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Leptin receptor 170 kDa (OB-R170) protein expression is reduced in obese human skeletal muscle: a potential mechanism of leptin resistance

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To examine whether obesity-associated leptin resistance could be due to down-regulation of leptin receptors (OB-Rs) and/or up-regulation of suppressor of cytokine signalling 3 (SOCS3) and protein tyrosine phosphatase 1B (PTP1B) in skeletal muscle, which blunt janus kinase 2-dependent leptin signalling and signal transducer and activator of transcription 3 (STAT3) phosphorylation and reduce AMP-activated protein kinase (AMPK) and acetyl-coenzyme A carboxylase (ACC) phosphorylation. Deltoid and vastus lateralis muscle biopsies were obtained from 20 men: 10 non-obese control subjects (mean ± s.d. age, 31 ± 5 years; height, 184 ± 9 cm; weight, 91 ± 13 kg; and percentage body fat, 24.8 ± 5.8%) and 10 obese (age, 30 ± 7 years; height, 184 ± 8 cm; weight, 115 ± 8 kg; and percentage body fat, 34.9 ± 5.1%). Skeletal muscle OB-R170 (OB-R long isoform) protein expression was 28 and 25% lower (both $P < 0.05$) in arm and leg muscles, respectively, of obese men compared with control subjects. In normal-weight subjects, SOCS3 protein expression, and STAT3, AMPKα and ACCβ phosphorylation, were similar in the deltoid and vastus lateralis muscles. In obese subjects, the deltoid muscle had a greater amount of leptin receptors than the vastus lateralis, whilst SOCS3 protein expression was increased and basal STAT3, AMPKα and ACCβ phosphorylation levels were reduced in the vastus lateralis compared with the deltoid muscle (all $P < 0.05$). In summary, skeletal muscle leptin receptors and leptin signalling are reduced in obesity, particularly in the leg muscles.

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Human obesity is characterized by increased leptin concentration in plasma, as well as leptin (Steinberg *et al.* 2002*b*; Bates & Myers, 2003; Anubhuti & Arora, 2008; Myers *et al.* 2008) and insulin resistance (Olefsky *et al.* 1982). Insulin resistance has been associated with raised plasma leptin concentrations independent of body fat mass (Sørensen *et al.* 1996). Leptin resistance in skeletal muscles could be caused by a down-regulation and/or desensitization of leptin receptors (OB−Rs), among other mechanisms.

Upon binding to the long form of its receptor (OB-Rb), leptin stimulates janus kinase 2 (JAK2), which autophosphorylates, and phosphorylates several tyrosine residues (Tyr) of OB-Rb (Bjørbæk & Kahn, 2004). The signal transducer and activator of transcription 3 (STAT3) binds to the phosphorylated Tyr^{1138} in OB-Rb, and this interaction is required for tyrosine phosphorylation and activation of STAT3 by JAK2 (Banks *et al.* 2000; Bates *et al.* 2003). Phosphorylation of STAT3 on Tyr^{705}, mediated by Tyr^{1138}, is required for leptin regulation of energy balance and body weight (Bates *et al.* 2003). Moreover, reduce Tyr^{705}-STAT3 phosphorylation in the presence of increased leptin concentrations is indicative of leptin resistance (Hosoi *et al.* 2008).

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Leptin promotes fatty acid (FA) oxidation in skeletal muscle through activation of AMP-activated protein kinase (AMPK) which, in turn, phosphorylates and inhibits acetyl-coenzyme A carboxylase (ACC), leading to reduced malonyl-coenzyme A and increased FA flux into the mitochondria via carnitine palmitoyl transferase-1 (Ruderman *et al.* 1999). In men, skeletal muscle leptin resistance may be accompanied by decreased basal Thr^{172}-AMPKα and Ser^{221}-ACCβ phosphorylation (Steinberg *et al.* 2002*a*; Bandyopadhyay *et al.* 2006). It remains unknown whether the obesity-associated reduction in basal Thr^{172}-AMPKα and Ser^{221}-ACCβ phosphorylation is general or is limited only to certain skeletal muscles.

The elevated leptin levels observed in obesity could down-regulate leptin receptors, since mRNA levels of the long (OB-Rb) and short isoforms (OB-Ra) of the leptin receptor are markedly reduced in the hypothalamus and liver of obese rats, which have enhanced plasma leptin concentration (Liu *et al.* 2007). Leptin may also down-regulate leptin signalling in the target tissues by inducing the protein suppressor of cytokine signalling 3 (SOCS3), which blunts JAK2/STAT3-dependent leptin signalling (Bjørbæk *et al.* 2000) and causes leptin resistance in the skeletal muscle (Steinberg *et al.* 2006*c*). Furthermore, overexpression of SOCS3 inhibits leptin activation of AMPK and ACCβ phosphorylation in skeletal muscle cells (Steinberg *et al.* 2006*a*; Steinberg & Jorgensen, 2007).

Protein tyrosine phosphatase 1B (PTP1B) is also a negative regulator of leptin and insulin signalling (Dube & Tremblay, 2005) that may be increased in skeletal muscle by inflammation (Zabolotny *et al.* 2008). Protein tyrosine phosphatase 1B blunts leptin signalling by causing dephosphorylation of the leptin receptor-associated JAK2 (Dube & Tremblay, 2005).

We hypothesized that the high level of circulating leptin observed in obese humans may lead to down-regulation of leptin receptor protein expression in skeletal muscle, and increased SOCS3 and PTP1B protein levels, which may cause leptin resistance and reduced basal levels of Tyr^{705}-STAT3 and Thr^{172}-AMPKα/Ser^{221}-ACCβ phosphorylation.

Therefore, the main aim of this study was to determine whether there is a down-regulation of leptin receptor protein expression in skeletal muscles of obese human and to investigate whether this down-regulation is related to serum leptin concentration. Another aim was to determine whether the high circulating levels of leptin in obese subjects are associated with increased SOCS3 and PTP1B protein expression and reduced Tyr^{705}-STAT3, Thr^{172}-AMPKα and Ser^{221}-ACCβ basal phosphorylation levels in skeletal muscles of both the upper and lower extremities. The reason for studying arm and leg muscles is that metabolic differences between arm and leg muscles have been described in humans (Olsen *et al.* 2005). For example, Olsen *et al.* (2005) showed that glucose clearance during an insulin clamp is higher in the arm than in the leg in healthy control subjects and in type 2 diabetics.

