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Leptin Expression in Human Primary Skeletal Muscle Cells Is Reduced During Differentiation

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Abstract We found leptin to be strongly expressed in undifferentiated human myoblasts derived from biopsies of the thigh (Musculus vastus lateralis). Both mRNA expression and secretion of leptin were reduced during in vitro differentiation into primary myotubes. However, the expression of the leptin receptor (OB-Rb) mRNA, was unchanged during differentiation of the muscle cells. Administration of recombinant leptin had no effect on leptin, myogenin, myoD, or GLUT4 mRNA expressions during the period of cellular differentiation. A functional leptin receptor was demonstrated by an acute leptin-induced 1.5-fold increase in ERK activity ($P = 0.029$). Although mRNA expression of regulation of suppressor of cytokine signaling-3 (SOCS-3) mRNA expression was unaltered, leptin significantly stimulated fatty acid oxidation after 6 h measured as acid soluble metabolites (ASM). Palmitic acid (PA), oleic acid (OA), and eicosapentaenoic acid (EPA), known to modulate leptin expression in other tissues, had no effect on mRNA expression or secretion of leptin from human myotubes. In conclusion, we demonstrate that leptin is highly expressed in undifferentiated human myoblasts and the expression is reduced during differentiation to mature myotubes. The role of leptin in these cells needs to be further characterized. J. Cell. Biochem. 96: 89–96, 2005. © 2005 Wiley-Liss, Inc.

Key words: leptin; leptin receptor; human skeletal muscle cells; differentiation; fatty acids

Although leptin initially was acknowledged as an adipocyte-derived hormone [Zhang et al., 1994] regulating food intake and energy expenditure via specific receptors located in the hypothalamus [Stephens et al., 1995], the picture is now recognized as more complicated. Leptin may be viewed as a multi-potent cytokine with several central as well as peripheral effects in different organs, tissues, and cells. Whereas circulating leptin in adult individuals is mainly secreted from adipose tissue [Zhang et al., 1994; Stephens et al., 1995], the leptin polypeptide and mRNA are detected in several tissues of mesenchymal origin, for example, bone [Reseland et al., 2001], cartilage [Figenschau et al., 2001], and skeletal muscle [Wang et al., 1998]. In rat skeletal muscle cells (L6 cells), leptin expression was shown to be induced by glucosamine, indicating that the hexosamine biosynthetic pathway may function as an energy-sensing device that is linked to regulation of leptin gene expression and secretion [Wang et al., 1998].

The leptin receptor (OB-R) exhibits considerable homology with the interleukin-(IL) 6 receptor and belongs to the cytokine class I receptor family [Tartaglia et al., 1995]. The long form of the leptin receptor (OB-Rb) is the most abundantly expressed isoform in the hypothalamus and probably mediates most of the signaling events [Bjorbak et al., 1997]. However, the leptin receptor is also expressed in other areas of the central nervous system, and in many other organs and cell types [Bennett et al., 1996]. Signals via OB-Rb receptor are initiated by activation of one or more members of the Janus tyrosine kinases [Heschka and Jones, 2001]. One pathway leads to phosphorylation

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and activation of signal transducers and activators of transcription (STATs) [Vaisse et al., 1996], and subsequent SOCS-3 expression [Bjorbak et al., 2000]. Another pathway leads to ERK/MAPK activation and nuclear transcription of c-fos [Heschka and Jones, 2001].

In this report, we demonstrate how leptin is expressed during differentiation of human primary myoblasts into mature myotubes.

