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Signaling Specificity of Interleukin-6 Action on Glucose and Lipid Metabolism in Skeletal Muscle

Lubna Al-Khalili, Karim Bouzakri, Stephan Glund, Fredrik Lönnqvist, Heikki A. Koistinen, and Anna Krook

Departments of Molecular Medicine and Surgery (L.A.-K., K.B., S.G., F.L.) and Physiology and Pharmacology (A.K.), Karolinska Institutet, S-171 77 Stockholm, Sweden; Department of Biology (F.L.), Biovitrum AB, SE-112 76 Stockholm, Sweden; and Department of Medicine (H.A.K.), Division of Cardiology, Helsinki University Central Hospital and Biomedicum, FI-00029 HUS Helsinki, Finland

We identified signaling pathways by which IL-6 regulates skeletal muscle differentiation and metabolism. Primary human skeletal muscle cells were exposed to IL-6 (25 ng/ml either acutely or for several days), and small interfering RNA gene silencing was applied to measure glucose and fat metabolism. Chronic IL-6 exposure increased myotube fusion and formation and the mRNA expression of glucose transporter 4, peroxisome proliferator activated receptor (PPAR)α, PPARδ, PPARγ, PPARγ coactivator 1, glycogen synthase, myocyte enhancer factor 2D, uncoupling protein 2, fatty acid transporter 4, and IL-6 ($P < 0.05$), whereas glucose transporter 1, CCAAT/enhancer-binding protein-α, and uncoupling protein 3 were decreased. IL-6 increased glucose incorporation into glycogen, glucose uptake, lactate production, and fatty acid uptake and oxidation, concomitant with increased phosphorylation of AMP-activated protein kinase (AMPK), signal transducer and activator of transcription 3, and ERK1/2. IL-6 also increased phosphatidylinositol (PI) 3-kinase activity (450%; $P < 0.05$), which was blunted by subsequent insulin-stimulation ($P < 0.05$). IL-6-mediated glucose metabolism was suppressed, but lipid metabolism was unaltered, by inhibition of PI3-kinase with LY294002. The small interfering RNA-directed depletion of AMPK reduced IL-6-mediated fatty acid oxidation and palmitate uptake but did not reduce glycogen synthesis. In summary, IL-6 increases glycogen synthesis via a PI3-kinase-dependent mechanism and enhances lipid oxidation via an AMPK-dependent mechanism in skeletal muscle. Thus, IL-6 directly promotes skeletal muscle differentiation and regulates muscle substrate utilization, promoting glycogen storage and lipid oxidation. (*Molecular Endocrinology* 20: 3364–3375, 2006)

SKELETAL MUSCLE SECRETES several circulating factors such as IGF I and II, IGF binding proteins 1–5 (1), ILs (2–4), TNF-α (5), myostatin (6), and interferon-γ (7). However, the metabolic impact of growth factor and cytokine production by skeletal muscle is incompletely resolved. At the whole-body level in the rested state, adipocytes, monocytes/macrophages, fibroblasts, and vascular endothelial cells are important sources of IL-6 production, with adipocytes secreting up to 35% of circulating IL-6 (8, 9). However, after exercise, IL-6 levels rise due to increased local production and release from skeletal muscle (10) and this has been suggested to enhance substrate metabolism and whole body glucose homeostasis (11–15). Targeted over-expression of IL-6 increases the extent of myogenic differentiation and myotube development in C2C12 cells and this effect is reduced by depletion of IL-6 using small interfering RNA (siRNA) (16). Thus, evidence is emerging that IL-6 has positive and negative effects on whole body metabolism and gene regulatory responses (17). However, the effect of IL-6 on cellular metabolism is unclear.

IL-6 is classified as a proinflammatory cytokine, which, in concert with other factors such as TNF-α, IL-1, IL-8 and granulocyte-macrophage colony-stimulating factor, is responsible for aggravating inflammation. Clinical studies have established IL-6 as a marker of an inflammatory response after surgery (18), transplant rejection (19), viral infection (20), congestive heart failure (21), obesity (22), and autoimmune disorders (23). Correlative evidence links elevated IL-6 levels with the development of insulin resistance in obesity and diabetes mellitus (24). In cultured hepatocytes (25–28), preadipocytes, and mature adipocytes (29), IL-6 directly impairs insulin action. However, in human subjects, an *in vivo* IL-6 infusion does not alter splanchnic glucose output (30, 31). In rodents, divergent IL-6 effects have also been noted. IL-6 infusion in

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Abbreviations: AMPK, AMP-activated protein kinase; CEBPα, CCAAT/enhancer-binding protein-α; FBS, fetal bovine serum; GLUT, glucose transporter; IRS, insulin receptor substrate; MEF2D, myocyte enhancer factor 2D; PGC1, PPARγ coactivator 1; PI, phosphatidylinositol; PKB, protein kinase B; PPAR, peroxisome proliferator activated receptor; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription; UCP, uncoupling protein.

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mice has been reported to reduce the effect of insulin to suppress hepatic glucose output and reduce insulin-stimulated skeletal muscle glucose uptake (32), whereas others report no effect of IL-6 on whole-body glucose disposal (33). Mice treated with a monoclonal anti-IL-6 antibody display increased levels of TNF (34), suggesting that IL-6 may in fact counter regulate TNF levels.

Skeletal-muscle-derived IL-6 has been proposed as an exercise factor that integrates metabolic responses between multiple organs such as adipose tissue, liver, and brain (35). Thus, the role of IL-6 on insulin action and metabolism is unresolved and warrants further investigation.

IL-6 infusion in humans is correlated with increased lipolysis and fat oxidation (36), providing clinical support for a role of IL-6 in lipid metabolism. In rodents, IL-6 has also been shown to activate AMP-activated protein kinase (AMPK) (37), a key enzyme involved in the activation of acetyl-coenzyme A carboxylase and lipid oxidation (38). AMPK is expressed in most mammalian tissues (39, 40), and signal transduction via this pathway plays an important role in stimulation of skeletal muscle fatty acid oxidation and muscle glucose uptake (40–42). Activation of AMPK mediates insulin-independent stimulation of glucose uptake in resting skeletal muscle (43, 44). Furthermore, AMPK is a critical regulator of glucose and fat metabolism in skeletal muscle during exercise (38, 45). Whether intact AMPK signaling is necessary for positive or negative effects of IL-6 on metabolism is unknown.

Here we identify the signaling pathways mediating IL-6 effects on differentiation and metabolism in primary human skeletal muscle cell cultures. We hypothesize that phosphatidylinositol (PI) 3-kinase and AMPK pathways may play a role.

