericin B, and no added insulin. The cells were grown on culture wells or flasks coated with extracellular matrix gel (19). After 1–2 weeks at ~80% confluence, growth medium was replaced by αMEM with 2% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1.25 μg/ml amphotericin B to induce the differentiation of myoblasts into multinucleated myotubes. The cells were cultured in a humidified 5% CO_2 atmosphere at 37°C, and medium was changed every 2–3 days. All cells used in the experiments were subcultured three to six times. All myotube cultures were used for analysis on day 8 after the onset of differentiation.

Pretreatment of myotubes with fatty acids

On day 7, differentiated myotubes were pretreated with 0.6 mM fatty acids, either OA or EPA, in αMEM with 2% FCS for 24 h. Fatty acids (0.6 mM) were bound to 0.24 mM fatty acid-free albumin (BSA) (FA/BSA = 2.5:1). BSA (0.24 mM) was used as fatty acid-free control.

Lipid distribution

Myotubes were incubated on six-well plates for 4 h in αMEM with [1-^{14}C]OA (0.5 μCi/ml, 0.6 mM) and 0.24 mM fatty acid-free albumin (BSA) with or without 100 nM insulin to study insulin-mediated or basal cellular oleate distribution, respectively. After incubation, myotubes were placed on ice, washed twice with ice-cold PBS, harvested into a tube in two additions of 250 μl of distilled water, and stored at −20°C. The cells were later sonicated and assayed for protein content, and cellular lipids were extracted. The homogenized cell fraction (400 μl) was mixed with 20 volumes of chloroform-methanol (2:1, v/v) (20) with FCS (50 μl) as a carrier. After 30 min, 4 volumes of 0.9% NaCl (pH 2) was added, and the mixture was centrifuged (1,000 g for 5 min). The organic phase was evaporated under a steam of nitrogen at 45°C. The residual lipid extract was redissolved in 200 μl of n-hexane and separated by TLC, using hexane-diethylether-acetic acid (65:35:1, v/v/v) as mobile phase. The various lipid bands were visualized by iodine and excised, and the radioactivity was quantified by liquid scintillation.

Fatty acid oxidation

β-Oxidation products (acid-soluble metabolites). Myotubes were exposed to αMEM supplemented with [1-^{14}C]OA (0.5 μCi/ml, 0.6 mM), 0.24 mM BSA, 0.5 mM L-carnitine, and 20 mM HEPES with or without 100 nM insulin to study insulin-mediated or basal oleate oxidation, respectively. After 4 h, the incubation medium was transferred to new tubes and assayed for labeled acid-soluble metabolites (ASMs) (21). An aliquot, 250 μl of the cell medium, was precipitated with 100 μl of 6% BSA and 1.0 ml of 1 M perchloric acid. After centrifugation (1,800 g for 10 min), 500 μl of

TABLE 1. Characteristics of the PCR primers

Assay	Sequence	Accession Number
β-Actin	F: ACC GAG CGC GGC TAC A	NM_001101
	R: TCC TTA ATG TCA CGC ACG ATT T	
Diacylglycerol acyltransferase-1	F: TCC ACT CCT GCC TGA ATG C	AF059202
	R: GGT GAC AGA CTC GGA GTT CCA	
CD36/FAT (fatty acid transporter)	F: AGT CAC TGC GAC ATG ATT AAT GGT	L06850
	R: CTG CAA TAC CTG GCT TTT CTC AA	
Glucose transporter GLUT1	F: CAG CAG CCC TAA GGA TCT CTC A	K03195
	R: CCG GCT CGG CTG ACA TC	
Glucose transporter GLUT4	F: GCT ACC TCT ACA TCA TCC AGA ATC TC	M20747
	R: CCAGAA ACA TCG GCC CA	

F, forward primer; R, reverse primer. The accession numbers in GenBank were used to design the primers in the Primer Express® program.

the supernatant was counted by liquid scintillation. No-cell controls were included.

Carbon dioxide. Cells were cultured in 12.5 cm^2 flasks, and the myotubes were exposed to αMEM supplemented with [1-^{14}C]OA (0.5 μCi/ml, 0.6 mM), 0.24 mM BSA, 0.5 mM L-carnitine, and 20 mM HEPES with or without 100 nM insulin. Flasks were made airtight with stopper tops. After 4 h, 300 μl of phenyl ethylamine-methanol (1:1, v/v) was added with a syringe to a center well containing a folded filter paper. Subsequently, 300 μl of 1 M perchloric acid was added to the cells through the stopper tops using a syringe. The flasks were placed for a minimum of 4 h at room temperature to trap labeled CO_2. No-cell controls were included to correct for unspecific CO_2 trapping.

Deoxyglucose transport

Myotubes were incubated for 60 min in serum-free αMEM with or without insulin (1–100 nM) at 37°C before the addition of 2-[^{3}H(G)]deoxy-D-glucose (1 μCi/ml) with or without insulin (100 nM). Deoxyglucose uptake was measured for 15 min in the presence of 10 μM unlabeled deoxyglucose. After incubation, the cells were washed three times with ice-cold PBS and lysed with 0.05 M NaOH, and the radioactivity was counted by liquid scintillation. Noncarrier-mediated glucose transport was determined in the presence of cytochalasin B (5 μM) and subtracted. The protein content of each cell sample was determined according to Bradford (22) using BSA as the reference protein. The insulin-stimulated glucose uptake was linear within 2 h, and in the presence of 100 nM insulin, glucose uptake was increased by 54% (mean response).