MATERIALS AND METHODS

Materials

The skeletal muscle cell growth medium SkGM Bullet kit was obtained from Clonetics (BioWhittaker, Verviers, Belgium), whereas Ham's F-10 medium, α-MEM, trypsin/EDTA, fetal calf serum (FCS), penicillin/streptomycin (10.000 IE/10.000 μg/ml), fungizone, L-glutamine, and α-MEM were from Gibco BRL (Paisly, UK). Extra cellular matrix (ECM) gel, cytochalasin B, bovine serum albumin (BSA essentially fatty acid free), L-carnitine, oleic acid (OA), and palmitic acid (PA) were purchased from Sigma-Aldrich (St. Louis, MO). [1-^{14}C] OA (53 mCi/mmol) was provided by American Radiolabeled Chemicals (St. Louis, MO), and Instagel scintillation liquid was from Packard Bioscience Company (Groningen, The Netherlands). The primers for leptin, OB-Rb, SOCS-3, and α-tubulin were delivered by Eurogenetics (Seraing, Belgium), whereas primers for myogenin, myoD, GLUT4, GAPDH, and 36B4 from Invitrogen Corp. (www.invitrogen.com). Recombinant human leptin was obtained from BIOMOL Research Labs, Inc. (Plymouth Meeting, PA). [^{3}H] thymidine was delivered by Amersham International (Buckinghamshire, UK). Monoclonal antibody against phosphorylated ERK (#9106, mouse) was purchased from Cell Signaling Technology (Beverly, MA).

Isolation and Culturing of Human Skeletal Muscle Cells

Muscle biopsies were taken from M. vastus lateralis of young healthy volunteers with normal fasting plasma glucose and insulin levels (Table I). The study was approved by the Regional Committee for Research Ethics, Oslo, Norway. Fibroblast-free muscle cell cultures were established by the methods of Henry et al. [1995] with minor modifications. Briefly, the muscle tissue was dissected in Ham's F-10 media at 4°C, and dissociated by three successive incubations with 0.05% trypsin/EDTA. Satellite cells were resuspended in muscle cell growth medium (SkGM) with 2% FCS and no insulin. The cells were grown in culture wells coated with ECM gel [Gaster et al., 2001]. After 2–3 weeks, at about 80% confluence, fusion, and differentiation of myoblasts into multinucleated myotubes were initiated by changing the medium to α-MEM with 2% FCS and incubated for 8 days. Experiments were performed on cells subcultured 3–6 times. The differentiated cells were free of fibroblasts, expressed the glucose transporter GLUT4, and responded to insulin as evaluated by glucose uptake and glycogen synthesis [Aas et al., 2002, 2004].

TABLE I. Clinical Characteristics of the Skeletal Muscle Cell Donors

Donors	Mean (±SEM)
Numbers (F/M)	6 (2/4)
Age (years)	24.5 (0.7)
BMI (kg/m^2)	23.6 (1.1)
Fasting plasma glucose (mmol/L)	4.8 (0.2)
Fasting plasma insulin (pmol/L)	91 (16)

F = female, M = male. Data is presented as mean (SEM).

Protein Analysis

The amount of leptin secreted by the cells into the media was measured by a competitive radioimmunoassay (Linco Research, St. Charles, MO) using recombinant ^{125}I-leptin as tracer.

RNA Isolation

For mRNA-isolation, cultured muscle cells were washed twice with ice-cold PBS and lysed in lysis/binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% SDS, 0.5 mM dithiothreitol). Isolation of mRNA was carried out using oligo(dT)$_{25}$ coated magnetic Dynabeads (Dynal AS, Oslo, Norway) as described by the manufacturer. Briefly, prewashed oligo(dT)$_{25}$ (5 mg/ml) Dynabeads and lysates from myotubes were rotated 5 min at room temperature. After extensive washing, the beads/mRNA were resuspended in 10 mM Tris-HCl, pH 8.0, and stored at −70°C before use.

For total RNA-isolation, the cells were lyzed and total RNA isolated by RNeasy Mini kit (Quiagen Sciences, MD). The RNA samples treated with RNase-free DNase (Quiagen Sciences) as previously described [Aas et al., 2004].