RESULTS

IL-6 Increases Glucose Incorporation into Glycogen

To determine the effect of IL-6 on glucose metabolism, primary skeletal muscle cultures were exposed to 5, 25, or 100 ng/ml IL-6 for 3 h. IL-6 increased glucose incorporation into glycogen approximately 1.5-fold ($P < 0.05$), with no dose-response effect noted (Fig. 1A). To assess whether IL-6 elicits an additive effect with insulin on glucose incorporation into glycogen, cells were exposed to 25 ng/ml IL-6 for 3 h with 6 or 60 nM insulin included for the last 30 min. IL-6 and 60 nM insulin increased glucose incorporation into glycogen in additive manner ($P < 0.05$; Fig. 1B). Similar effects were seen using IL-6 concentrations of 5 or 100 ng/ml (data not shown). We next determined whether 8 d exposure to 25 ng/ml of IL-6 would affect insulin-stimulated glucose incorporation into glycogen. Cells exposed to IL-6 for 8 d increased glycogen synthesis approximately 2-fold ($P < 0.05$ Fig. 1C), with similar

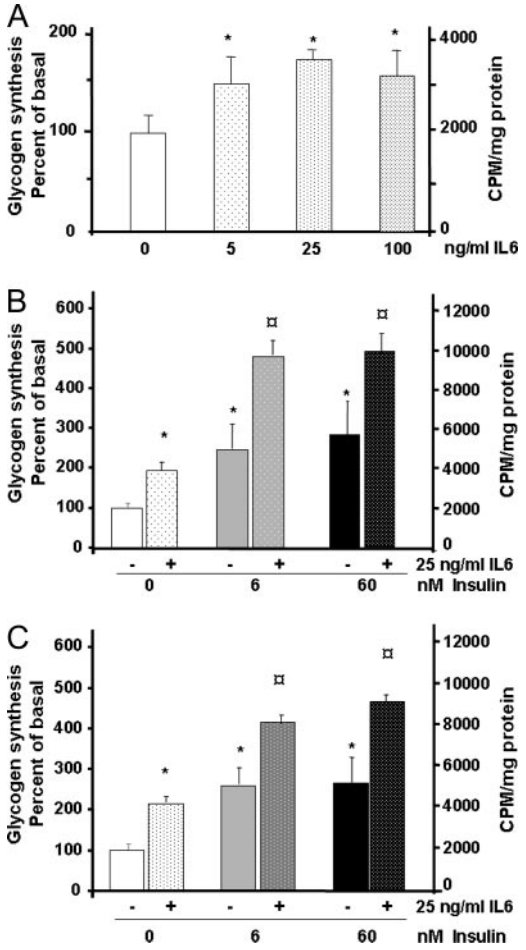

Fig. 1. Glycogen Synthesis

A, Dose response of acute (3 h) treatment with different concentrations of IL-6 (0, 5, 25, and 100 ng/ml) on differentiated primary human skeletal muscle myotubes (8 d) (n = 4). B, Insulin-stimulated glucose incorporation to glycogen after 3 h of treatment with 25 ng/ml IL-6 on differentiated primary human skeletal muscle myotubes (8 d) (n = 6). C, Insulin-stimulated glucose incorporation to glycogen after 8 d of treatment with 25 ng/ml IL-6 on differentiated primary human skeletal muscle myotubes (n = 6). Glycogen synthesis was calculated as counts per minute per milligram of protein. Data are shown as mean ± SE (n = 4–6). *, $P < 0.05$ over basal; ⋄, $P < 0.05$ over insulin without IL-6.

effects noted under insulin-mediated conditions as seen after 3 h of IL-6 treatment (Fig. 1, B and C).

IL-6 Increases Glucose Uptake and Increases Production of Lactate

Glycogen synthesis provides a combined measure of glucose uptake and subsequent storage as glycogen. To further dissect the effects of IL-6 on glucose metabolism, we determined IL-6 effects on glucose uptake. Acute stimulation of primary human skeletal muscle cultures with 25 ng/ml of IL-6 for 3 h resulted in a small but significant ($P < 0.05$) increase in glu-

cose uptake (Fig. 2A). Interestingly, 8 d of exposure to IL-6 profoundly increased glucose uptake, possibly due to IL-6-mediated effects on skeletal muscle differentiation.

The effect of IL-6 exposure on production of lactate was also determined. Primary differentiated human skeletal muscle cultures were exposed to 25 ng/ml of IL-6, either alone or in combination with insulin, and total lactate production was assessed after 2, 24, 48, and 72 h (Fig. 2B). Several time points were selected to assess how insulin and IL-6 affects the expected accumulation of lactate over time. As expected, insulin profoundly increased lactate production in the cultured muscle cells. IL-6 also potently increased lactate production, although the magnitude of this increase was less than that observed with insulin. Interestingly, a combined exposure to insulin and IL-6 resulted in a reduction of the insulin effect to the levels observed with IL-6 alone (Fig. 2B).

IL-6 Increases β-Oxidation of Lipids

To determine the effect of IL-6 on lipid metabolism, primary human skeletal muscle cells were exposed to 25 ng/ml IL-6 for 3 h, and the intracellular accumulation of labeled palmitic acid [$^{1-14}$C] and subsequent oxidation were determined. IL-6 increased basal and insulin-stimulated intracellular accumulation of labeled palmitic acid (Fig. 3A). Insulin reduced skeletal muscle palmitic acid oxidation 20% ($P < 0.05$; Fig. 3B). In contrast, IL-6 robustly increased palmitate oxidation 2.5-fold ($P < 0.05$). Insulin partially suppressed IL-6-mediated palmitate oxidation. Similar results were noted in skeletal muscle myotubes after chronic (8 d) exposure to IL-6. IL-6 had comparable effects on [$^{1-14}$C] oleate oxidation (data not shown).

IL-6-Mediated Increase in Protein Phosphorylation

IL-6-mediated intracellular signaling was assessed in differentiated primary human skeletal muscle cultures. Cells were exposed to 25 ng/ml IL-6 for 20 min or 3 h in the presence or absence of insulin (60 nM). IL-6 increased phosphorylation of signal transducer and activator of transcription (STAT)3, with phosphorylation peaking at 20 min and returning to basal levels after 3 h (Fig. 4a). Insulin did not increase phosphorylation of STAT3. Insulin robustly increased phosphor-

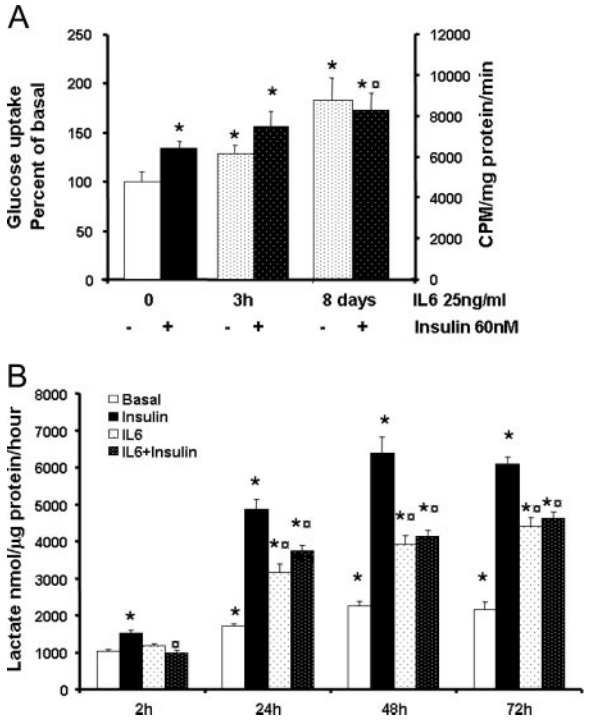

Fig. 2. Glucose Uptake and Lactate Production

A, Glucose uptake in myotubes treated with IL-6 (25 ng/ml) for 3 h or 8 d with or without acute insulin stimulation (60 nM) (n = 4). B, Lactate production after 2, 24, 48, and 72 h treatment with 25 ng/ml IL-6 on differentiated primary human skeletal muscle myotubes (8 d) (n = 6). Data are shown as counts per minute per milligram of protein, mean ± SE. *, $P <$ 0.05 over basal (2 h) ✧, $P < 0.05$ compared with insulin.