Methods

Materials

The complete protease inhibitor cocktail was obtained from Roche Diagnostics (Mannheim, Germany). The polyclonal rabbit anti-human leptin receptor antibody that recognizes three isoforms of the human leptin receptor present in skeletal muscle (Guerra *et al.* 2007) was obtained from Linco Research (St Charles, MO, USA). The polyclonal rabbit anti-perilipin A antibody was kindly provided by Dr Andrew S. Greenberg (Jean Mayer USDA Human Nutrition Research Center, Boston, MA, USA). The polyclonal rabbit anti-human SOCS3 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal mouse anti-α-tubulin antibody was obtained from Biosigma (Madrid, Spain). The polyclonal rabbit anti-Tyr^{705}-STAT3 and the monoclonal mouse anti-STAT3 antibodies were from Cell Signaling Technology (Barcelona, Spain). The monoclonal mouse-anti-PTP1B was from Calbiochem (San Diego, CA, USA). The polyclonal rabbit anti-pThr^{172}-AMPKα, anti-AMPKα and anti-acetyl-coenzyme A carboxylase (ACC) antibody were obtained from Cell Signaling Technology (Barcelona, Spain). The polyclonal rabbit anti-phospho-acetyl-coenzyme A carboxylase (Ser^{79}) antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). The secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit and donkey anti-mouse antibodies were from Jackson ImmunoReseach (West Grove, PA, USA). The Hybond-P transfer membranes, Hyperfilm ECL and the ECL plus Western Blotting Detection System were from Amersham Biosciences (Little Chalfont, UK). The ChemiDoc XRS System and the image analysis software Quantity One© were obtained from Bio-Rad Laboratories (Hemel Hempstead, UK).

Subjects

Twenty young male subjects participated in this investigation. Body composition, basal serum glucose and endocrine variables are shown in Table 1. Written informed consent was obtained from each subject after they received a full explanation about the nature and the possible risks associated with the study procedures. The study was approved by the Copenhagen Ethics Committee (KF 01 304792), and the experiments conformed to The Declaration of Helsinki of 1975.

General procedures

Subjects reported to the laboratory after an overnight fast on 3 days over a 3 week period, and the order

of the experiments performed on the two last days was randomized. Before each of the experimental days subjects fasted overnight, and on the first experimental day, after an initial 15 min rest, height and weight were measured, whereafter subjects underwent a standard 120 min oral glucose tolerance test (OGTT), ingesting a solution of 75 g glucose dissolved in 300 ml of water. Blood samples were taken before and after 2 h for measurement of plasma glucose concentrations (ABL, series 700; Radiometer, Copenhagen, Denmark). Body composition was determined by dual-energy X-ray absorptiometry scanning using a Lunar Prodigy Advance bone densitometer (Lunar Corporation, Madison, WI, USA). Finally, a graded incremental exercise protocol was used to establish the maximal oxygen uptake ($\dot{V}_{O_2max}$) on a normal bicycle ergometer (Ergometrics 800, Jaeger, Würzburg, Germany). Before every test, a volume calibration and a calibration of the gas analysers using gases of known composition was performed.

On the second and the third day, a needle biopsy from the deltoid or the vastus lateralis muscle was obtained using Bergstrom's technique with suction, as described elsewhere (Lundby *et al.* 2006). The muscle specimen was cleaned to remove any visible blood, fat or connective tissue. The muscle tissue was frozen within 15 s in liquid nitrogen, and stored at –80°C for later analysis. On one of the days, venous blood was sampled from an antecubital vein.

Analytical procedures (glucose, insulin and leptin measurements)

Blood was transferred into iced tubes containing 0.3 M EDTA (10 μl ml^{-1} blood) and immediately centrifuged at 2480*g* at 4°C for 10 min. A small fraction of the blood was transferred into tubes containing ethylene glycol tetraacetic acid (15 μl (ml blood)$^{-1}$), and this was later used to determine insulin concentrations. The plasma was stored at −80°C until analysis. Plasma glucose was analysed using a conventional, commercially available assay on an automatic analyser (Hitachi, 612 Automatic Analyzer, Roche, Switzerland). Plasma insulin was determined using a radioimmunoassay kit (Insulin RIA100, Pharmacia, Uppsala, Sweden). Plasma leptin was measured using a specific high-sensitive human ELISA kit (R&D Systems, MN, USA). The leptin assay had an intra-assay coefficient of variation of 3.2%.

Assessment of insulin resistance

In each subject, the degree of insulin resistance was estimated by the homeostasis model assessment (HOMA). In brief, fasting plasma insulin and fasting plasma glucose values were used to calculate an index of insulin resistance. The HOMA index was calculated as fasting insulin concentration (in μU ml^{-1}) × fasting glucose concentration (in mmol l^{-1})/22.5, assuming that normal young subjects have an insulin resistance of 1.

Total protein extraction, electrophoresis and Western blot analysis

For total protein extraction from human skeletal muscle, a piece of frozen tissue was homogenized as described elsewhere (Guerra *et al.* 2007). After centrifugation at 20 000*g* at 16°C for 15 min to remove tissue debris, total protein extracts were transferred to clean tubes, and an aliquot of each extract was preserved for protein quantification by bicinchoninic acid assay (Smith *et al.* 1985). Proteins were solubilized in sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.001% (w/v) Bromophenol Blue. Equal protein amounts (50 μg) of each sample were electrophorezed on 7.5–10% SDS-PAGE using the system of Laemmli (1970) and transferred to Hybond-P membranes according to the method of Towbin *et al.* (1979). For immunoblotting, membranes were pre-incubated with 5% blotting grade blocker non-fat dry milk (Bio-Rad Laboratories, Hercules, CA, USA) in Tris-buffered saline (TBS) with 0.1% Tween 20 (blotto blocking buffer) for 1 h at room temperature (20–22°C). To detect the leptin receptor isoforms (OB-Rs), membranes were incubated with a rabbit polyclonal specific anti-human OB-R (long form) antibody. To detect SOCS3 protein expression, membranes were incubated with a rabbit polyclonal specific anti-human SOCS3 antibody. To detect PTP1B protein expression, membranes were incubated with a mouse monoclonal specific anti-human PTP1B antibody. To detect Tyr^{705}-STAT3 phosphorylation, membranes were incubated with a rabbit polyclonal antibody that recognizes this kinase only when the residue Tyr^{705} is phosphorylated. To detect total STAT3, membranes were incubated with a mouse monoclonal antibody that recognizes both forms (phosphorylated and non-phosphorylated) of this kinase. To detect Thr^{172}-AMPKα phosphorylation, membranes were incubated with a rabbit polyclonal antibody that recognizes this kinase only when the residue Thr^{172} is phosphorylated. To detect total AMPKα, membranes were incubated with a rabbit polyclonal antibody that recognizes the both forms of AMPK, namely AMPKα1 and AMPKα2. To detect ACC phosphorylation, membranes were incubated with a rabbit polyclonal antibody raised against a peptide corresponding to the sequence in rat liver ACCα around the Ser^{79} phosphorylation site, which recognizes the equivalent Ser^{221} in human ACCβ in the phosphorylated state. A single band was detected at ∼280 kDa in human