PCR Analyses

Semiquantitative analyses of mRNA expression were performed directly on the mRNA attached to the Dynabeads using GeneAmp EZ rTth RNA PCR kit (Perkin Elmer, Applied Biosystem, Foster City, CA). One and a half microliters of the mRNA/beads and 2 μCi ^{32}P-dCTP were added for each reaction. The one-step RT-PCR reactions were performed at the following temperature cycles: 60°C for 30 min, 94°C for 1 min followed by 25 to 40 cycles at 94°C for 30 sec and 60°C for 1.5 min. Finally the samples were incubated at 60°C for 7 min. Oligonucleotide sequence of sense (S) and antisense (A) primers used for PCR are shown in Table II.

The reaction mixtures were separated on 2% agarose gels and stained with ethidium bromide before the bands were visualized on a UV screen, excised, eluted for 2 h in scintillation liquid and counted for 1 min in a liquid Packard 1900 TR scintillation counter (Packard, Chicago, IL). Relative abundance of mRNAs was calculated as the ratio between leptin, OB-Rb or SOCS-3 versus α-tubulin for each sample, and presented as percentage of control.

The total RNA was reversely transcribed as previously described [Aas et al., 2004]. Real-time PCR was performed using specific primers (Table II) and an ABI PRISM 7000 Detection System (Applied Biosystems). DNA expression was determined by SYBR Green, and each target was quantified in triplicate and carried out in 25 μl reaction volume. All assays were run for 40 cycles (95°C for 12 s followed by 60°C for 60 s). The transcription levels of leptin, MyoD, myogenin, and GLUT 4 were normalized to the housekeeping control genes 36B4, GAPDH, and α-tubulin.

Measurement of ERK Activity

ERK kinase activity was measured as previously described [Anderson et al., 1991; Thoresen et al., 1998]. Shortly, the cells were stimulated with 100 ng/ml leptin for 0, 15, 30, and 60 min, rinsed twice in saline, and harvested into a buffer containing 25 mM Tris pH 7.4, 25 mM NaCl, 1.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 mM dithiotreitol, 1 mM Na_3O_4 V, and 10% (v/v) ethylene glycol. The lysate was centrifuged at 15,000*g* for 10 min. The supernatant was mixed with phenyl-sepharose (Pharmacia, Sweden), washed twice with each of buffers containing 10% or 35% ethylene glycol, before elution of the ERK kinases with 60% ethylene glycol. The eluate was assayed for kinase activity in the presence of a protein kinase A inhibitor (protein kinase inhibitor, Sigma P-0300) using myelin basic protein as substrate.

Immunoblotting

Immunoblotting was performed as previously described [Thoresen et al., 1998] with antibodies recognizing ERK1 and ERK2 ($p44^{mapk}$ and $p42^{mapk}$) in activated forms, that is, specific for ERK phosphorylated at Thr202 and Tyr204.

TABLE II. Primers Used in the PCR Reactions

Gene	Primer sequence	GeneBank accession number
Leptin	S 5′-GGCTTTGGCCCTATCTTTTC-3′ A 5′-GGATAAGGTCAGGATGGGGT-3′	NM_000230
OB-Rb	S 5′-GCCAGAGACAACCCTTTGTTAAA-3′ A 5′-TGGAGAACTCTGATGTCCGTGAA-3′	NM_002303
SOCS-3	S 5′-CTCAAGACCTTCAGCTCCAA-3′ A 5′-TTCTCATAGGAGTCCAGGTG-3′	NM_003955
Myogenin	S 5′-GGACTGGACGCCCTCATTC-3′ A 5′-CGC TCT GGT CCC CTG CTT-3′	X_17651
MyoD	S 5′-GCGCCCAAAAGATTGAACTTA-3′ A 5′-CCGCCTCTCCTACCTCAAGA-3′	BC_064493
GLUT4	S 5′-GCTACCTCTACATCATCCAGAATCTC-3′ A 5′-CCAGAAACATCGGCCCA-3′	M_20747
α-tubulin	S 5′-CACCCGTCTTCAGGGCTTCTTGGTTT-3′ A 5′-CATTTCACCATCTGGTTGGCTGGCTC-3′	NM_006082
36B4	S 5′-CCATTCTATCATCAACGGGTACAA-3′ A 5′-AGCAAGTGGGAAGGTGTAATCC-3′	M_17885
GAPDH	S 5′-TGCACCACCAACTGCTTAGC-**3**′ A 5′-GGCATGGACTGTGGTCATGAG-3′	J_04038

Oligonucleotide sequences of sense (S) and antisense (A) primers used in the PCR of target and housekeeping genes and their corresponding gene bank accession numbers.