Glycogen synthesis

Myotubes were incubated for 60 min in serum-free αMEM with or without insulin (1–100 nM) at 37°C, before the addition of D-[^{14}C(U)]glucose (1 or 2 μCi/ml, 5.5 mM) with or without insulin (100 nM). After 60–120 min, the cells were washed three times with ice-cold PBS and lysed with 1 M KOH. Synthesized glycogen was measured as described by Franch, Aslesen, and Jensen (23). The response to insulin (100 nM) was a >2-fold increase in glycogen formation compared with unstimulated cells, and glycogen synthesis was linear within 4 h.

Glucose oxidation

Myotubes were incubated in 12.5 cm^2 flasks made airtight by stopper tops in serum-free αMEM with [1-^{14}C]D-glucose (0.5 μCi/ml, 5.5 mM) and 20 mM HEPES with or without 100 nM insulin. After 4 h, 300 μl of phenyl ethylamine-methanol (1:1, v/v) was added with a syringe to a center well containing a folded filter paper. Subsequently, 300 μl of 1 M perchloric acid was added to the cells through the stopper tops using a syringe. The flasks were placed for a minimum of 4 h at room temperature to trap labeled CO_2. The filter paper was counted by liquid scintillation. No-cell controls were included to correct for unspecific CO_2 trapping.

RNA isolation and analysis of gene expression by real-time PCR

RNA was obtained as either total RNA or mRNA according to the following protocols. For isolation of total RNA, human skeletal muscle cells grown on 60 mm dishes were washed, trypsinized, and pelleted before total RNA was isolated with the RNeasy Mini kit according to the supplier total RNA isolation protocol. RNA samples were incubated with RNase-free DNase for a minimum of 15 min in an additional step during the RNA isolation procedure. For isolation of mRNA, myotubes grown on

six-well plates were washed twice with ice-cold PBS and lysed by adding 200 μl of lysis/binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% Lithium dodecyl sulphate, and 5 mM dithiothreitol). The Dynabeads mRNA DIRECT kit was used for isolation of mRNA according to the manufacturer's procedure. Oligo(dT)$_{25}$-Dynabeads (5 mg/ml) were prewashed in lysis/binding buffer before cell lysates were added. The tubes were gently rotated for 3–5 min at room temperature before they were placed on a magnet for 2 min. The supernatants were removed, and the beads/mRNA were washed twice with washing buffer 1 (10 mM Tris-HCl, pH 8.0, 0.15 mM LiCl, 1 mM EDTA, and 0.1% Lithium dodecyl sulphate) and twice with washing buffer 2 (10 mM Tris-HCl, pH 8.0, 0.15 mM LiCl, and 1 mM EDTA). Finally, the beads were washed once in diethyl-pyrocarbonate-treated H_2O, resuspended in 20 μl of diethylpyrocarbonate-treated H_2O, and stored at −70°C.

Total RNA and mRNA were reverse-transcribed with oligo primers using a Perkin-Elmer Thermal Cycler 9600 (25°C for 10 min, 37°C for 1 h, and 99°C for 5 min) and a TaqMan reverse transcription reagents kit. One microgram of total RNA or 1 μl of mRNA was added per 10 μl of total TaqMan reaction solution. Real-time PCR was performed using an ABI PRISM® 7000 Detection System (Applied Biosystems). RNA expression was determined by SYBR® Green, and primers were designed using Primer Express® (Applied Biosystems). Each target were quantified in triplicate and carried out in a 25 μl reaction volume according to the supplier protocol. All assays were run for 40 cycles (95°C for 12 s followed by 60°C for 60 s). The housekeeping control genes α-tubulin and β-actin were both measured, and transcription levels are presented relative to β-actin.

Immunoblotting

Total cell lysates prepared in Laemmli buffer were electrophoretically separated on 10% (w/v) polyacrylamide gels (acrylamide/N′N′-bis-methylene acrylamide = 30:0.8) followed by immunoblotting with antibodies recognizing extracellular signal-regulated kinase (ERK), p38, or Akt kinases in activated form: specific for ERK (p44^{mapk} and p42^{mapk}; ERK1 and ERK2) phosphorylated at Thr202 plus Tyr204; specific for p38 phosphorylated at Thr180 plus Tyr182; specific for Akt1 [protein kinase B (PKB)] phosphorylated at Ser478 and Akt2 and Akt3 when phosphorylated at equivalent sites (Cell Signaling Technology, Beverly, MA). Immunoreactive bands were visualized with enhanced chemiluminescence and quantified with Gel-Pro Analyzer (version 2.0) software.

Statistics and data presentation

All values are reported as means ± SEM. The value n represents the number of different donors used. All experiments were run with duplicate or triplicate samples unless stated otherwise. Statistical analyses were performed with StatView® (SAS Institute, Inc.). Comparisons of different treatments were evaluated by ANOVA and Fisher's protected least significant difference. $P < 0.05$ was considered significant.