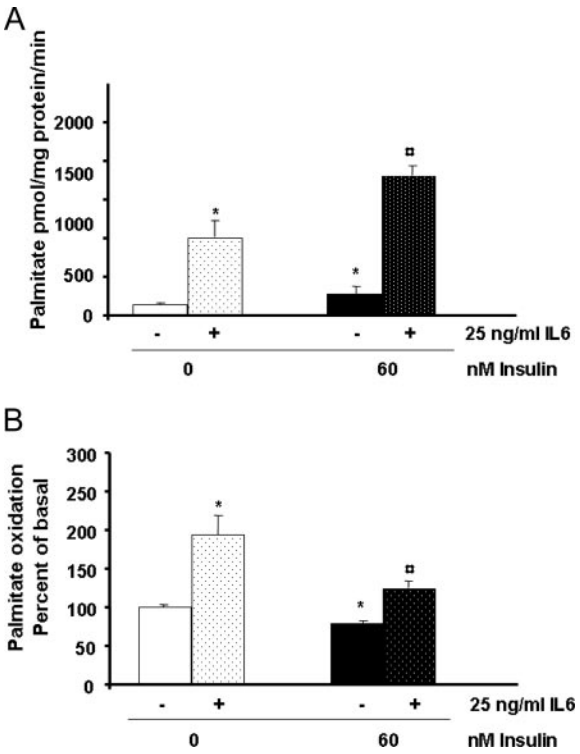

Fig. 3. Effect of IL-6 and Insulin on Accumulation of Labeled Palmitate and Oxidation

Effect of 3 h of treatment with 25 ng/ml IL-6 on (A) intracellular accumulation of labeled palmitate and (B) oxidation with or without insulin (60 nM) on differentiated primary human skeletal muscle myotubes (n = 4). Palmitate oxidation and uptake were calculated as palmitate pmol/mg protein·min. Data are shown as percentage over basal condition ± SE. *, $P < 0.05$ with respect to basal; ✧, $P < 0.05$ with respect to insulin without IL-6.

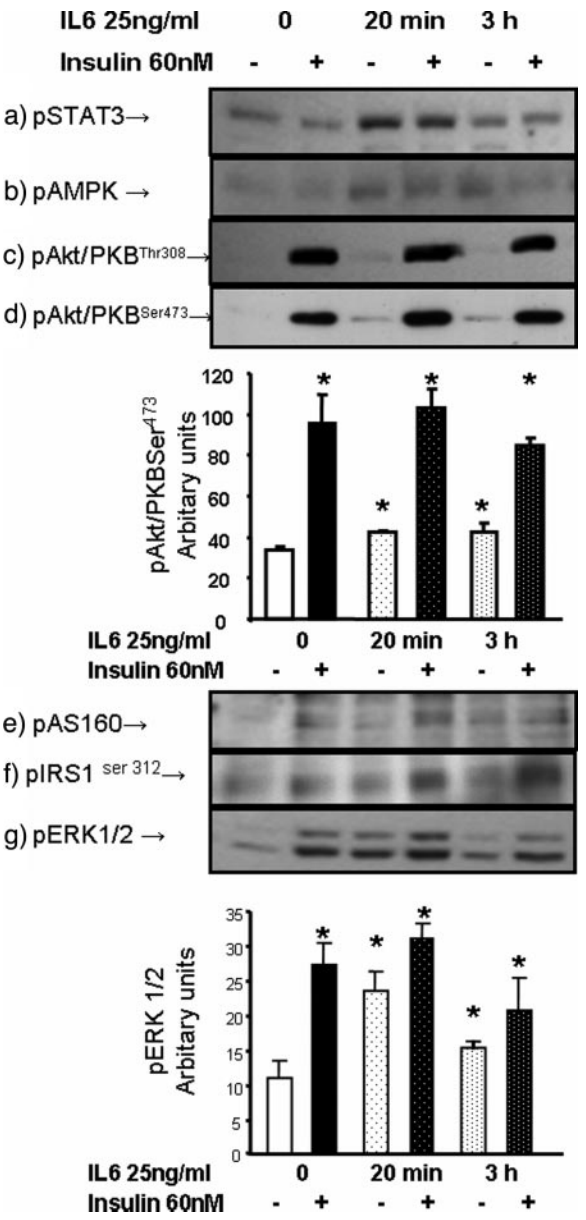

Fig. 4. Western Blot Results

Western blot showing the IL-6 effect on the phosphorylation of (a) STAT3, (b) AMPK, (c) PKB/Akt kinase Thr 308, (d) PKB/Akt kinase Ser 473, (e) AS160, (f) IRS1ser312, and (g) EKR1/2 MAPK in myotubes after 20 min or 3 h of treatment with 25 ng/ml IL-6 and/or insulin as indicated. Graphs shown summarized values (mean ± SEM) of four subjects. *, $P < 0.05$ *vs.* basal.

ylation of AKT/protein kinase B (PKB) on Thr^{308} and Ser^{473} (Fig. 4, c and d). IL-6 induced a weak (15%; $P < 0.05$; Fig. 4c), although detectable, increase in AKT/PKB Thr^{308} phosphorylation. IL-6 increased AKT/PKB Ser^{473} phosphorylation 20% ($P < 0.05$; Fig. 4d). Insulin, but not IL-6, increased phosphorylation of the AKT substrate AS160 (Fig. 4e). In primary human skeletal muscle cultures, insulin, but not IL-6, increased phosphorylation of insulin receptor substrate (IRS)1 Ser^{312} (Fig. 4f). Insulin and IL-6 independently increased ERK1 and ERK2 phosphorylation, and a combined exposure to insulin and IL-6 showed a trend toward an additive effect (Fig. 4g).

IL-6 Increases PI3-Kinase Activity

IRS1-associated PI3-kinase activity was determined in cells in response to 25 ng/ml IL-6 for 20 min, 3 h, or 8 d. Twenty minutes of IL-6 exposure increased PI3-kinase activity approximately 3-fold ($P < 0.05$; Fig. 5A), and this activation was reduced at longer exposures. IRS1-associated PI3-kinase activity was then determined in cells in response to 25 ng/ml IL-6 and/or insulin for 20 min. Insulin increased PI3-kinase activity 2-fold ($P < 0.05$; Fig. 5B). Treatment of cells with 25 ng/ml IL-6 increased PI3-kinase activity 4.5-fold ($P < 0.05$). A combined exposure of insulin and IL-6 attenuated the PI3-kinase signal, as compared with IL-6 alone (Fig. 5). Primary human skeletal muscle cells were next exposed to 25 ng/ml IL-6 for 3 h in the