Fatty Acid Oxidation

For measurement of ASM (fatty acid β-oxidation products), cells were grown in 6-well plates and loaded with [1-^{14}C] OA (OA; 0.5 μCi/ml, 0.2 mM) for 24 h. The cells were washed twice with PBS and incubated with α-MEM containing 1.0 mM L-carnitine and leptin (100 ng/ml). After 2, 6, or 24 h an aliquot of 250 μl of the cell media was precipitated with 100 μl 6% BSA and 1.0 ml 1 M perchloric acid, and centrifuged at 1,800*g* for 10 min. ASM were measured by liquid scintillation of 500 μl of the supernatant.

Fatty Acid Incubations

Differentiated cells were incubated for 24 and 48 h with 0.1 or 0.6 mM of PA, OA, or eicosapentaenoic acid (EPA, 20:5, n-3), respectively. The fatty acids were bound to fatty acid-free BSA (2.5:1).

DNA Synthesis

[^{3}H]-thymidine was added to the cultures (1 μCi/ml, 0.125 Ci/mmol) at day 5 and 6. DNA synthesis was measured by determining the amount of radioactivity incorporated into cellular DNA. The cellular material was dissolved with 1.5 ml 0.5 N NaOH at 37°C, collected, mixed with 1.5 ml H_2O, and precipitated with 0.75 ml 50% trichloroacetic acid (TCA). The acid-precipitable material was transferred to glass fiber filters (GF/C Whatman) and washed twice with 5 ml 5% TCA, followed by liquid scintillation counting of the filters.

Statistics

Data are presented as means ± SEM for experiments performed on three different donors. Statistical comparison between different incubations was performed by SPSS® Base 12.0 for Windows®. When comparing different incubations, Mann–Whitney Rank Sum Test and ANOVA on ranks were used. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Differentiation of Human Primary Skeletal Muscle Cell Cultures

During a differentiation period of 8 days, single myoblasts fused into multinucleated myotubes (Fig. 1A). No fibroblastic cells appeared to be present in the culture. The proliferation potential of the cells was lost at day 5 of differentiation, as no changes in ^{3}H-thymidine incorporation was found after this time point (data not shown). The myotubes showed striation and spontaneous contractions as evaluated by light microscopy, indicating functionally in vitro differentiated cells (data not shown).

Expression of Leptin and OB-Rb During Differentiation

Primary myoblasts expressed leptin, verified both at the mRNA level and as protein secretion to the medium. The expression level of leptin was high for 48 h prior to start of differentiation (data not shown). During the first 6 days of differentiation the expression of leptin mRNA was reduced to 47 ± 5% of basal level, and this reduced expression was maintained through the 8 days differentiation period (Fig. 1B,C). Concomitant with this, leptin secretion was reduced from 102 ± 8 pg/ml the initial 2 days to 43 ± 2 pg/ml at the end of the differentiation period. OB-Rb mRNA expression (Fig. 1B) was not altered during the differentiation period when compared to control (data not shown).

Effect of Recombinant Leptin on Skeletal Muscle Cells

There were no visual morphological differences in the cells grown with or without the addition of recombinant leptin during the differentiation period from day 0 to 8 after plating of the cells.

No significant changes were found of mRNA expressions of myogenin and myoD (Table III), which are regulators of muscle differentiation [Cornelison and Wold, 1997]. Neither was the expression of the glucose transporter GLUT4 altered (Table III).