RESULTS

Effect of 24 h pretreatment with fatty acids on [^{14}C]OA uptake, distribution into cellular lipids, and oxidation

Skeletal muscle cells from young, healthy subjects were differentiated for 8 days before measurement of OA uptake, esterification to lipids, and oxidation. Lipid metabolism was examined after 24 h of exposure of myotubes to
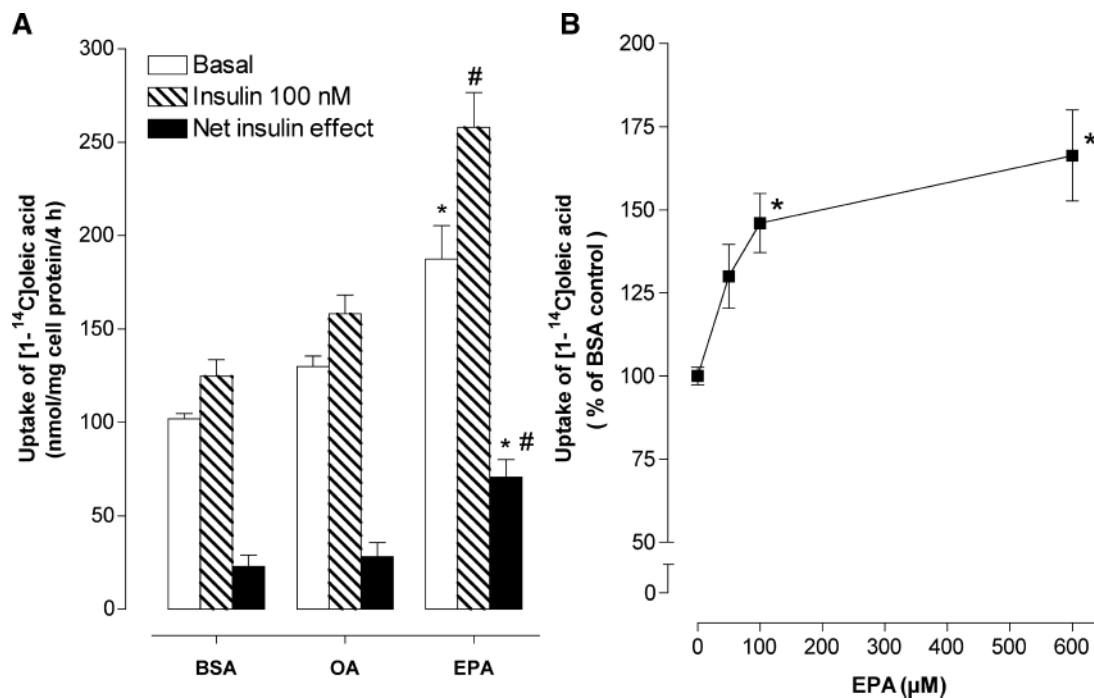
Fig. 1. Cellular uptake of labeled oleic acid ([^{14}C]OA) after pretreatment with fatty acids (0.6 mM) (A), and dose response of eicosapentaenoic acid (EPA) on [^{14}C]OA uptake (B). Differentiated myotubes were pretreated with various fatty acids (0.05–0.6 mM) in minimum essential medium α (αMEM) with 2% fetal calf serum (FCS) for 24 h and then incubated in αMEM containing [1-^{14}C]OA (0.5 μCi/ml, 0.6 mM), L-carnitine (0.5 mM), and HEPES (20 mM) with or without insulin (100 nM) for 4 h. The results are presented as means ± SEM (A, n = 4; B, n = 2–6). OA was bound to BSA in the ratio 2.5:1. * Significantly different from BSA control at $P < 0.05$; # EPA significantly different from OA at $P < 0.05$ (ANOVA with Fisher's protected least significant difference).

various fatty acids, after which the cells were incubated with [1-^{14}C]OA (0.6 mM) for another 4 h. Uptake of labeled OA (the sum of cell-associated lipids and oxidized oleate) was significantly increased by 84% after pretreatment with 0.6 mM EPA compared with the fatty acid-free BSA control and by 44% compared with OA pretreatment (**Fig. 1A**). Pretreatment with OA did not affect the subsequent uptake of labeled OA (Fig. 1A). EPA pretreatment dose-dependently increased OA uptake, demonstrating that EPA also significantly increased fatty acid uptake at lower concentrations (Fig. 1B).

Incorporation of labeled OA into various lipid classes after treatment with EPA showed that cellular TAG and phospholipids (PLs) were increased 2.5- to 3-fold compared with the BSA control (**Fig. 2A**). In addition, cellular free OA (FFA) was also increased by 87% versus the BSA control after EPA treatment (Fig. 2A). After exposure of myotubes to OA for 24 h, a >2-fold increase in TAG was observed, whereas the other lipid classes remained unchanged (Fig. 2A).