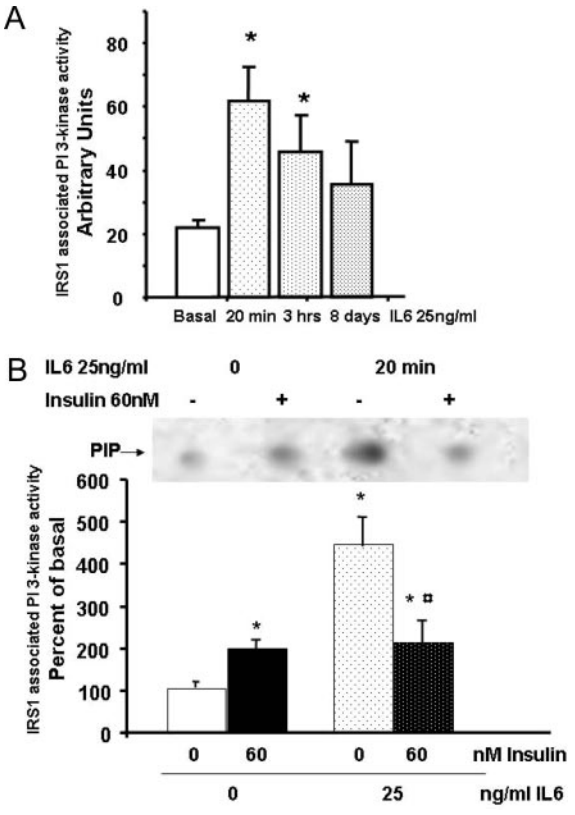

Fig. 5. Effect of IL-6 on IRS-1-Associated PI3-Kinase Activity in Differentiated Primary Human Skeletal Muscle Myotubes

A, Time course showing IRS-associated PI3-kinase activity after 20 min, 3 h, or 8 d (n = 4). B, Effect of 25 ng/ml IL-6 treatment (20 min) on IRS-associated PI3-kinase activity on differentiated primary human skeletal muscle myotubes (n = 4). Data are shown as percentage over basal condition ± SE. *, $P < 0.05$ w.rt. basal; ⋄, $P < 0.05$ with respect to IL-6 only. PIP, Phosphatidylinositolphosphate.

presence or absence of 10 μM LY294002, an inhibitor of PI3-kinase. The effect of IL-6 on glucose incorporation into glycogen was prevented in the presence of LY294002 (Fig. 6). In contrast, β-oxidation was unaltered by LY294002 (data not shown). Thus, IL-6 effects on glucose incorporation into glycogen and fatty acid oxidation appear to be mediated via separate pathways.

AMPK siRNA Reduces IL-6-Mediated Fatty Acid Oxidation But Not Glycogen Synthesis

IL-6 exposure increased phosphorylation of AMPK 3.8-fold ($P < 0.05$; Fig. 4b). Insulin did not increase phosphorylation of AMPK. To determine the consequence of the IL-6-mediated activation of AMPK on metabolic endpoints, we silenced the catalytic α-subunits of AMPK using siRNA. The sequences employed to silence AMPK α1- and α2-subunits are shown in Table 1. Sequences targeted at AMPK α1 silenced the α1- and α2-isoform, as evidenced by protein and mRNA expression analysis (Fig. 7). Sequences targeted at AMPK α2 specifically reduced α2 but not α1 RNA and protein. IL-6-mediated fatty acid uptake was prevented by silencing of AMPK α-subunits (Fig. 8A). Similar results were noted for IL-6-mediated increase in palmitate oxidation (Fig. 8B). In contrast, silencing of AMPK α-subunits did not affect the IL-6-mediated glucose incorporation into glycogen (Fig. 8C).

Chronic IL-6 Exposure Enhances Skeletal Muscle Differentiation

Primary cultures exposed to 25 ng/ml IL-6 for 8 d during the differentiation process displayed increased myotube formation and fusion rate (15 $\pm$ 4% over basal, myotubes with $>$5 nucleus/myotubes/μm^2) (Fig. 9). The expression of multiple key genes involved in glucose and lipid metabolism was assessed in primary human cultures exposed to IL-6 for 1, 3, or 8 d (Fig. 10) or only 8 d (Table 2). IL-6 exposure for 8 d increased mRNA expression of glucose transporter (GLUT)4, peroxisome proliferator activated receptor (PPAR)α, PPARδ, PPARγ, PPARγ coactivator 1 (PGC1)α, glycogen synthase, myocyte enhancer factor 2D (MEF2D), uncoupling protein (UCP)2, fatty acid transporter 4, and IL-6 itself ($P < 0.05$). Conversely, expression of GLUT1, CCAAT/enhancer-binding protein-α (CEBPα) and UCP3 was decreased ($P < 0.05$; Table 2). To determine whether IL-6 was mediating these gene-expression effects via AMPK, we used siRNA to silence AMPK and determined mRNA expression in myotubes after 1 and 3 d of exposure to IL-6. siRNA targeted against both the α1 and α2 catalytic subunits of AMPK were introduced into myotubes at d 2 after the start of muscle cell differentiation (if AMPK α-subunits are silenced before initiation of differentiation, myoblasts will not differentiate; Al-Khalili, L., and A. Krook, unpublished observation) and were harvested for mRNA as indicated. Interestingly, IL-6-mediated increase in PGC1α was blocked in cells in which AMPK α-subunit expression was reduced by siRNA. In contrast, IL-6 effects on mRNA expression of GLUT4, GLUT1, UCP3, and glycogen synthase was not dependent on AMPK, suggesting that IL-6 effects on mRNA expression are mediated via several distinct signaling pathways.

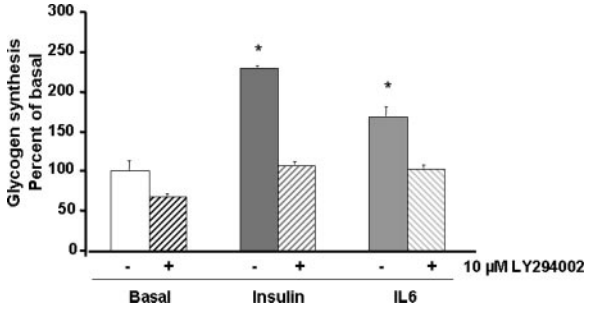

Fig. 6. Effect of PI3-Kinase Inhibitor on Glycogen Synthesis

Effect of the PI3-kinase inhibitor (LY294002, 10 μM) on IL-6 (25ng/ml)-stimulated glycogen synthesis (3 h) with or without insulin stimulation (60 nM) in human skeletal muscle myotubes (d 8). All values are shown as mean $\pm$ SEM; n = 5 subjects. *, $P < 0.05$ *vs.* basal.