In the initial phase of cell differentiaton (day 2), recombinant leptin (100 ng/ml) tended to reduce ($P = 0.08$) in leptin mRNA expression as compared to untreated cells. However, the expression of leptin mRNA was not altered by the addition of recombinant leptin (1, 10, and 100 ng/ml) in the late phase of differentiation (day 4–8) (Fig. 2A).

Furthermore, we observed no change in SOCS-3 mRNA expression during leptin administration (Fig. 2B), whereas leptin acutely induced a 1.5-fold increase ($P = 0.029$) in ERK activity as compared to untreated cells (Fig. 2C). The rise in ERK activity was verified by Western

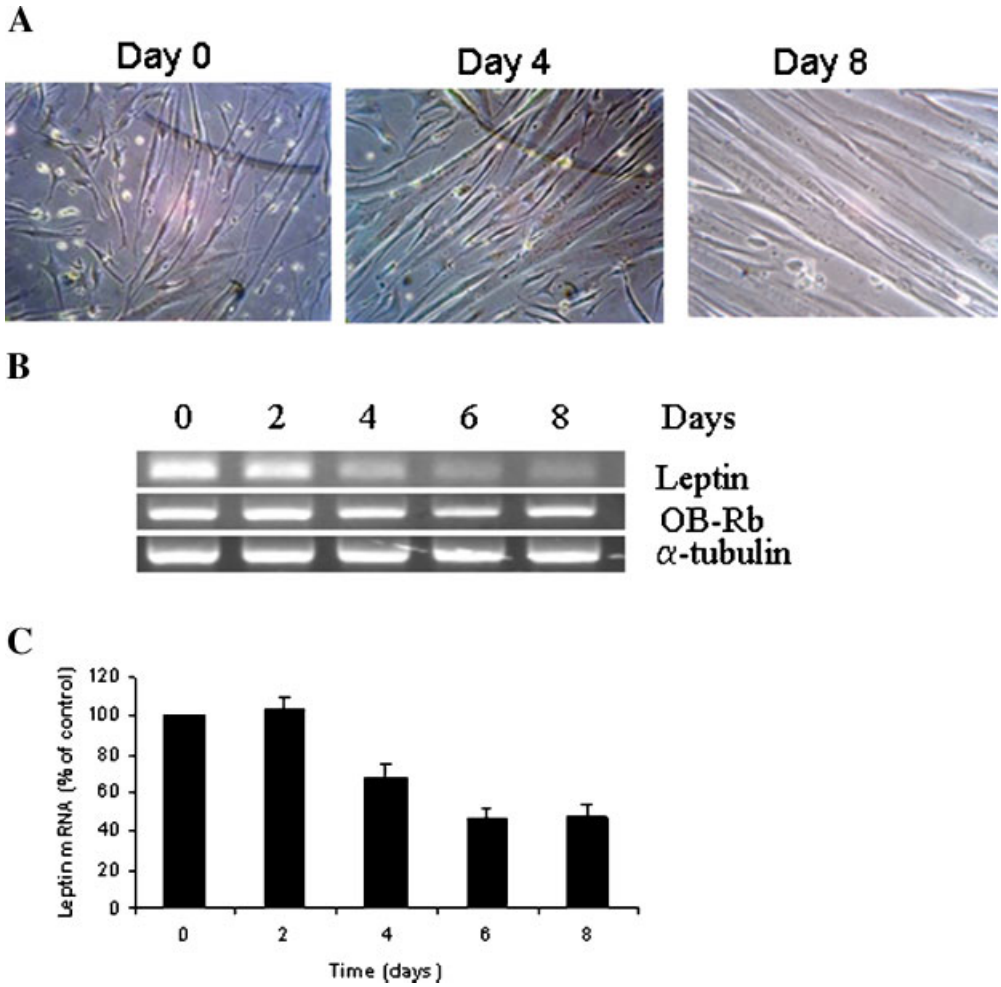

Fig. 1. Expression of leptin and OB-Rb during differentiation of human skeletal muscle cells. **A**: Light microscopy picture of undifferentiated myoblasts (left), after 4 days of differentiation (middle), and fused multinucleated myotubes after 8 days of differentiation (right) (40× magnification). **B**: Semiquantitative analyses of mRNA expression of leptin, OB-Rb, and α-tubulin after 0, 2, 4, 6, and 8 days of differentiation in vitro. One representative agarose gel of one experiment (n = 4) is shown. **C**: The expression of leptin mRNA (in B) is related to α-tubulin, and presented as percentage of untreated cells (control). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE III. Effects of Recombinant Leptin (100 ng/ml) on the Relative mRNA Expressions of the Skeletal Muscle Cell Markers Myogenin, MyoD, and GLUT4

	Days after start of differentiation			
Gene	2	4	6	8
Myogenin	0.81 (0.18)	0.85 (0.30)	1.19 (0.44)	0.65 (0.30)
MyoD	0.88 (0.19)	1.46 (0.51)	1.02 (0.38)	1.48 (0.69)
GLUT4	0.82 (0.18)	1.00 (0.35)	1.63 (0.61)	1.10 (0.51)

Data are based on the expression in three donors corrected against the expression of α-tubulin, 36B4, and GAPDH, and presented as the mean (SEM) ratio to untreated cells.

blotting using an antibody against phosphorylated ERK (data not shown).

After 6 h of incubation, leptin (100 ng/ml) increased fatty acid oxidation measured as acid soluble metabolites (ASM) in the cell culture medium. ASM was increased from 31.3 ± 1.0 nmol/mg protein in untreated cells to 34.1 ± 0.7 nmol/mg protein after leptin incubation ($P = 0.041$). No significant differences were observed after 2 or 24 h of leptin incubation, respectively (data not shown).

Effects of Fatty Acids and Glucose

Neither glucose (5.5 mM vs. 20 mM) (data not shown) nor fatty acids (0.1 or 0.6 mM of PA,