skeletal muscle, which coincides with the molecular mass reported for ACCβ (Thampy, 1989). We also verified that this antibody recognizes the two phosphorylated ACC isoforms (ACCα at 265 kDa and ACCβ at 280 kDa) in protein extracts obtained from subcutaneous adipose tissue (data not shown). To assess total ACC protein content, membranes were incubated with a rabbit polyclonal antibody that recognizes both forms of ACC. In additional experiments using human subcutaneous adipose tissue, we detected two bands with the ACC antibody corresponding to the α and β isoforms of the ACC (data not shown). In human skeletal muscle extracts, however, only one band at 280 kDa was detected, which corresponded to the β isoform (data not shown). To control for differences in loading and transfer efficiency across membranes, an antibody directed against α-tubulin was used to hybridize with the same samples. Membrane incubations with polyclonal rabbit anti-OB-R (diluted 1:1500 in blotto blocking buffer), polyclonal rabbit anti-Tyr^{705}-STAT3 (diluted 1:500 in 5% bovine serum albumin in TBS with 0.1% Tween 20; BSA blocking buffer), monoclonal mouse anti-STAT3 (diluted 1:750 in BSA blocking buffer), polyclonal rabbit-anti-Thr^{172}-AMPKα (diluted 1:1000 in BSA blocking buffer), polyclonal rabbit-anti-AMPKα (diluted 1:1000 in BSA blocking buffer), polyclonal rabbit-anti-Ser^{221}-ACCβ (diluted 1:400 in BSA blocking buffer), polyclonal rabbit-anti-ACCβ (diluted 1:400 in BSA blocking buffer) and the monoclonal mouse anti-PTP1B (diluted 1:1000 in blotto blocking buffer) were performed overnight at 4°C. Membrane incubations with polyclonal rabbit anti-SOCS3 (diluted 1:500 in blotto blocking buffer) and with monoclonal mouse anti-α-tubulin (diluted 1:50 000 in blotto blocking buffer) were performed for 1 h at room temperature. As a control for the presence of adipose tissue protein in the muscular tissue, a polyclonal rabbit anti-perilipin A antibody was used (Guerra *et al.* 2007). To explore the expression of this protein in human skeletal muscle, membranes were blocked with BSA blocking buffer for 1 h at room temperature. Membrane incubations with polyclonal rabbit anti-perilipin A antibody (diluted 1:1500 in BSA blocking buffer) were performed for 1 h at room temperature. Antibody-specific labelling was revealed by incubation with a HRP-conjugated goat anti-rabbit antibody (1:20 000) or a HRP-conjugated donkey anti-mouse antibody (1:10 000), both diluted in blotto blocking buffer and visualized with the ECL chemiluminiscence kit (Amersham Biosciences). Specific bands were visualized with the ECL chemiluminiscence kit, visualized with the ChemiDoc XRS system (Bio-Rad Laboratories) and analysed with the image analysis program Quantity one© (Bio-Rad Laboratories). The densitometry analysis was carried out immediately before saturation of the immunosignal. Data are reported as band intensity of immunostaining values (arbitrary units) obtained for OB-R, perilipin, PTP1B or SOCS3 relative to those obtained for α-tubulin, or as arbitrary units of band density obtained for the phosphorylated form of STAT3, AMPKα and ACCβ relative to those obtained for the total STAT3, AMPKα and ACCβ form, respectively. α-Tubulin, total STAT3, total AMPKα and total ACCβ protein content in the muscle biopsies of the two experimental groups was similar (data not shown; all $P > 0.05$). Western blot analysis of all proteins studied was performed in triplicate for each muscle biopsy, with a variation coefficient less than 10%. Samples from each subject were running in the same gel.

Table 1. Body composition, basal plasma glucose and endocrine variables

	Control group	Obese group
Age (years)	31.2 ± 4.8	30.4 ± 7.4
Height (cm)	184.3 ± 9.4	183.9 ± 8.2
Weight (kg)	90.9 ± 13.2	114.9 ± 8.2*
Body mass index (kg m^{-2})	26.6 ± 3.7	33.8 ± 2.3*
Whole body fat (kg)	22.3 ± 7.8	37.4 ± 9.0*
Percentage body fat	24.8 ± 5.8	34.9 ± 5.1*
$\dot{V}_{O_2max}$ (ml min^{-1} (kg whole body mass)$^{-1}$)	39.7 ± 6.1	29.8 ± 3.8*
$\dot{V}_{O_2max}$ (ml min^{-1} (kg lean mass)$^{-1}$)	54.5 ± 4.8	49.1 ± 8.7
Glucose (mmol l^{-1})	5.0 ± 0.2	5.5 ± 0.3*
Insulin (pmol l^{-1})	47.6 ± 24.7	102.7 ± 51.8*
Homeostasis model assessment	10.6 ± 5.2	25.1 ± 12.9*
Leptin (ng ml^{-1})	5.7 ± 5.2	20.1 ± 12.1*

Values are means ± S.E.M. *$P < 0.05$ *versus* control group.

Statistical analysis

Variables were checked for normal distribution by using a Kolmogorov–Smirnov test with the Lilliefors correction. Variables that deviated from the normal distribution where logarithmically transformed. Between groups, as well as between extremities, differences were determined with ANOVA, and with ANCOVA, using the percentage of body fat as covariate. The relationship between variables was determined using Pearson correlation analysis. Values are reported as the means ± S.E.M., and $P \leq 0.05$ was considered significant. Statistical analysis was performed using SPSS v.14.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Body composition and anthropometrics in both experimental groups

Body composition and anthropometrics are reported in Table 1. Both groups were comparable in age, height and lean mass, but the obese group had greater weight, body mass index (BMI), whole body fat mass, percentage of

body fat, leg fat mass, arm fat mass and trunk fat mass compared with the control subjects (all $P < 0.05$).