DISCUSSION

IL-6 is a proinflammatory cytokine generally believed to promote insulin resistance (46). However, the precise role of IL-6 in inflammation is currently being debated, and there are indications that IL-6 may in fact reduce inflammatory response. For example, IL-6 inhibits lipopolysaccharide-induced TNF and IL-1 production in freshly isolated human peripheral blood mononuclear cells (47). In humans, an *in vivo* IL-6 infusion leads to production of the antiinflammatory cytokines IL-1 receptor antagonist and IL-10 (31). Furthermore, IL-6 may be a primary inducer of antiinflammatory hepatocyte-derived acute-phase proteins, as well as soluble TNF receptors, which would neutralize TNF effects (48). The insulin-resistance-promoting effects of IL-6 have been partly inferred from the observation that IL-6 is elevated in the circulation and adipose tissue explants of patients with type 2 diabetes (reviewed in Ref. 49). IL-6 has also been shown to directly reduce insulin action *in vitro* in adipocytes (29). The increased circulating IL-6 observed in patients with type 2 diabetes may be related to increased adipose mass rather than impaired insulin responsiveness (49). Physical exercise and muscle contraction lead to increased production (50) and release (10) of IL-6 from skeletal muscle. Furthermore, exercise-induced IL-6 release is positively correlated with skeletal muscle glucose uptake during contraction (10, 11). Here we show that, in primary cultures of human skeletal muscle, IL-6 exposure increases differentiation

Table 1. Sequences of AMPKα1 and α2 siRNA Oligos

	AMPKα1	AMPKα2
Sense	AGU GAA GGU UGG CAA ACA Utt	UAU GAU GUC AGA UGG UGA Att
Antisense	AUG UUU GCC AAC CUU CAC Utt	UUC ACC AUC UGA CAU CAU Att

and myotube formation. These morphological changes are accompanied by increased mRNA expression IL-6, glycogen synthase, UCP2, MEF2D, PGC1, and GLUT4, as well as PPARα, PPARγ, and PPARδ. IL-6 directly increases basal and insulin-mediated glucose uptake and glycogen synthesis. IL-6 stimulation of muscle cultures increases production of lactate. IL-6 also increases skeletal muscle β-oxidation of fatty acids, thus directing substrate metabolism in favor of lipid utilization and glycogen storage.

We demonstrate that, in primary human cultured muscle, IL-6 has a direct effect on glucose metabolism. IL-6 increases glucose uptake and enhances glycogen synthesis. Compared with insulin, the IL-6-mediated increase in production of lactate is approximately 50% reduced, suggesting that, in response to IL-6, skeletal muscle shifts substrate utilization toward a more glucose sparing phenotype.

IL-6 mediates intracellular effects via binding to either the IL-6 transmembrane receptor (51) or the cleaved soluble version of the membrane-bound receptor (52). Activated IL-6 transmembrane receptor mediates signal transduction via activation of the gp130β transmembrane receptor (53), which in turns leads to activation of the Janus activated protein kinase/STAT pathway. STAT3 and ERK signaling have been shown to cooperatively activate the *c-fos* gene, (54), suggesting that IL-6-regulated gene expression

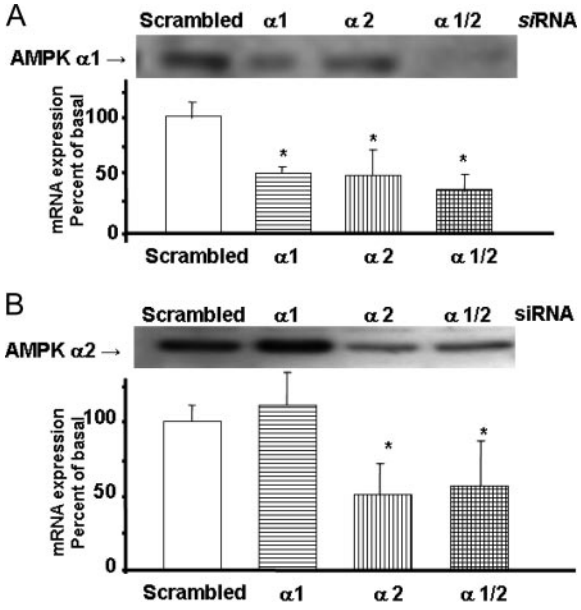

Fig. 7. Protein and mRNA Expression of AMPK after siRNA

Protein and mRNA expression of AMPK α1 (A) and AMPK α2 (B) in human skeletal muscle myotubes (d 4 of differentiation) after siRNA-mediated depletion of AMPK α1 and AMPK α2. *Top panel*, A representative immunoblot respective isoform. The mRNA levels of AMPK α1 and AMPK α2 were standardized by reference to 18s mRNA levels. *Bottom panel*, Mean ± SEM; n = 5 subjects. *, $P < 0.05$ *vs.* scrambled siRNA basal.

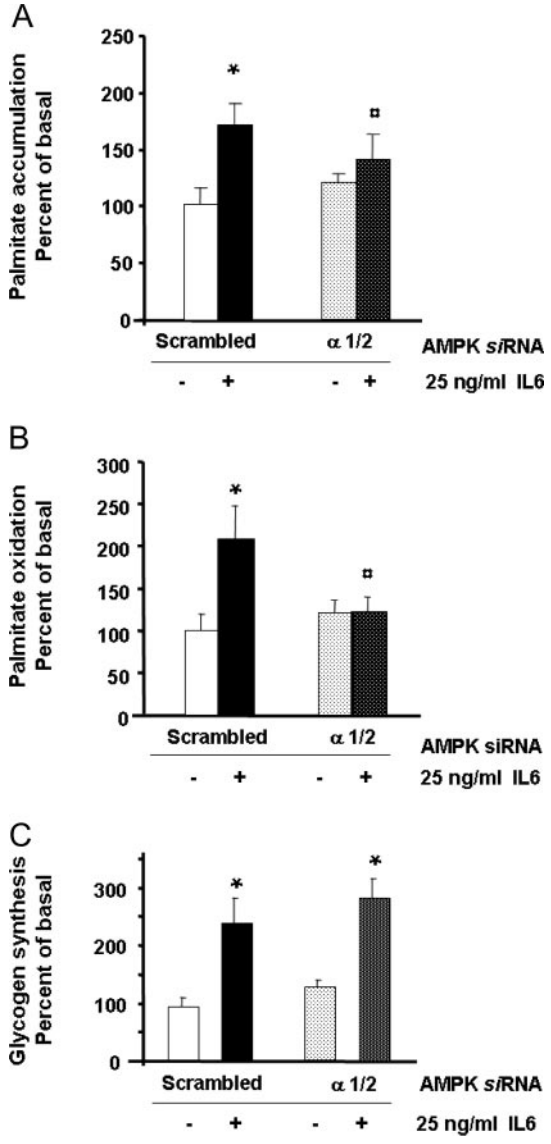

Fig. 8. Effect of AMPK α1 and AMPK α2 Depletion

Effect of AMPK α1 and AMPK α2 depletion by siRNA on intracellular accumulation of labeled palmitate (A), palmitate oxidation (B), and glycogen synthesis (C) in human skeletal muscle myotubes (d 5). All values are shown as mean ± SEM; n =5 subjects. *, $P < 0.05$ *vs.* scramble siRNA basal; ⋄, $P < 0.05$ *vs.* scrambled siRNA IL-6.