Oxidation of oleate after the 24 h fatty acid exposure was determined by measurement of ASM (β-oxidation products) (Fig. 2A) and CO_2 released from the cells. There was no change in the formation of ASM in EPA-treated myotubes compared with the BSA control, despite a much higher oleate uptake (Fig. 2A). On the other hand, production of CO_2 was decreased by 50% after pretreatment with EPA (4.9 ± 0.3 vs. 9.9 ± 0.4 nmol/mg cell protein/4 h for EPA vs. BSA, respectively). Finally, ASM

formation was decreased by 25% for OA compared with the BSA control (Fig. 2A).

Short-term effects of insulin on OA uptake and metabolism

Insulin-mediated [^{14}C]oleate metabolism after fatty acid pretreatment of myotubes was also studied. In the presence of insulin (100 nM) for 4 h, total [^{14}C]oleate uptake was similarly increased above basal level by 22% for OA and the BSA control, whereas it was 38% above basal for EPA (Fig. 1). The net insulin effect was thus 2.5- to 3-fold higher for EPA than the BSA control and OA (Fig. 1). Figure 2B shows the acute effects of insulin on the metabolism of oleate into various cellular lipids and ASM. Insulin markedly increased the incorporation of labeled oleate into all lipid classes in EPA-treated myotubes compared with control and OA-treated cells, which had similar insulin responses as control cells. Fatty acid oxidation (ASM), on the other hand, was not significantly changed by short-term insulin treatment (Fig. 2B).

Effect of pretreatment with fatty acids on [^{14}C]OA partitioning

For further insight into how fatty acid-treated myotubes metabolize labeled OA, partitioning of OA between oxidation (ASM) and complex lipids was calculated. Fatty acid pretreatment changed fatty acid distribution in favor of lipid synthesis and decreased fatty acid oxidation (**Fig. 3**). Insulin-mediated effects on partitioning showed a further
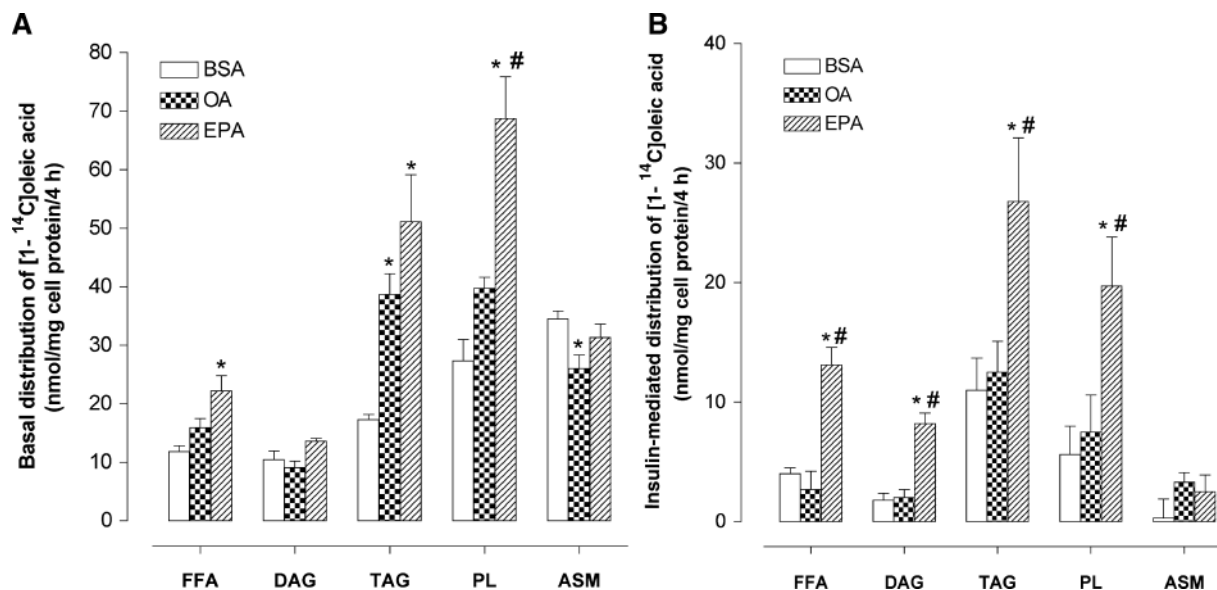
Fig. 2. Incorporation of [^{14}C]OA into cellular lipids (A), and acute effects of insulin on [^{14}C]OA metabolism (B) after pretreatment with fatty acids. Differentiated myotubes were pretreated with various fatty acids (0.6 mM) in αMEM with 2% FCS for 24 h and incubated in αMEM containing [1-^{14}C]OA (0.5 μCi/ml, 0.6 mM), L-carnitine (0.5 mM), and HEPES (20 mM) without (A) or with (B) insulin (100 nM) for 4 h, and lipids were extracted and separated by TLC. OA was bound to BSA in the ratio 2.5:1. The results are presented as means ± SEM (n = 4). ASM, acid-soluble metabolite; DAG, diacylglycerol; PL, phospholipid; TAG, triacylglycerol. * Significantly different from BSA control at $P < 0.05$; # EPA significantly different from OA at $P < 0.05$ (ANOVA with Fisher's protected least significant difference).

significant change from fatty acid oxidation toward the synthesis of complex cellular lipids for EPA and control myotubes (Fig. 3).