Serum leptin concentrations, HOMA and $\dot{V}_{O_2max}$ in both experimental groups

Serum leptin concentration was 3.5-fold higher in the obese compared with the control group ($P < 0.05$; Table 1), and this difference remained significant after accounting for the differences in percentage of body fat ($P < 0.05$). In both groups, serum leptin concentration was related to the BMI ($r = 0.72$ and $r = 0.80$ in the control and obese group, respectively; both $P < 0.05$) and to the whole body fat mass ($r = 0.77$ and $r = 0.67$ in the control and obese group, respectively; both $P < 0.05$).

The value of HOMA was 2.4-, insulin 2.2- and glucose 1.1-fold higher in the obese compared with the control group (all $P < 0.05$; Table 1). In all subjects studied, there was a linear relationship between HOMA and the serum leptin concentration ($r = 0.64$, $P < 0.01$), BMI ($r = 0.71$, $P < 0.001$) and whole body fat mass ($r = 0.57$, $P < 0.01$).

The $\dot{V}_{O_2max}$ expressed as millilitres per minute per kilogram of whole body mass was 25% lower in the obese compared with the control group ($P < 0.005$; Table 1). However, when the $\dot{V}_{O_2max}$ was expressed as millilitres per minute per kilogram of lean body mass, it was similar in both groups ($P = 0.1$; Table 1). In all subjects studied, $\dot{V}_{O_2max}$ expressed as millilitres per minute per kilogram of whole body mass was inversely related to serum leptin concentration ($r = -0.70$, $P < 0.01$), even after accounting for differences in lean mass ($r = -0.50$, $P < 0.05$).

Expression of OB-R170 protein is reduced in the arm and leg muscles of obese subjects

Skeletal muscle OB-R170 protein expression was 28 and 25% lower (both $P < 0.05$) in arm and leg muscles (Fig. 1*A* and *B*), respectively, of the obese men compared with the control subjects. However, the expression of OB-R128 and OB-R98 (Fig. 1*C* and *D*), as well as the expression of perilipin A (Fig. 1*E*) was similar in control and obese groups, both in the arms and in the legs.

Skeletal muscle SOCS3 and PTP1B protein expression was comparable in obese and control subjects

There were no significant between-group differences in SOCS3 and PTP1B protein expression in arm

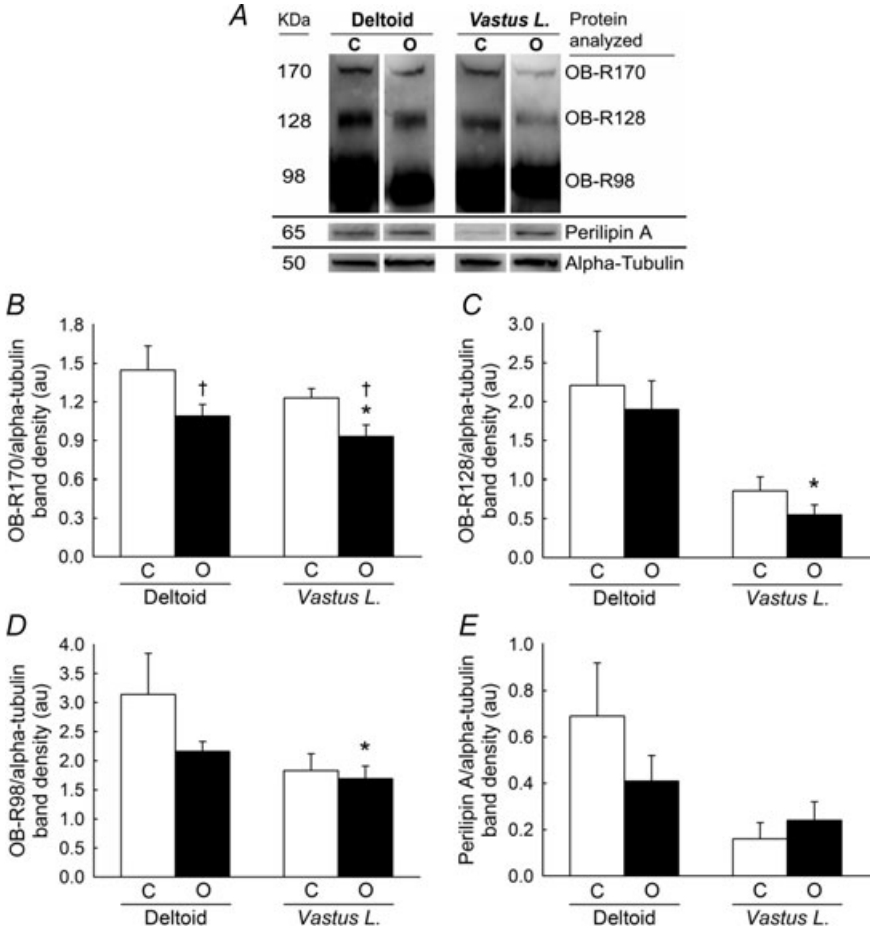

Figure 1. Determination of the leptin receptor (OB-R) and perilipin A protein expression in the deltoid and vastus lateralis muscle biopsies obtained from the control (C) and the obese subjects (O)
A, representative Western blot assays to determine OB-R, perilipin A and α-tubulin protein expression levels in deltoid and *vastus lateralis* muscle biopsies obtained from both the control and the obese subjects. The figure also shows densitometric immunosignal values (arbitrary units of band densities) of OB-R170 (*B*) OB-R128 (*C*) OB-R98 (*D*) and perilipin A bands (*E*) relative to those obtained for α-tubulin. $^*P < 0.05$ *versus* deltoid muscle. †$P < 0.05$ compared with the control group.

and leg muscles ($P > 0.05$; Fig. 2*A* and *B*). The ratio OB−R170/SOCS3 was 36% lower in the vastus lateralis of the obese subjects compared with the control subjects ($P < 0.05$). However, there were no significant between-group differences in the ratio of OBR-170/PTP1B in arm and leg muscles.