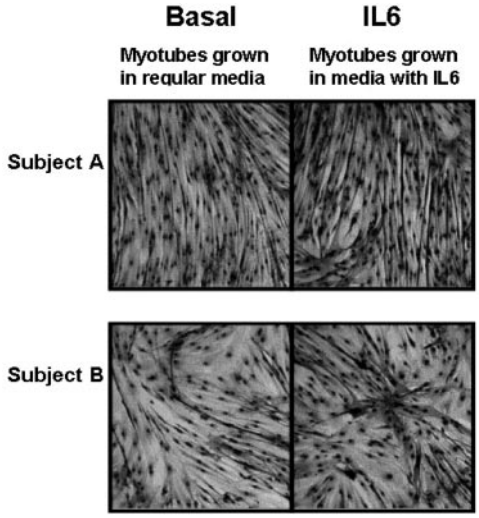

Fig. 9. Morphological Changes of Human Skeletal Muscle Myotube Formation after Long-Term (8 d) Treatment with 25 ng/ml IL-6

Photomicrographs are shown at ×10 magnification for myotubes that were stained with Giemsa/Wright at 8 d after initiation of differentiation. Results are two representative experiments; n = 6.

occurs at least in part via the STAT3/ERK1/2-mediated pathway. Exposure of primary human skeletal muscle cells to IL-6 increased phosphorylation of STAT3, ERK1/2, and AMPK, in a time-dependent manner, with maximum phosphorylation noted at 20 min. A role for PI3-kinase in IL-6 signaling has also been suggested in a basal cell carcinoma cell line, in which mRNA expression of basic fibroblast growth factor and cyclooxygenase-2 were shown to be dependent on PI3-kinase/Akt pathway (55). Here we report that IL-6 increases IRS1-associated PI3-kinase activity in human skeletal muscle cells. This is in line with a recent report showing that IL-6 induces a rapid recruitment of IRS-1 to the IL-6 receptor complex in cultured C2C12 skeletal muscle cells (56). Furthermore, we demonstrate that inhibition of PI3-kinase activity reduced both insulin- and IL-6-mediated glycogen synthesis. Thus, IL-6 appears to mediate glycogen synthesis via a PI3-kinase-dependent signaling pathway.

Treatment of primary cultured muscle cells with IL-6 increased fatty acid oxidation. *In vivo* experiments in humans indicates that IL-6 infusion stimulates lipolysis and rates of whole-body fat oxidation (36), suggesting that IL-6 is a regulator of whole-body lipid metabolism. Likewise, acute IL-6 treatment increases β-oxidation in skeletal muscle *in vivo* in elderly subjects and *in vitro* in cultured cells (30). In isolated rat soleus strips, IL-6 directly stimulates exogenous and endogenous fatty acid oxidation and attenuates the lipogenic effects of insulin (57). Thus, there is increasing evidence that IL-6 is involved in the regulation of skeletal muscle fat metabolism. Here we show that the IL-6-mediated increase in fatty acid uptake and oxidation is dependent on AMPK, because siRNA mediated down-regulation of AMPK α1 and α2 reduces both the IL-6-mediated β-oxidation and palmitate uptake. In

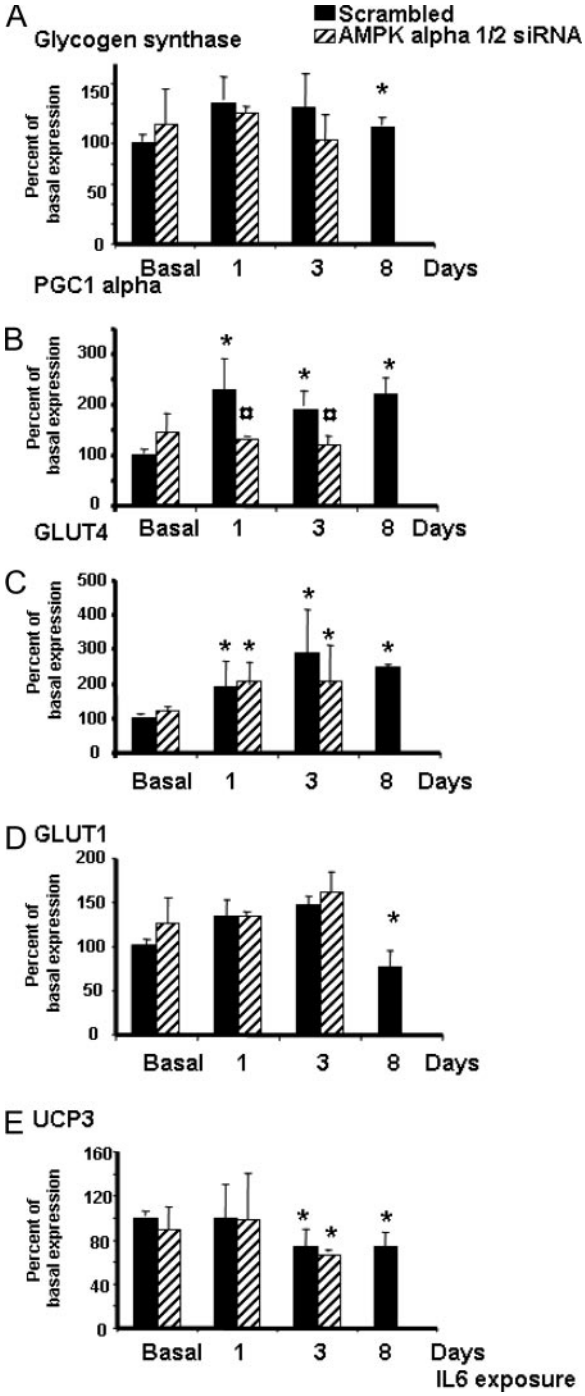

Fig. 10. Changes in mRNA Expression

Changes in mRNA expression of (A) glycogen synthase, (B) PGC1α, (C) GLUT4, (D) GLUT1, and (E) UCP3 in primary human skeletal muscle cultures after exposure to 25 ng/ml IL-6 at d 1, 3, and 8with or without AMPK α1/2 siRNA (only for d 1 and 3). All values are shown as mean ± SEM; n = 4–6 subjects. ✧, $P < 0.05$ *vs.* scrambled IL-6; *, $P < 0.05$ *vs.* respective scrambled siRNA basal.

Table 2. mRNA Expression in Primary Human Skeletal Muscle Cultures after 8 d of IL-6 (25 ng/ml) Exposure

Gene	mrna Expression in Relation to Untreated Cultures (Untreated 100%)
FATP4	172 ± 21[a]
MEF2A	108 ± 7
MEF2C	114 ± 11
MEF2D	122 ± 1[a]
PPARα	131 ± 11[a]
PPARδ	156 ± 19[a]
PPARγ	156 ± 6[a]
CEBPα	82 ± 11[a]
UCP2	145 ± 13[a]
AdipoR1	120 ± 17
IL-6	183 ± 25[a]

[a] $P < 0.05$ compared with untreated cells.

contrast, siRNA-mediated down-regulation of AMPK did not affect the IL-6-mediated increase in glycogen synthesis. This latter observation was unexpected, because several lines of evidence implicate AMPK in the regulation of skeletal muscle glycogen synthesis. For example, mutations in the γ3-subunit of AMPK lead to a constitutively active enzyme, which leads to enhanced glycogen accumulation (58, 59). Thus, signaling specificity of IL-6 action on glucose and lipid metabolism is observed in skeletal muscle.