Effect of 24 h pretreatment with fatty acids on glucose transport and metabolism

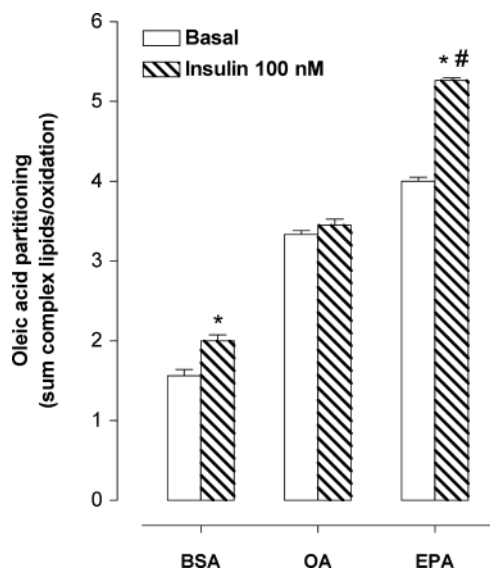Glucose transport in the absence of insulin (basal) was increased 2.4-fold after preincubation of the myotubes

Fig. 3. Partitioning of labeled oleate. Data are taken from Fig. 2 and are shown as the sum of complex lipids/ASM (oxidation) (means ± SEM, n = 4). Complex lipids are OA-labeled cellular lipids except for FFA. * Insulin significantly different from basal at $P < 0.05$; # EPA significantly different from OA at $P < 0.05$ (ANOVA with Fisher's protected least significant difference).

for 24 h with 0.6 mM EPA compared with the fatty acid-free control (BSA) and OA (**Fig. 4A**). Acute treatment of the myotubes with insulin (100 nM) further increased ($P < 0.05$) glucose transport for control and EPA, whereas insulin failed to increase glucose transport after OA treatment (Fig. 4A). Glucose transport was also significantly ($P < 0.05$) increased after pretreating myotubes with a lower concentration of EPA (0.1 mM) for 24 h: basal by 19 ± 7% and insulin-stimulated (100 nM) by 70 ± 8% of the BSA control. Glucose transport measured after shorter pretreatment (4 h) of myotubes with 0.6 mM EPA was 113 ± 16% for basal and 114 ± 6% with insulin for the BSA control, demonstrating that the effect on glucose transport was dependent on long-term exposure to this fatty acid.

To further study intracellular glucose metabolism, glycogen synthesis and glucose oxidation were determined after fatty acid pretreatment. Basal glycogen synthesis was significantly decreased by 52% with EPA compared with the BSA control, whereas OA had no effect (Fig. 4B). Acute insulin treatment increased ($P < 0.05$) glycogen synthesis >2-fold in control, EPA-treated, and OA-treated cells (Fig. 4B). However, the net insulin effects were markedly lower after exposure of the myotubes to EPA, when a reduction of 53% was observed (Fig. 4B). Glycogen synthesis was also measured after exposure to 0.1 mM EPA for 24 h; basal as well as insulin-stimulated (100 nM) synthesis were significantly ($P < 0.05$) decreased by 33 ± 2% and 35 ± 2%, respectively, of the BSA control.