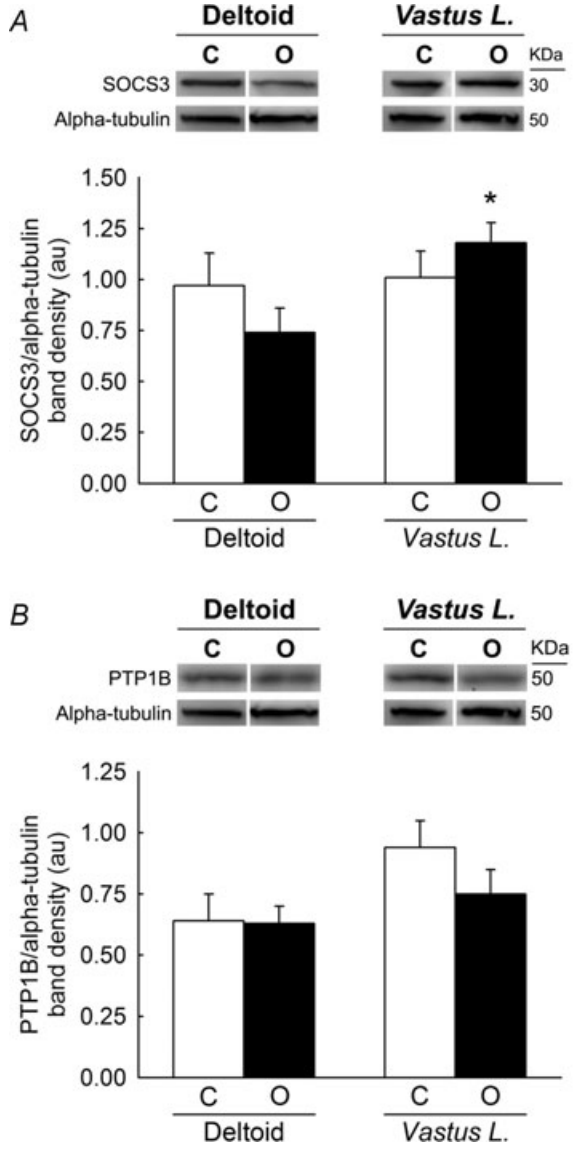

Figure 2. Determination of SOCS3 and PTP1B protein expression in the deltoid and vastus lateralis muscle biopsies obtained from the control (C) and the obese subjects (O)
A, top panel, representative Western blot assay to determine SOCS3 protein expression level in deltoid and vastus lateralis muscle biopsies obtained from both the control and the obese subjects; bottom panel, densitometric immunosignal values (arbitrary units of band densities) of SOCS3 bands relative to those obtained for α-tubulin. *B*, top panel, representative Western blot assay to determine PTP1B protein expression level in deltoid and vastus lateralis muscle biopsies obtained from both the control and the obese subjects; bottom panel, densitometric immunosignal values (arbitrary units of band densities) of PTP1B bands relative to those obtained for α-tubulin. *$P < 0.05$ *versus* deltoid muscle.

Deltoid but not vastus lateralis Tyr^{705}-STAT3 phosphorylation level was increased in the obese subjects

Deltoid muscle Tyr^{705}-STAT3 phosphorylation level was 373% higher in obese men than in control men ($P < 0.01$; Fig. 3). However, there were no significant between-group differences in vastus lateralis Tyr^{705}-STAT3 phosphorylation (Fig. 3).

Skeletal muscle Thr^{172}-AMPKα phosphorylation but not Ser^{221}-ACCβ phosphorylation levels were comparable in obese and control subjects

There were no significant between-group differences in Thr^{172}-AMPKα phosphorylation levels in arm and leg muscles (Fig. 4*A*). However, deltoid muscle Ser^{221}-ACCβ phosphorylation level was 67% higher and vastus lateralis Ser^{221}-ACCβ phosphorylation level was 36% lower in obese compared with control subjects (both $P < 0.05$; Fig. 4*B*). The pThr^{172}-AMPKα/SOCS3 and pSer^{221}-ACCβ/SOCS3 ratios were 45 and 49% lower, respectively, in the vastus lateralis of the obese compared with the control subjects (both $P < 0.05$).

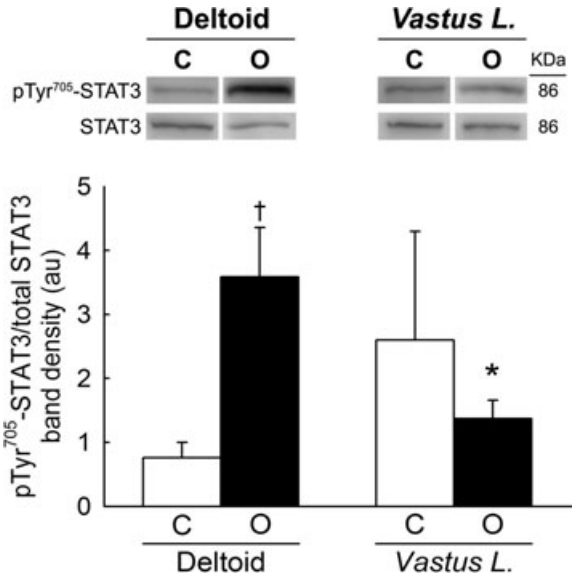

Figure 3. Determination of pTyr^{705}-STAT3 phosphorylation level in the deltoid and vastus lateralis muscle biopsies obtained from the control (C) and obese subjects (O)
Top panel, representative Western blot assay to determine pTyr^{705}-STAT3 phosphorylation level in deltoid and vastus lateralis muscle biopsies obtained from both the control and the obese subjects. Bottom panel, densitometric analysis of pTyr^{705}-STAT3 immunoblots (arbitrary units of band densities). Values are relative to total STAT3. *$P < 0.05$ *versus* deltoid muscle. †$P < 0.05$ compared with the control group.

Control group

Protein expression of the three leptin receptor isoforms (OB-R170, OB-R128 and OB-R98) (Fig. 1*A*) was similar in leg and arm muscles ($P = 0.27$, $P = 0.1$ and $P = 0.06$, respectively; Fig. 1*B*, *C* and *D*, respectively). No relationship was observed between OB-Rs protein expression in arm or leg muscles and serum leptin concentration or HOMA. Perilipin A protein expression (Fig. 1*A*) was similar in both muscles ($P = 0.06$; Fig. 1*A* and *E*).

Protein expression levels of SOCS3 ($P = 0.93$; Fig. 2*A*) and PTP1B ($P = 0.09$; Fig. 2*B*) were similar in arm and leg muscles. In the deltoid, OB-R98 and SOCS3 protein expression were related ($r = 0.76$, $P < 0.05$).

The phosphorylation level of Tyr^{705}-STAT3 was similar in arm and leg muscles ($P = 0.30$; Fig. 3). In the arms, but not in the legs, STAT3 phosphorylation level was directly related to the OB-R170 ($r = 0.80$, $P < 0.05$) and OB-R128 ($r = 0.70$, $P < 0.05$). The STAT3 phosphorylation level and SOCS3 protein content were related in the leg ($r = 0.64$, $P < 0.05$), but not in the arm ($r = 0.36$, $P = 0.34$). The mean of the STAT3 phosphorylation in both limbs was related to OB-R170 ($r = 0.91$, $P < 0.001$), OB-R128 ($r = 0.98$, $P < 0.001$) and OB-R98 ($r = 0.85$, $P < 0.01$).