Exposure of isolated adipocytes to IL-6 impairs differentiation (60). In contrast, IL-6 enhances skeletal muscle differentiation. Similar to our findings in primary human skeletal muscle cultures, increased formation of myotubes and mRNA expression of myogenic genes such as creatine kinase and Myf-5 were seen in rat L6 cells after 10 d of IL-6 treatment (61). Thus, IL-6 appears to facilitate the differentiation of myoblasts. In skeletal muscle cultures exposed to IL-6 for 8 d, mRNA expression of several key genes is increased. When AMP kinase was reduced by siRNA, only the IL-6 effects on increased PGC-1 expression were affected in the subset of genes that were analyzed. PI3-kinase has previously been reported to be important for mediating the IL-6-dependent increase in basic fibroblast growth factor and cyclooxygenase-2 mRNA (55). Thus, IL-6 appears to mediate effects on mRNA expression via at least two separate signaling pathways, one dependent on AMP kinase and one possibly dependent on PI3-kinase. Expression of GLUT4, PGC-1, and MEF2D was significantly increased in cultured human muscle cells exposed to IL-6. We have previously shown that GLUT4 and PGC1 are linked to insulin sensitivity, as assessed by glucose incorporation into glycogen in cultured human muscle (62). MEF2D is a key myogenic transcription factor, which has also been implicated in the regulation of GLUT4 (63). As additional evidence of IL-6-increased differentiation to myotubes, the mRNA expression of CEBPα, a transcription factor primarily involved in adipose cell differentiation, was significantly decreased in cultured human muscle cells after IL-6 treatment. IL-6 exposure also increased mRNA expression of a number of genes important for lipid metabolism including UCP2, PPAR isoforms (α, δ, γ), and fatty acid transporter 4, whereas the expression of UCP3 and GLUT1 mRNA expression decreased. These genes may reflect the role of IL-6 to modulate skeletal muscle substrate utilization to favor fatty acid oxidation. However, the magnitude of the IL-6-mediated increase in β-oxidation was similar after acute and long-term IL-6 exposure and, thus, was independent of changes in gene expression.

Exercise and skeletal muscle contraction lead to a number of metabolic and gene regulatory adaptations. In response to exercise training, skeletal muscle growth is increased and metabolism is altered due to changes in gene expression profiles. Exercise training increases mRNA expression of PGC1, PPARα, and PPARδ (64, 65). In response to exercise training, insulin sensitivity is increased and the reliance on fatty acids, a major fuel substrate, is increased. After cessation of exercise, there is an increase in glycogen storage, a phenomenon referred to as glycogen supercompensation (66, 67). Exposure of primary cultured human skeletal muscle cells to IL-6 *in vitro* leads to many of these classical exercise-induced changes in metabolism and gene expression. Thus, IL-6 is a potent mediator of metabolism in skeletal muscle by directly promoting skeletal muscle differentiation and regulating substrate utilization to promote glycogen storage and lipid oxidation.

MATERIALS AND METHODS

Materials

DMEM, Ham's F-10 medium, fetal bovine serum (FBS), penicillin, streptomycin, and fungizone were obtained from Invitrogen (Stockholm, Sweden). Recombinant human IL-6 was from Sigma (St. Louis, MO). General laboratory reagents were obtained from Sigma, and radioactive reagents were purchased from Amersham (Uppsala, Sweden).

Subjects' Characteristics

Muscle biopsies were obtained with the informed consent of the donors during scheduled abdominal surgery. Subjects (four male and three female) had no known metabolic disorders. Mean age was 52.5 ± 8 yr, body mass index was 25.3 ± 3.0 kg/m^2, and fasting blood glucose was 5.6 ± 0.5 mM. The research ethical committee Nord at the Karolinska Institutet approved the study protocols.

Cell Culture and Differentiation

Muscle biopsies (rectus abdominus, ~1–3 g) were collected in cold PBS supplemented with 1% PeSt (100 U/ml penicillin and 100 μg/ml streptomycin). Satellite cells were isolated and cultured as described (68). Myotubes were treated for 20 min, 3 h, or 8 d without or with IL-6 (5, 25, or 100 ng/ml). Media (including fresh IL-6) were changed every day and before each experiment. Myotubes were incubated with serum-free DMEM for 6 h before use.

Giemsa/Wright Staining

Myotubes used for long-term (8 d) incubation with 25 ng/ml IL-6 were grown on six-well plates. To assess the extent of differentiation, myotubes were fixed in methanol (10 min), 1:10 Giemsa (15 min), and 1:10 Wright (20 min). Cells were washed with double-distilled H_2O, and mono- or multinucleated cells were observed under a phase contrast invert light microscope. Cells were placed over a Bürker-chamber, and the total number of myotubes was counted in 20 squares (0.04 mm^2) using the 40× objective. Myotube formation and fusion rate after IL-6 stimulation was measured as the percentage of myotubes with more than five nuclei. This was assessed by determining myotubes with greater than five nuclei/total myotubes/μm^2.

Western Blot Analysis

Myoblasts were grown on 100-mm dishes and were treated for 20 min, 3 h, or for 8 d with 5, 25, and 100 ng/ml IL-6 with or without acute (60 nM) insulin (20 min) and were then scraped into 400 μl ice-cold homogenizing buffer [50 mM HEPES, pH 7.6; 150 mM NaCl; 1% Triton X-100; 1 mM Na_3VO_4; 10 mM NaF; 30 mM $Na_4P_2O_7$; 10% (vol/vol) glycerol; 1 mM benzamidine; 1 mM dithiothreitol, 10 μg/ml leupeptin; 200 mM phenylmethylsulfonyl fluoride; and 1 μM microcystin]. Homogenates were rotated for 60 min at 4 C and were subjected to centrifugation (20,000 × *g* for 10 min at 4 C). After protein determination, aliquots of lysates were mixed with 4× Laemmli-sample buffer, and proteins were separated by SDS-PAGE. The results were quantified by densitometry.

PI3-Kinase Activity

Myoblasts were grown on 100-mm dishes, exposed to 25 ng/ml IL-6 for 8 d or myotubes for 3 h, and then scraped into ice-cold homogenizing buffer (see above). After protein determination, an aliquot of 300 μg protein was immunoprecipitated overnight (4 C) with anti-IRS1 antibody coupled to protein A-Sepharose (Sigma). PI3-kinase activity was assessed directly on the protein A-Sepharose beads as described (69). Reaction products were resolved by thin-layer chromatography and quantified using a PhosphorImager (Bio-Rad Laboratories, Richmond, CA).

Glycogen Synthesis

Glycogen synthesis as assessed by incorporation of ^{14}C-labeled glucose into glycogen was determined essentially as previously described (68). Myotubes, in six-well dishes, were treated as indicated above and were serum starved for 6 h before the assay. Thereafter, the myotubes were stimulated with or without 25 ng/ml IL-6 for 90 min with 0, 6, or 60 nM insulin included at the last 30 min before adding the radioactive glucose. Thereafter, cells were incubated with 5 mM glucose DMEM, supplemented with D-[U-^{14}C] glucose (final specific activity, 0.18 μCi/μmol) for another 90 min. Each experiment was carried out on duplicate wells.

Lactate Concentration

Day-7 differentiated myotubes were stimulated with 25 ng IL-6 with or without 60 nM insulin for 0, 2, 24, 48, and 72 h in serum-free DMEM. Media (100 μl) were collected in duplicates, and lactate concentration in media was determined using a lactate kit (catalog no. A-108; Biochemical Research Service Center, University at Buffalo, New York).

Glucose Uptake

Glucose uptake was determined essentially as previously described (70). In brief, overnight serum-starved myotubes were stimulated with or without 25 ng/ml IL-6 for 60 min with 0, 6, or 60 nM insulin in KREBS buffer (20 mM HEPES, pH 7.4; 140 mM NaCl; 5 mM KCl; 2.5 mM $MgSO_4$; 1 mM $CaCl_2$). Thereafter, cells were incubated with 10 μM 2-deoxy-[^3H]glucose (1μCi/ml) for another 10 min. Each experiment was carried out on duplicate wells.