Basal glucose oxidation (CO_2 formation) was increased 2-fold after preincubation of the myotubes with 0.6 mM EPA for 24 h compared with control and OA-treated cells (Fig. 4C). Glucose oxidation in the presence of insulin (100 nM) was further increased ($P < 0.05$) to the same
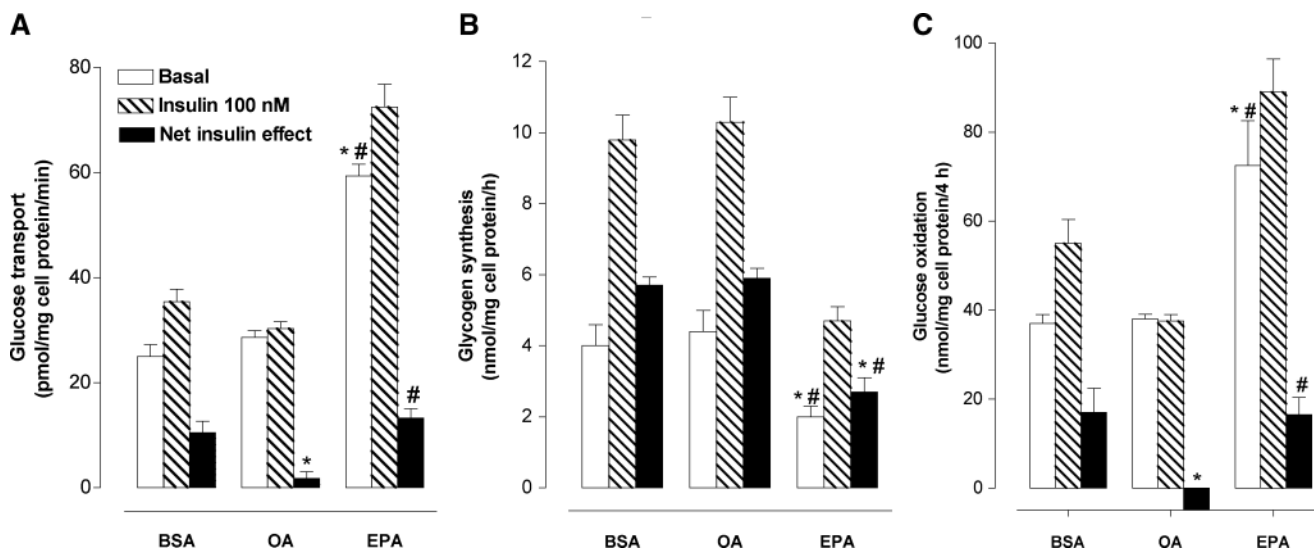
Fig. 4. Effects of pretreatment with fatty acids on basal and insulin-stimulated glucose transport (A), glycogen synthesis (B), and glucose oxidation (C). A: Differentiated myotubes (7 days) were treated with various fatty acids (0.6 mM) in αMEM with 2% FCS for 24 h, and the cells were further incubated for 1 h at 37°C in αMEM with or without insulin (100 nM). The cells were finally incubated in uptake buffer (see Experimental Procedures) containing [³H]deoxyglucose (1 µCi/ml, 10 µM) with or without insulin (100 nM) for 15 min. B: Differentiated myotubes were pretreated with fatty acids (0.6 mM) in αMEM with 2% FCS for 24 h and then incubated in αMEM containing [U-¹⁴C]D-glucose (2 µCi/ml, 5.5 mM) with or without insulin (100 nM) for 60 min. C: Differentiated myotubes were pretreated with fatty acids (0.6 mM) in αMEM with 2% FCS for 24 h and then incubated in αMEM containing [1-¹⁴C]D-glucose (2 µCi/ml, 5.5 mM) and HEPES (20 mM) without (basal) or with insulin (100 nM) for 4 h. The fatty acids were bound to BSA at the ratio of 2.5:1. Data are given as means ± SEM (n = 3–5). * Significantly different from BSA control at $P < 0.05$; # EPA significantly different from OA at $P < 0.05$ (ANOVA with Fisher's protected least significant difference).

extent for both EPA and control myotubes and was totally abolished by OA pretreatment (Fig. 4C).

Expression of genes involved in fatty acid and glucose metabolism

Because pretreatment with EPA had profound effects on OA uptake and lipid synthesis, we studied the effect on some genes of the central role in lipid metabolism. The expression of the fatty acid transporter CD36/FAT was significantly increased by 65% after EPA and was increased 2-fold after OA preincubation compared with the BSA control (**Fig. 5**). Diacylglycerol acyltransferase-1, the terminal enzyme involved in TAG biosynthesis, was not changed by any of the treatments (Fig. 5). Because pretreatment with EPA had profound effects on glucose transport and glucose oxidation, we also studied mRNA levels of the glucose transporters GLUT1 and GLUT4. After 24 h of pretreatment with EPA, GLUT1 mRNA expression was increased 2.5-fold, whereas the levels of GLUT4 were not changed after exposure of the myotubes to either EPA or OA (Fig. 5).

Effect of pretreatment with fatty acids on PKB/Akt, ERK, and p38 activity

The signaling pathways affected by EPA are poorly explored. Previously, we found that pretreatment with the saturated fatty acid palmitate for 24 h reduced the activation of PKB/Akt of the phosphatidylinositol 3-kinase pathway and increased the activation of the p38 mitogen-activated protein kinase (24), indicating that these signaling pathways may be influenced by long-term exposure

to fatty acids. Pretreatment with EPA for 24 h did not affect the activity of PKB/Akt (data not shown). OA compared with EPA and the BSA control decreased the phosphorylation (e.g., activation) of both the ERK and p38 mitogen-activated protein kinases (**Fig. 6**).

DISCUSSION

The present study shows that pretreatment of differentiated human myotubes with EPA promotes increased uptake and metabolism of [¹⁴C]oleate to cellular lipids, and the increased oleate uptake was not accompanied by increased [¹⁴C]oleate oxidation. Interestingly, after EPA pretreatment, the net insulin responses on [¹⁴C]oleate metabolism were enhanced despite an increased lipid synthesis compared with control cells (BSA) and cells exposed to OA. Moreover, EPA also increased basal as well as insulin-mediated glucose uptake and oxidation compared with myotubes pretreated with OA, which showed an abolished insulin action on these two processes.