Basal phosphorylation levels of Thr^{172}-AMPKα ($P = 0.34$) and Ser^{221}-ACCβ ($P = 0.48$) were similar in arm and leg muscle (Fig. 4*A* and *B*). There was no correlation between Thr^{172}-AMPKα and Ser^{221}-ACCβ basal phosphorylation and plasma leptin concentration.

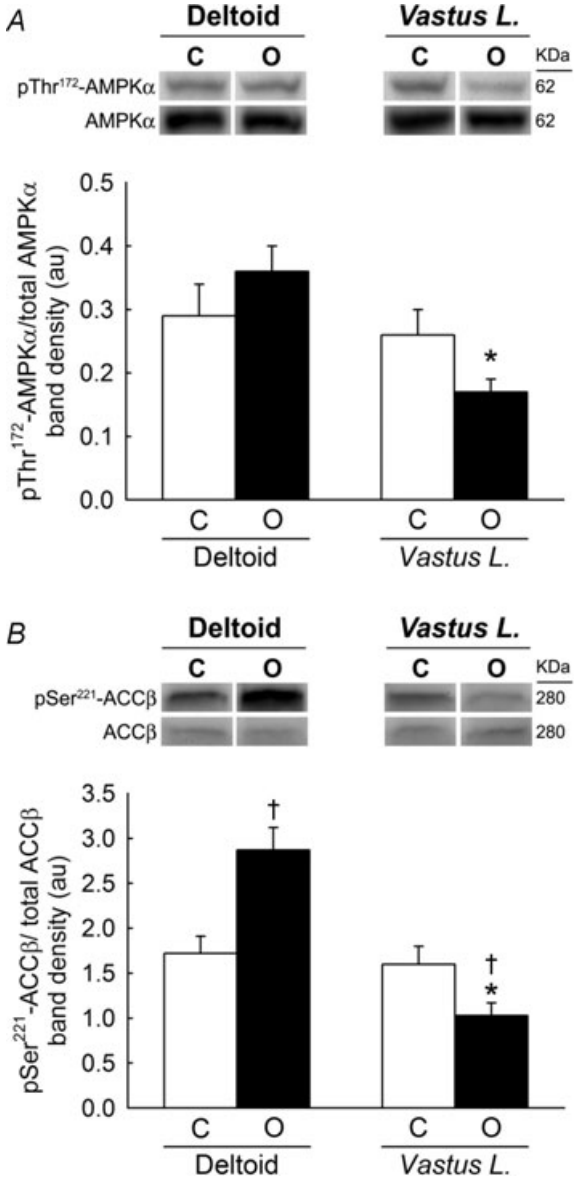

Figure 4. Determination of Thr^{172}-AMPKα and Ser^{221}-ACCβ phosphorylation level in the deltoid and vastus lateralis muscle biopsies obtained from the control (C) and the obese subjects (O)

A, top panel, representative Western blot assay to determine pThr^{172}-AMPKα phosphorylation level in deltoid and vastus lateralis muscle biopsies obtained from both the control and the obese subjects; bottom panel, densitometric analysis of pThr^{172}-AMPKα immunoblots (arbitrary units of band densities). Values are relative to total AMPKα. *B*, top panel, representative Western blot assay to determine pSer^{221}-ACCβ phosphorylation level in deltoid and vastus lateralis muscle biopsies obtained from both the control and the obese subjects; bottom panel, densitometric analysis of pSer^{221}-ACCβ immunoblots (arbitrary units of band densities). Values are relative to total ACCβ. *$P < 0.05$ *versus* deltoid muscle. †$P < 0.05$ compared with the control group.

Obese group

The expression of the three isoforms of the leptin receptor (Fig. 1*A*) was reduced by 15, 70 and 22% in the leg compared with the arm muscles (OB-R170, OB-R128 and OB-R98, respectively, all $P < 0.05$; Fig. 1*B*, *C* and *E*, respectively). Expression of OB-R170 did not correlate with serum leptin concentration or HOMA in either muscle. The expression of perilipin A was similar in both extremities (Fig. 1*A* and *E*; $P = 0.34$).

The protein expression of SOCS3 was 59% higher in leg than in arm muscles ($P < 0.05$; Fig. 2*A*). However, PTP1B protein content was similar in arm and leg muscles ($P = 0.3$; Fig. 2*B*). In the arm muscles, neither SOCS3 nor PTP1B protein expression was significantly related to OB-Rs protein expression, serum leptin concentration or HOMA. Neither SOCS3 nor PTP1B protein content in leg muscles was significantly related to serum leptin concentration.

The phosphorylation level of Tyr^{705}-STAT3 was 62% lower in leg than in arm muscles ($P < 0.05$; Fig. 3). The phosphorylation level of Tyr^{705}-STAT3 in vastus lateralis and deltoid muscles was not related to serum leptin concentration.

Basal phosphorylation levels of Thr^{172}-AMPKα and Ser^{221}-ACCβ were 53 and 65% lower in leg than in arm muscles, respectively (both $P < 0.001$; Fig. 4).

Discussion

In agreement with our hypothesis, we have shown that leptin receptor content is reduced in the skeletal muscle of obese subjects. This effect is exclusive to the long isoform of the leptin receptor (OB-R170), which is the main OB-R isoform involved in intracellular signalling (Kamikubo *et al.* 2008). In addition, we have shown that SOCS3, which blunts JAK2-dependent leptin signalling, is increased, whereas pTyr^{705}-STAT3 phosphorylation, which regulates gene expression in response to leptin signalling, and Thr^{172}-AMPKα and Ser^{221}-ACCβ phosphorylation levels, which regulate skeletal muscle fatty acid oxidation in response to leptin stimulation, are reduced in the vastus lateralis compared with the deltoid muscle in obese subjects. Moreover, in non-obese subjects there is a tight coupling between the amount of long isoform present in the skeletal muscles of the extremities and STAT3 phosphorylation, while this relationship is lost in obesity. Thus, these findings essentially confirm our hypothesis, i.e. obesity-induced leptin resistance in human skeletal muscle is associated with reduced availability of leptin receptors combined with reduced leptin signalling, as reflected by the lower levels of Tyr^{705}-STAT3, Thr^{172}-AMPKα and Ser^{221}-ACCβ basal phosphorylation levels, probably caused by increased SOSC3 protein expression in the leg muscles. However, our study also provides evidence for higher leptin resistance in the leg than in the arm muscle of obese people.