Determination of Free Fatty Acid Oxidation

Assessment of fatty acid oxidation was adapted from Petersen *et al.* (30) with some modification. In brief, myoblasts were grown on a 25-cm^2 cell culture flask in 5% CO_2-95% O_2 humidified air in growth medium (Ham's 10 medium supplemented with 20% FBS) and differentiated to myotubes at greater than 80% confluence. Before starting the experiment, a 2-mm hole was made on the lid of each flask and two sheets of 24-mm Whatman filter (catalog no. 108340-24; VWR International AB, Stockholm, Sweden) were encircled with a gauze bandage compass. The filter-compass was then pressed into the inside of the culture flask lid. Overnight serum-starved myotubes (8 d after differentiation) were treated for 180 min with 0.4 μCi of [^{1-14}C] palmitate (or oleate) in 2 ml serum-free DMEM with or without 25 ng/ml IL-6 and with or without insulin (60 nM) at 37 C in 5% CO_2-95% O_2 and were then tightly closed with the filter-lid. After treatment, 200 μl of Solvable reagent (benzethonium hydroxide; Packard Biosciences, Meriden, CT) was added drop-wise through the hole of flask-lid to soak the filter. Thereafter, 300 μl of 70% perchloric acid was injected through the hole and filter. The lids were then sealed with flexible film (Parafilm; Nordic EM Supplies, Espoo, Finland) to avoid escape of gas from the flasks through the hole. Flasks were then laid down with slight agitation for 1 h at room temperature. The filter-compass was moved to a scintillation tube with 10 ml scintillation liquid, and 200 μl of ice-cold methanol was added. The trapped $^{14}CO_2$ in the filter was then counted in a liquid scintillation counter.

Accumulation of Intracellular Labeled Palmitate

As an indication of free fatty acid uptake, flasks from the fatty acid oxidation experiment described above were washed five times with TBS-Tween 20 and cells were lysed with 2 ml of 0.03% sodium dodecyl sulfate for 2 h at room temperature with slight agitation. Lysates (400 μl) were then transferred to 4 ml scintillation fluid, and the accumulated [^{14}C] in the lysate was determined by liquid scintillation counting

Real-Time PCR Analysis of Gene Expression

Myoblasts were cultured in 100-mm dishes, and at greater than 90% confluence the differentiation was initiated together with the long-term stimulation with 25 ng/ml IL-6. Eight days after differentiation, myotubes were washed three times with ribonuclease-free PBS and were harvested directly for RNA extraction (RNAeasy mini kit; QIAGEN, Crawley, UK). All RNA was deoxyribonuclease treated before reverse transcription (RQ1 RNase-free DNase; Promega, Southampton, UK). The mRNA concentrations of target genes were determined, and cDNA was prepared from total RNA samples using the TaqMan reverse transcription reagent. The quantification of PCR products was analyzed by real-time PCR (TaqMan; Applied Biosystems, Foster City, CA) using a standard curve method (User Bulletin No. 2, ABI PRISM 7700 Sequence Detection System). All samples were analyzed in triplicate. The ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) was used for analysis. The sequences of the primers and the probes were either designed using PubMed-published data, Primer Express software (PerkinElmer, Wellesley, MA), or acquired by Assays-on-Demand (Applied Biosystems). The sequences for myocyte enhancers A, C, and D and adiponectin receptor

Table 3. Primer and Probe Sequences Used for Real-Time PCR Analysis

	Sense	Probe	Antisense
MEF2A	CAGCACCACCTAGGACAAGCA	CTCAGCTCTCTTGTTGCTGGAGGGCA	TGGATAAATTGGAACCCTGAGATAA
MEF2C	CCT ACA TAA CAT GCC ACC ATC TG	TCA GTC AGT TGG GAG CTT GCA CTA GCA C	AGG GAG AGA TTT GAA CTC TGA GAT AAA
MEF2D	CGCTCTTTGCCGTGACAAC	CCGGGCTGGAGGCTGTGC	GTTCATGGTCTGCAGGATACCTT
AdipoR 1	GCCAACCCACCCAAAGCT	AAACATGCCCAGTGCCCCAGG	CCGCACCTCCTCCTCTTCT

The primers and probe concentrations ranged from 300–900 nmol/liter.

primer and probes are given in Table 3. Hs18, PPARα, PPARδ, PPARγ, PGC1, glycogen synthase, fatty acid transporter 4, human IL-6, human GLUT1, CEBPα, human UCP2, and human UCP3 primer/probe complexes were obtained from Assays-on-Demand (Applied Biosystems).

To verify lack of contamination by genomic DNA, an identical sample in which the reverse transcription reagent had been omitted from the cDNA synthesis was run in parallel under identical conditions. The control samples indicated that there was no genomic contamination in the total RNA preparation (data not shown). Data were analyzed using the values of the 18s gene levels as a baseline. Two other housekeeping genes (β2-microglubolin and glyceraldehyde-3-phosphate dehydrogenase) were also assessed, but the 18s gene levels showed least variation between different conditions.

AMPKα1 and α2 siRNA Design and Transfection

siRNA Design. Constructs of AMPKα1 and α2 siRNA oligos (Table 1) were designed with 3′ overhanging thymidine dimers. Primers were purchased from Ambion (Austin, TX). Target sequences were aligned to the human genome database in a BLAST search to eliminate sequences with significant homology to other genes. Sense and antisense RNAs were annealed following the manufacturer's recommended procedures.

siRNA Transfection. siRNA transfection on myoblasts was described previously (71). Because the transfection of AMPK siRNA impaired myocyte differentiation, we applied this technique to differentiated myotubes. In brief, individual siRNAs (1 μg/ml) were mixed in serum/antibiotic-free DMEM (final vol, 50 μl/ml) for 5 min, and 1 μl of the transfection agent, lipofectamine 2000 (Invitrogen), was mixed and incubated with 49 μl DMEM in a separate tube for 5 min. The two mixtures were combined and mixed gently with agitation at room temperature for 30 min. Differentiated myotubes (2 d) were washed with sterile PBS twice, and 1 ml of serum/antibiotic-free DMEM was added to each well and incubated at 37 C. siRNA transfection complexes (100 μl) were added to each well and incubated for more than 16 h. Myotubes were washed with sterile PBS, and 2 ml/well 4% FBS-supplemented DMEM was added without or with IL-6 (25 ng/ml). Myotubes were used 4 d after transfection. Control cultures were similarly prepared but without addition of siRNA or scramble siRNA. We observed no further cell death in cultures exposed to siRNA/Lipofectamine 2000 compared with those exposed to Lipofectamine 2000 alone. Transfected myotubes were used to determine glycogen synthesis and β-oxidation as described above.

Statistics

Data are presented as mean ± SEM. Statistical differences were determined by Student's *t* test or ANOVA using Fisher's least significant difference test for *post hoc* determination.

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Address all correspondence and requests for reprints to: Anna Krook, Ph.D, Integrative Physiology, Department of Physiology and Pharmacology, Karolinska Institutet, 171 77 Stockholm, Sweden. E-mail: Anna.Krook@ki.se.

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