Exposure of myotubes to EPA increased basal as well as insulin-mediated [¹⁴C]oleate uptake and also the expression of the fatty acid transporter CD36/FAT compared with control cells. CD36/FAT is an important factor involved in fatty acid uptake and intracellular transport. This protein is ubiquitously expressed, and its expression correlates with long-chain fatty acid uptake into both heart and muscle (25, 26). It was shown that peroxisome proliferator-activated receptor γ (PPARγ) activation by thiazolidinedione treatment increased palmitate uptake and
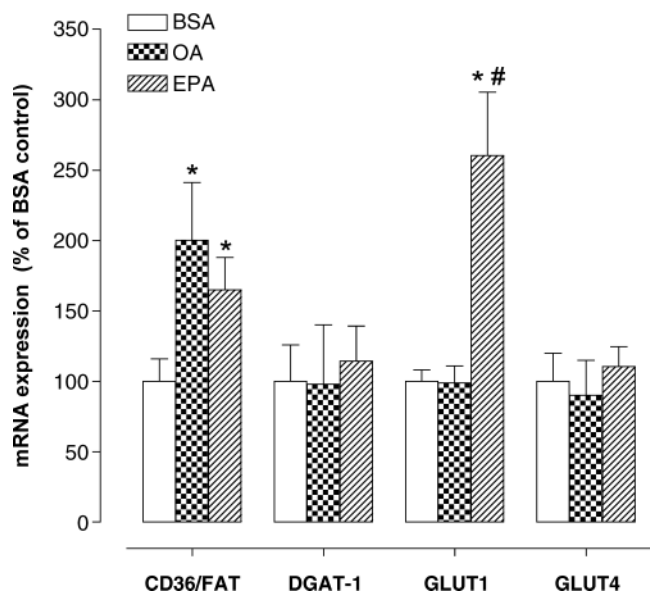
Fig. 5. mRNA expression of CD36/FAT (fatty acid transporter), diacylglycerol acyltransferase-1 (DGAT-1), and glucose transporters GLUT1 and GLUT4 after pretreatment with fatty acids. Differentiated myotubes (7 days) were pretreated with BSA (0.24 mM) and fatty acids (0.6 mM) for 24 h. The cells were harvested, and mRNA was isolated with the RNeasy Mini kit or oligo(dT)$_{25}$-Dynabeads. Reverse transcription and real-time PCR were performed. Levels of CD36/FAT, DGAT-1, GLUT1, and GLUT4 were quantified relative to the housekeeping control gene β-actin (means ± SEM, n = 4–6). * Significantly different from BSA control at $P <$ 0.05; [#] EPA significantly different from OA at $P <$ 0.05 (ANOVA with Fisher's protected least significant difference).

Fig. 6. Effects of pretreatment with fatty acids on the phosphorylation of extracellular signal-regulated kinase (ERK) and p38. Differentiated myotubes (7 days) were pretreated with BSA (0.24 mM) and fatty acids (0.6 mM) for 24 h. Immunoblot analysis was performed with antibodies against phosphorylated ERK and p38. The data represent means ± SEM for ERK (n = 5) and p38 (n = 4), presented as percentages of the EPA response. * Significantly different from BSA control at $P <$ 0.05; [#] EPA significantly different from OA at $P <$ 0.05 (ANOVA with Fisher's protected least significant difference).

oxidation in cultured human skeletal muscle cells in concert with the upregulation of CD36/FAT expression (26). EPA has previously been shown to induce PPARγ mRNA levels in isolated human adipocytes (27). We also observed a 2.3-fold increase in PPARγ expression after exposure of myotubes to EPA (0.6 mM, 24 h) compared with OA (data not shown). PPARγ shows a strong preference for binding to PUFAs, such as EPA, compared with monounsaturated or saturated fatty acids (28). The upregulation of CD36/FAT mRNA levels seen after EPA treatment observed in this study may be important for the increased [14C]oleate uptake. Pretreatment of the myotubes with OA did not significantly increase fatty acid uptake, but the expression of CD36/FAT increased similarly to that in EPA-exposed cells. It has been observed that PUFAs and their oxidation products stimulate the expression of CD36/FAT in human macrophages (29), most likely through the activation of PPARγ, and the activation of p38 mitogen-activated protein kinase has been shown to be involved in the regulation of CD36/FAT through PPARγ (30). On the other hand, activation of CD36/FAT has been shown to induce the phosphorylation of both ERK and p38 (31–33). Activities of p38 and ERK were decreased significantly in cells treated with OA compared with EPA-treated and control cells; however, whether this is a consequence of CD36 activation or contributes to CD36 regulation and fatty acid uptake is unknown. In cardiac myotubes, PKB/
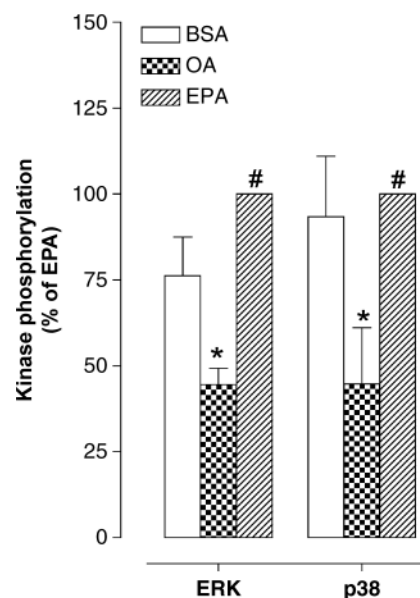
Akt is reported to be involved in the insulin-induced regulation of CD36/FAT (34), but pretreatment with EPA did not affect the activity of this kinase in human myotubes (data not shown).