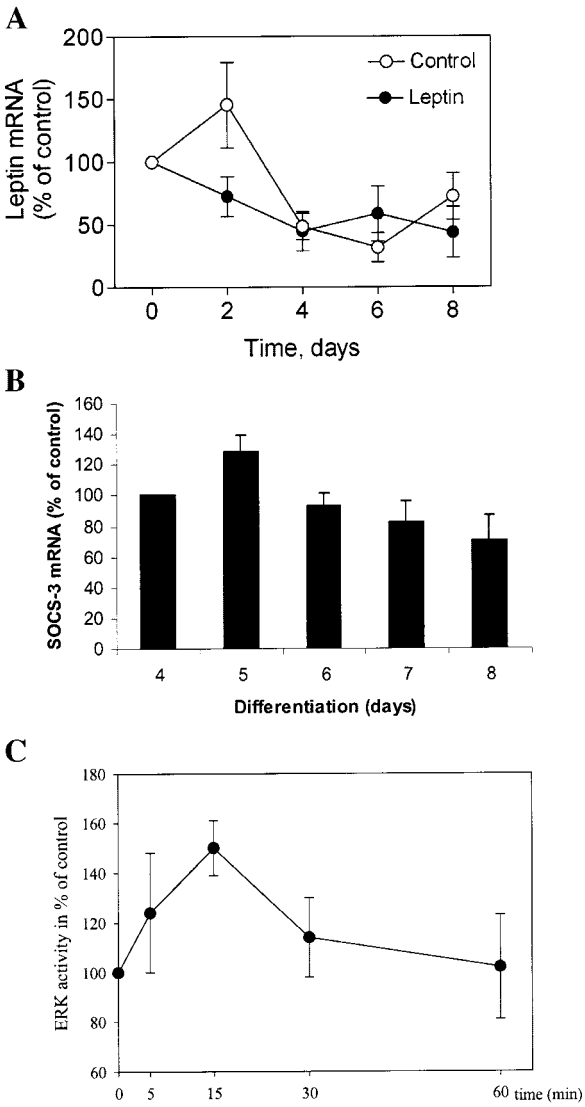

Fig. 2. Effects of recombinant leptin (100 ng/ml) on leptin (**A**) and SOCS-3 (**B**) mRNA expressions, and ERK activity (**C**). A: The effect of leptin (closed circles) on thex expression of leptin mRNA from day 0 to 8 during cell differentiation. Data are based on the expression in three donors normalized to the mean of three housekeeping genes; α-tubulin, 36B4, and GAPDH, and presented as percentage of the expression in untreated cells (control; open circles). B: The effect of leptin on SOCS-3 mRNA expression. Data are related to α-tubulin and presented as percentage of untreated cells (control). C: The effect of leptin on ERK activity after 0, 5, 15, 30, and 60 min incubation and presented as percentage of control cells. The experiments were performed on day 7 of differentiation.

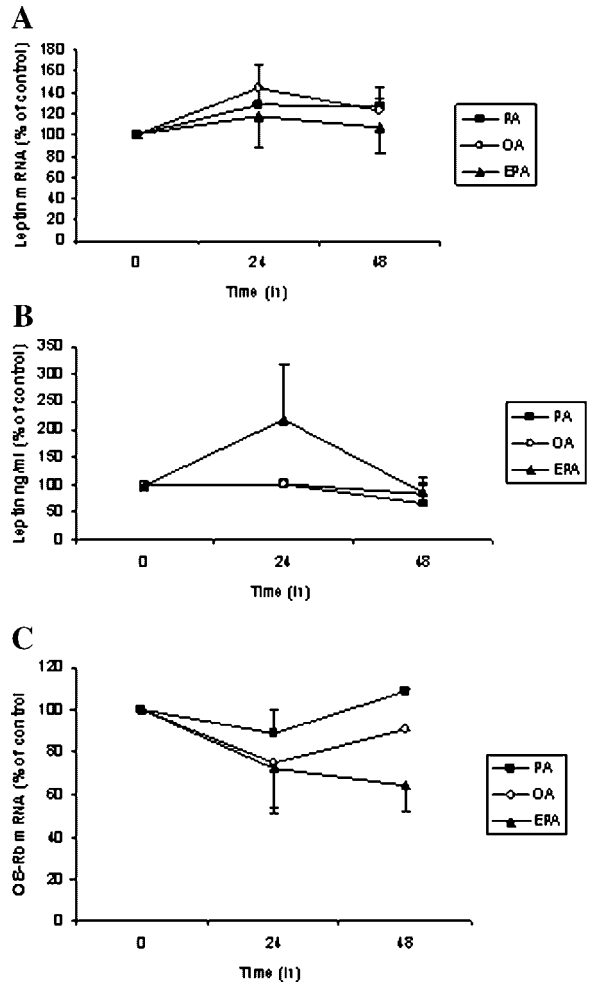

Fig. 3. Effects of fatty acids on leptin mRNA expression (**A**), leptin secretion to the medium (**B**), and OB-Rb mRNA expression (**C**) in differentiated myotubes. Differentiated myotubes (day 6) were incubated with 0.6 mM of palmitic acid (PA), oleic acid (OA), or eicosapentaenoic acid (EPA) for 24 and 48 h, respectively. Data are expressed as percentage of BSA control.

OA, and EPA) had any effect on leptin mRNA expression (Fig. 3A) or secretion of the protein to the culture medium from mature myotubes (Fig. 3B). OA and EPA (0.6 mM), but not PA, caused a reduction ($P = 0.029$ for both) in OB-Rb mRNA expression relative to control after 24 h (Fig. 3C). The effect of OA was temporarily, whereas EPA showed a lasting reduction ($P = 0.037$) in OB-Rb expression pattern after 48 h (Fig. 3C). No significant effects were observed using a lower concentration (0.1 mM) of OA or EPA (data not shown).