Our findings concur with previous studies showing a down-regulation of gene expression of the short and long isoforms of the leptin receptor (OB-Ra and OB-Rb, respectively) in the hypothalamus and liver in obesity (Hikita *et al.* 2000; Liu *et al.* 2007). Cell-culture studies have shown that leptin receptor expression is controlled by leptin (Hikita *et al.* 2000; Liu *et al.* 2004). Acute leptin administration causes an acute reduction in the expression of leptin receptors in cell lines (Hikita *et al.* 2000; Liu *et al.* 2004). Moreover, a reduction of circulating leptin levels by prolonged fasting in humans increases OB-R mRNA in peripheral mononuclear cells (Chan *et al.* 2002), while administration of human recombinant leptin in fasting humans blunts the increase in OB-R in mononuclear cells (Chan *et al.* 2002). The reduction of OB-R170 protein content in obesity might have been caused by the hyperleptinaemia observed in this experimental group. However, no relationship was observed in the present study between the basal levels of leptin, which were chronically elevated in obesity, and the expression of leptin receptors in skeletal muscle, except for the expression of OB-R128 in the arm muscles, which was inversely related to serum leptin concentration. Similar to the findings of the present investigation, no relationship between skeletal muscle OB-R protein expression and serum leptin concentrations has been reported in normal-weight subjects, including women (Guerra *et al.* 2007; Guerra *et al.* 2008). In contrast, a negative relationship between plasma leptin concentration and both OB-Ra and OB-Rb gene expression (mRNA) in hypothalamus and liver has been reported in rats (Liu *et al.* 2007). However, in that study the effect hyperleptinaemia on the amount of leptin receptor protein was not reported. Thus, our findings indicate that the amount of muscle OB-R170, in addition to circulating leptin levels, must be regulated by other mechanisms (Guerra *et al.* 2007, 2008).

Expression of OB-R and muscle leptin resistance

The OB-R170 has a molecular weight which corresponds well to the glycosylated form of the OB-R long isoform (OB-Rb) and is expressed in human skeletal muscle and hypothalamus (Guerra *et al.* 2007). The OB-R128 could correspond to the non-glycosylated form of the long isoform of the leptin receptor (Aparicio *et al.* 2005). The OB-R170 isoform could very well be the main ligand for leptin in skeletal muscle (Bjørbæk *et al.* 2000). It has also been shown that this isoform phosphorylates in response to leptin binding (Bates & Myers, 2003), and this phosphorylation has been linked to the activation of intracellular cascades with subsequent effects on fatty acid transport and metabolism (Steinberg *et al.* 2002*b*). In theory, down-regulation of the OB-R170 receptor number could account for some of the accumulation of triglycerides, lipotoxicity and altered insulin signalling typical of obesity.

Central leptin resistance has been associated with hypothalamic OB-R mRNA and protein down-regulation (Martin *et al.* 2000). Peripheral leptin resistance could also be caused by a reduction of the OB-Rs mRNA (Liu *et al.* 2007). Therefore, the reduced amount of OB-R170 protein in the human obese skeletal muscle observed in our study might be a potential mechanism of muscular leptin resistance. Previous data have shown that muscle leptin sensitivity is reduced in obesity, since the hormone is unable to increase the fatty acid oxidation in human obese skeletal muscle *in vitro* (Steinberg *et al.* 2002*b*), and chronic leptin administration decreases fatty acid uptake and fatty acid transporters in rat skeletal muscle (Steinberg *et al.* 2002*a*).

Protein expression of SOCS3 and PTP1B in human skeletal muscle

Cellular leptin resistance also could be caused by an attenuation of the OB-Rb signalling (Munzberg *et al.* 2005). Induction of SOCS3 expression has been implicated as a potential mechanism of leptin resistance and of leptin-induced insulin resistance (Bjørbæk *et al.* 1999). Expression of SOCS3 is increased in hypothalamus, white

adipocytes and skeletal muscle of leptin-resistant rodents (Wang *et al.* 2000, 2001; Steinberg *et al.* 2004*b*; Eguchi *et al.* 2007). Additionally, in mice, decreasing SOCS3 expression in the whole body or deleting SOCS3 in neurons increases the amplitude of OB-Rb signalling, resulting in animals that are leaner than wild-types at baseline, and that are resistant to diet-induced obesity (Myers *et al.* 2008). Furthermore, Steinberg *et al.* (2006*b*) reported that SOCS3 mRNA is up-regulated in human myotubes cultured from skeletal muscle of obese humans, which inhibits the leptin-induced AMPK activation in these obese myotubes. Moreover, Steinberg *et al.* (2006*b*) showed that overexpression of SOCS3 via adenovirus-mediated infection in lean myotubes to a similar degree as observed in obese myotubes prevented leptin activation of AMPK. In the present investigation, we have measured, for the first time, the SOCS3 protein levels in human skeletal muscle of obese and control subjects. Our data indicate similar skeletal muscle SOCS3 protein content in obese and control subjects. Moreover, muscle SOCS3 protein expression was not related to leptin serum concentrations.

Our results also imply that differences in leptin sensitivity could hardly be explained uniquely by differences in SOCS3 protein content. In contrast with our results, Eguchi *et al.* (2007) reported that SOCS3 mRNA and protein expression are up-regulated by leptin in rat skeletal muscle in a time-dependent manner. Moreover, endurance training restored the ability of leptin to increase the muscular fatty acid oxidation in obese rats with high muscular SOCS3 mRNA expression, but this effect of exercise was not mediated by a decrease of the muscular SOCS3 mRNA expression (Steinberg *et al.* 2004*b*). Therefore, while in certain circumstances increased SOCS3 expression may be an important regulator of leptin and insulin sensitivity, our data show that SOCS3 protein expression is not increased in human obese skeletal muscle, but it is differentially distributed, with increased SOCS3 levels in leg compared with arm muscles. These regional differences in SOCS3 protein expression between arm and leg muscle of obese subjects may, at least partly, explain why there is better-preserved insulin sensitivity in arm than leg muscle in humans with type 2 diabetes (Olsen *et al.* 2005).

Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of leptin and insulin signalling (Dube & Tremblay, 2005) and is overexpressed in multiple insulin- and leptin-responsive tissues in mice with diet-induced obesity, including the arcuate nucleus and medial hypothalamus, important sites of PTP1B action on body weight regulation, and in peripheral tissues, such as skeletal muscle, adipose tissue and liver (Zabolotny *et al.* 2002; Dube & Tremblay, 2005). Moreover, PTP1B overexpression in muscle of transgenic mice causes impaired insulin signalling in muscle and whole body insulin resistance (Zabolotny *et al.* 2004). Protein tyrosine phosphatase 1B is overexpressed in obese rodent skeletal muscle, and this PTP1B overexpression is promoted by inflammation (Zabolotny *et al.* 2008). Reports of PTP1B expression in tissues of insulin-resistant, obese and/or diabetic humans are inconsistent. Several studies have reported that PTP1B levels are increased in skeletal muscle and adipose tissue of obese humans (Ahmad *et al.* 1997*a*,*b*; Cheung *et al.* 1999). However, other studies have shown that PTP1B expression is unchanged or decreased in obese and/or diabetic humans compared with control subjects (Kusari *et al.* 1994; Ahmad *et al.* 1997*a*; Worm *et al.* 1999). In the present investigation, we have measured PTP1B protein levels in human skeletal muscle of obese and control subjects. Our data indicate similar skeletal muscle PTP1B protein content in obese and control subjects in arm and leg muscles. Moreover, muscle PTP1B protein expression was not related to leptin serum concentrations, implying that differences in human muscle leptin sensitivity could hardly be explained by differences in PTP1B protein content.

Phosphorylation of STAT3 in skeletal muscle

The STAT3 signalling pathway in human skeletal muscle is the signal transducer of numerous stimuli in addition to leptin signalling (Stepkowski *et al.* 2008) and is involved in the regulation, among other processes, of cellular proliferation, differentiation, programmed cell death, inflammation, muscle hypertrophy and the immune response (Akira, 2000; Judd *et al.* 2006). Thus, the lack of correlation between leptin concentrations and Tyr^{705}-STAT3 phosphorylation levels, and the fact that Tyr^{705}-STAT3 phosphorylation is not related to the amount OB-R170 in the arm muscles of obese subjects, could simply reflect the influence of other signals overruling the effects of leptin in the deltoid muscle. In contrast, since SOCS3 is elevated in the vastus lateralis of obese subjects, several signals eliciting Tyr^{705}-STAT3 phosphorylation may be blunted (Murray, 2007), explaining the lower basal STAT3 phosphorylation in the leg muscles of obese subjects. Reduced basal levels of Tyr^{705}-STAT3 phosphorylation, in turn, may attenuate lipid oxidation, leading to triglyceride intramyocellular accumulation. In agreement, it has been shown that leptin administration increases lipid oxidation in the mouse, which was blocked by a JAK2 inhibitor and STAT3 small interfering RNA (Akasaka *et al.* 2009).

Phosphorylation of AMPKα and ACCβ in skeletal muscle

Basal Ser^{221}-ACCβ phosphorylation level and AMPK activity (but not Thr^{172}-AMPKα phosporylation level) are significantly reduced in obese compared with control muscle biopsies obtained from the vastus lateralis muscle (Bandyopadhyay *et al.* 2006). In agreement,

we have shown that Ser^{221}-ACCβ but not Thr^{172}-AMPKα phosphorylation levels were reduced in the vastus lateralis muscle of obese compared with control subjects. The reduced Ser^{221}-ACCβ phosphorylation observed in obese leg muscle probably increases muscle malonyl-coenzyme A levels and reduces FA oxidation (Bandyopadhyay *et al.* 2006; Steinberg & Jorgensen, 2007). Another study found no differences between obese and control women in AMPK activity and Ser^{221}-ACCβ phosphorylation in the rectus abdominis muscle (Steinberg *et al.* 2004*a*). In contrast to our hypothesis, we found greater Ser^{221}-ACCβ phosporylation in the deltoid muscle of obese compared to control subjects. This finding may be a consequence of hyperleptinaemia in obesity, as supported by our results, which are compatible with lower leptin resistance in the arm compared with the leg muscles in obesity. Similarly, regional differences with greater insulin sensitivity in arm than in leg muscles have been reported in type 2 diabetes (Olsen *et al.* 2005). Another novelty from this study is that we have observed lower Thr^{172}-AMPKα and Ser^{221}-ACCβ phosphorylation levels in the vastus lateralis compared with the deltoid muscle of the obese subjects, which could be explained by the greater SOCS3 protein content found in the obese vastus lateralis.

Regional differences in the OB-R protein expression in obese muscle

Another interesting finding from our study is that there are regional differences in OB-R protein expression between the deltoid muscle and the vastus lateralis in obese humans. Since leg muscles are used frequently in ambulation, while arm muscles are used with a more intermittent pattern, this finding could indicate that muscle activity plays a role in the regulation of leptin receptors. More active muscles, such as vastus lateralis, could need less leptin receptors because exercise improves leptin sensitivity. Interestingly, the leptin receptors are up-regulated in the medial gastrocnemius after 4–11 days of cast immobilization or bed rest in humans (Chen *et al.* 2007). The greater OB-R protein expression detected in the deltoid muscles of obese subjects could facilitate leptin signalling and, in the presence of hyperleptinaemia, lead to increased STAT3 and AMPK signalling compared with the vastus lateralis. However, it remains to be determined whether regular exercise modifies the expression of skeletal muscle leptin receptors in humans.

Regional differences in the OB-R protein expression in obese muscle could relate to differences in muscle fibre type composition between deltoid and vastus lateralis. However, our obese and control subjects had a similar fibre type composition in their deltoid and vastus lateralis (I. Ara, S. Larsen, B. Stallknecht, B. Guerra, D. Morales-Alamo, J.L. Andersen, J.G. Ponce-González, A. Guadalupe-Grau, H. Galbo, J.A.L. Calbet & J.W. Helge, unpublished observations), ruling out this possible explanation.

Conclusion

In summary, this study shows that in obese humans there is a down-regulation of the OB-R170 protein expression in skeletal muscle, which cannot be explained by differences in circulating leptin or insulin between obese and control subjects. Moreover, we found that in obese humans the deltoid muscle, which has a fibre type composition similar to that of the vastus lateralis (Calbet *et al.* 2005; I. Ara, S. Larsen, B. Stallknecht, B. Guerra, D. Morales-Alamo, J.L. Andersen, J.G. Ponce-González, A. Guadalupe-Grau, H. Galbo, J.A.L. Calbet & J.W. Helge, unpublished observations), has more leptin receptors than the vastus lateralis. In normal-weight subjects, SOCS3 protein expression, and STAT3, AMPKα and ACCβ phosphorylation, are similar in the deltoid and vastus lateralis muscles. However, in obesity, SOCS3 protein expression is increased, and basal STAT3, AMPKα and ACCβ phosphorylation levels are reduced in the vastus lateralis compared with the deltoid muscle. In combination, these findings are compatible with marked regional differences in skeletal leptin resistance in obese humans.

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