There is evidence that insulin can activate fatty acid uptake in rat skeletal muscle by translocation of CD36/FAT to the plasma membrane (35). In human myotubes, insulin acutely increases the uptake of the saturated fatty acid palmitate and the formation of TAG (26, 36). We examined oleate uptake and subsequent metabolism in myotubes under basal conditions and in the presence of a short-term high concentration of insulin (100 nM). EPA pretreatment changed basal [14C]oleate distribution, with an increased level of free OA and enhanced incorporation into TAG and PLs compared with control cells, whereas diacylglycerol was unaltered. On the other hand, pretreatment with OA caused increased synthesis of TAG and decreased fatty acid β-oxidation. Moreover, after EPA pretreatment, insulin-induced changes for [14C]oleate uptake and incorporation into various lipids were enhanced compared with the BSA control and OA. On the contrary, β-oxidation of [14C]oleate as well as complete oxidation to CO_2 were decreased by EPA. Thus, after exposure of myotubes to EPA, cellular partitioning of oleate showed a distinct change from fatty acid oxidation toward increased incorporation into complex cellular lipids, especially PL, also independent of the short-term addition of insulin. In accordance with this finding, a similar change

in fatty acid distribution has been observed in rat hepatocytes after overexpression of mitochondrial glycerol-3-phosphate acyltransferase, which markedly decreased fatty acid oxidation and increased lipid formation, especially PL synthesis (37). The role of an increased formation of PL, and probably changed fatty acid composition, for modulating insulin action in myotubes after exposure to EPA needs to be further elucidated, but it could be related to changes in cellular membrane structure and function and lipid-derived signaling pathways (13). The fatty acid composition of skeletal muscle cell membrane PL, especially phosphatidylcholine, is known to influence insulin responsiveness in humans (38).

An increased intramyocellular content of lipids, especially TAG, is often associated with impaired insulin action (1–5); however, information on the regulatory processes involved in the synthesis and degradation of lipids within the muscle and their putative link to insulin resistance is at present fragmentary. In this study, we demonstrate that pretreatment with EPA positively influenced glucose metabolism and insulin action despite an increased fatty acid uptake and synthesis of complex lipid. In support of this finding, it has been demonstrated in rats that administration of EPA caused a reduction in plasma cholesterol and TAG, but it increased cholesterol and TAG contents in skeletal muscle without causing insulin resistance (39). Furthermore, it has also been shown in L6 myotubes pretreated with palmitic acid that overexpression of carnitine palmitoyltransferase-1, which accelerates fatty acid β-oxidation, exerts an insulin-sensitizing effect independent of changes in intracellular lipid content (40). It was also shown in C2C12 myocytes that overexpression of CD36 increased fatty acid uptake and channeling to a lipase-accessible TAG pool. This TAG pool was related to lipid content in the cells and insulin responsiveness, depending on the degree of futile cycling of fatty acids (41). In contrast to EPA, exposure of myotubes to OA showed decreased fatty acid β-oxidation and enhanced TAG synthesis only, and it also failed to respond to acute insulin stimulation on glucose transport and oxidation. In accordance with this fact, we have recently shown that exposure of human myotubes to palmitate caused increased fatty acid uptake and TAG formation, decreased fatty acid oxidation, and impaired insulin-mediated glucose uptake (42). Together, our data from human myotubes exposed to various fatty acids suggest that there is a complex relationship between the metabolism of intracellular lipids and glucose transport and utilization in skeletal muscle that needs to be addressed in future studies.

Pretreatment of myotubes with EPA for 24 h promoted increased basal glucose transport, whereas insulin-mediated transport was similar to that in control cells. An increased basal glucose uptake after prolonged incubation with other fatty acids has also been observed (42, 43). Moreover, the mRNA expression of the glucose transporter GLUT1 was increased 2.5-fold after EPA, whereas GLUT4 mRNA expression was unaltered. The increased expression of GLUT1 may explain the markedly increased basal uptake and oxidation of glucose after EPA treatment. The mechanisms behind the EPA-induced GLUT1 expression and increased basal glucose uptake are unknown.

It was also observed that EPA pretreatment decreased complete OA oxidation (CO_2) by the cells. Lower fatty acid oxidation might be a consequence of markedly increased glucose transport and oxidation as well as increased de novo lipogenesis. This process by which glucose suppresses fatty acid oxidation is often referred to as the inverse glucose-fatty acid cycle and is thought to occur via an increased formation of malonyl-CoA and inhibition of carnitine palmitoyltransferase-1 (44). It was shown recently that OA oxidation can be reduced during acute hyperglycemia in human myotubes (45). In addition, although de novo lipogenesis is low in human skeletal muscle, we have demonstrated that chronic glucose oversupply leads to increased lipid synthesis by human myotubes that might interfere with fatty acid metabolism (24).

In summary, our data show that chronic exposure of human myotubes to EPA promotes increased uptake of OA and uptake and oxidation of glucose, and the mechanisms may involve increased expression of CD36/FAT and GLUT1. Interestingly, despite an enhanced fatty acid uptake and synthesis of complex lipids, the insulin responses after EPA pretreatment were maintained for glucose uptake and oxidation and were even increased for oleate uptake and distribution into complex lipids. Thus, our data show that EPA directly modulates lipid and glucose metabolism in human skeletal muscle, and the observed changes may contribute to the beneficial effects of n-3 PUFAs in relation to peripheral insulin resistance and type 2 diabetes.⬛

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