DISCUSSION

We demonstrate in this study that leptin is expressed in and secreted from primary undifferentiated human skeletal myoblasts and that the expression decrease during differentiation into multinucleated myotubes. Cultured skeletal myoblasts expressed functional leptin receptor but administration of recombinant leptin

during differentiation caused no alternations in the expression of regulators of muscle differentiation. The expression of leptin was reduced, indicating that leptin expression might be autoregulated in myoblasts. Recently, leptin and its receptors were identified in the rat heart [Purdham et al., 2004], both in cardiomyocytes and in whole heart homogenates, suggesting the heart muscle as a target of leptin action via a paracrine or autocrine action.

The role of leptin in the early stage of myoblast differentiation is not known. Muscle cells, osteoblasts, and adipocytes differentiate from a common mesenchymal origin and express leptin [Wang et al., 1998; Reseland et al., 2001], although the gene expression pattern in the mature endpoint of these cells is different. In adipocytes and osteoblasts, leptin expression is induced and increases during differentiation, whereas we demonstrate the opposite in skeletal muscle cells. The cell model system used in this study is well established [Henry et al., 1995], and the cells hold all characteristics for mature muscle cell type expressing known muscle cell markers, changing glucose uptake and glycogen synthesis in response to insulin [Aas et al., 2002, 2004]. In addition, we observed striation and spontaneous contractions, which strengthens the conclusion that the differentiation end point is myotubes.

Exogenous leptin did not seem to alter the differentiation process of the cells since incubation with recombinant leptin had no effect on the mRNA expression of two regulators of muscle differentiation, myoD, and myogenin, neither was the expression of GLUT4 changed during the cellular differentiation. Although leptin reduced its own expression in the initial phase of differentiation, any endocrine or paracrine role of leptin during myoblast differentiation needs to be investigated further.

The regulation and detailed function of leptin in differentiated myotubes also remains obscure. Peripheral leptin infusions has previously been demonstrated to induce leptin expression in skeletal muscle samples from lean (wild-type) and Sprague–Dawley (S–D) rats, whereas leptin expression in skeletal muscle of Zucker (fa/fa) rats failed to be affected by exogenous leptin [Wang et al., 1999]. The OB-Rb receptor was found to be functional, as observed by an acute effect of leptin administration on ERK activation, and a small but consistent effect on fatty acid oxidation. A functional OB-Rb receptor was also found by Steinberg et al. [2002] who demonstrated that leptin increases fatty acid oxidation in human skeletal muscle from lean but not in obese.

Leptin mRNA expression may be induced in rodent skeletal muscle cells by providing glucosamine, a regulator of the hexosamine biosynthetic pathway [Wang et al., 1998]. We observed no change in leptin mRNA expression by adding fatty acids (PA, OA, and EPA) or high concentrations of glucose (20 mM). The low leptin mRNA expression level in mature myotubes might make any effect of nutrients on leptin expression hard to detect. Fatty acids, as well as thiazolidinediones, may induce the adipose differentiation program of C2C12N myoblasts, as well as satellite cells from newborn mouse muscle [Teboul et al., 1995], preventing myotube formation and inducing an adipocyte phenotype. This is in contrast to our system, where the cells differentiate into myotubes prior to addition of fatty acid. In contrast to incubation with leptin, unsaturated fatty acids (OA and EPA) reduced the OB-Rb mRNA expression.

Cultured L6 rat skeletal muscle cells may respond both to acute and chronic leptin incubation [Sweeney et al., 2001; Tajmir et al., 2003]. Sweeney et al. [2001] showed that 30-min incubation (acute) had no effect on ERK phosphorylation. These data are verified in our study showing that the ERK effect appears at 15 min and thereafter vanished to basic levels after 30 min. During myotube differentiation we found that SOCS-3 and OB-Rb mRNA expressions were unaffected, although leptin expression diminished.

In conclusion, we demonstrate that leptin is transcribed, translated, and secreted from primary cultured human myoblasts, and that leptin expression is reduced during differentiation to myotubes. The regulation and function of leptin in these cells need to be further characterized.

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