HSL serine phosphorylation and glycerol exchange across skeletal muscle in lean and obese subjects: effect of beta-adrenergic stimulation

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ABSTRACT

Objective: Increased intramuscular triacylglycerol (IMTG) storage is a characteristic of the obese insulin resistant state. We aimed to investigate whether a blunted fasting or beta-adrenergically mediated lipolysis contributes to this increased IMTG storage in obesity.

Research design and Methods: Forearm skeletal muscle (SM) lipolysis was investigated in thirteen lean and ten obese men using $[^2H_5]$-glycerol combined with the measurement of arterio-venous differences before and during beta-adrenergic stimulation using the non-selective beta-agonist isoprenaline (ISO). Muscle biopsies were taken from the vastus lateralis muscle before and during ISO to investigate hormone-sensitive lipase (HSL) protein expression and serine phosphorylation.

Results: Baseline total glycerol release across the forearm was significantly blunted in obese compared with lean subjects (P=0.045). This was accompanied by lower HSL protein expression (P=0.004), and HSL phosphorylation on PKA sites Ser^{563} (P=0.041) and Ser^{659} (P=0.09) and on the AMPK site Ser^{565} (P=0.007), suggesting a blunted skeletal muscle lipolysis in obesity. Total forearm glycerol uptake during baseline did not differ significantly between groups while higher net fatty acid uptake across the forearm was observed in the obese (P=0.064). ISO induced an increase in total glycerol release from SM, which was not significantly different between groups. Interestingly, this was accompanied by an increase in HSL Ser^{659} phosphorylation in obese subjects during ISO compared with baseline (P=0.008).

Conclusions: Obesity is accompanied by impaired fasting glycerol release, lower HSL protein expression and serine phosphorylation. It remains to be determined whether this is a primary factor or an adaptation to the obese insulin resistant state.

The obese insulin resistant state is characterized by increased triacylglycerol (TAG) storage in adipose and non-adipose tissues (ectopic fat), such as skeletal muscle (1). A strong link between increased intramuscular triacylglycerol (IMTG) stores and skeletal muscle insulin resistance has been shown in lean and obese subjects (2; 3). Recent studies have, however, indicated that intramuscular accumulation of lipid intermediates rather than TAG per se may be the direct cause of skeletal muscle insulin resistance, through interference with insulin signaling (4). Impaired uptake and a reduced oxidation of fatty acids has been reported in skeletal muscle under post-absorptive conditions, during beta-adrenergic stimulation and moderate-intensity exercise in obese subjects with type 2 diabetes (5-7). Besides impaired fatty acid handling, disturbances in the regulation of skeletal muscle lipolysis may contribute to the increased storage of IMTG and lipid metabolites. So far, little is known on the *in vivo* regulation of skeletal muscle lipolysis in obesity. Data from our laboratory indicate that the catecholamine-induced increase in interstitial glycerol concentration as well as local blood flow is blunted in obese subjects (8), a factor that may contribute to an increase in content of muscle TAG and diacylglycerol (DAG).

Although the molecular mechanisms that underlie muscle lipolysis are not known into detail, it has been shown that hormone-sensitive lipase (HSL) is expressed in skeletal muscle of rodents (9; 10) and humans (11). HSL activity appears to be regulated by site-specific phosphorylation on several serine residues. It has been demonstrated that skeletal muscle HSL can be phosphorylated on at least five serine residues (Ser[563], Ser[565], Ser[600], Ser[659] and Ser[660]) (11-13). Catecholamines increase intracellular cyclic AMP concentration resulting in the activation of protein kinase A (PKA). HSL Ser[563], Ser[659] and Ser[660] are major PKA phosphorylation sites, although Ser[563] may not affect HSL activity directly (14). It is still unclear which of the PKA phosphorylation sites on HSL are important in mediating the effect of catecholamines on *in vivo* muscle HSL activity. Ser[659] appears to be a likely candidate, since HSL Ser[659] phosphorylation and HSL activity show a similar response to exercise with concomitant increase in circulating epinephrine (13). *In vitro* studies on purified bovine adipocyte HSL have shown that AMP-activated protein kinase (AMPK) phosphorylates HSL on Ser[565] thereby abolishing PKA induced HSL activation (15). In human skeletal muscle, changes in AMPK activity during exercise were also associated with an increased HSL Ser[565] phosphorylation but this was not accompanied by an increased HSL activity, suggesting that AMPK can phosphorylate HSL on Ser[565] but that AMPK is of minor importance as a regulator of HSL activity in human skeletal muscle during exercise (11).

So far, limited data are available on the differences in *in vivo* regulation of skeletal muscle lipolysis between lean and obese subjects. The aim of the present study was to investigate whether *in vivo* baseline and/or catecholamine-induced lipolysis is impaired in skeletal muscle of obese compared with lean subjects. For this reason [^{2}H$_5$]-glycerol tracer methodology was used to investigate *in vivo* whole-body and regional forearm skeletal muscle lipolysis in lean and obese subjects after an overnight fast and during beta-adrenergic stimulation, using the non-selective beta-adrenergic agonist isoprenaline. To obtain more information on the underlying mechanism at the molecular level we measured skeletal muscle HSL protein expression and serine phosphorylation on Ser[563], Ser[565] and Ser[659].

MATERIAL AND METHODS

Subjects. Three healthy lean (2F/1M; Age 20±1yr; BMI 22.3±1.1 kg/m^2) subjects participated in a pilot experiment during which [^{2}H$_5$]-glycerol enrichment was investigated during 6h infusion in order to determine the time required to achieve an isotopic steady-state. Thirteen lean and ten obese non-smoking male subjects participated in the actual muscle lipolysis experiment during which [^{2}H$_5$]-glycerol was infused for 3h. Clinical characteristics of the subjects included in the experiment are summarized in Table 1. Body weight and body density (by hydrostatic weighing), used for calculations of percentage body fat (%BF), fat mass (FM) and fat free mass (FFM), were determined after an overnight fast, as previously described (16). All subjects were in good health as assessed by medical history, were free of any medication and spent no more than 3h a week in organized sports activities. The Medical Ethical Committee of Maastricht University approved the study protocol, and all subjects gave their written informed consent before participating in the study.

Experimental protocol. In a pilot study in 3 subjects, the time course of [^{2}H$_5$]-glycerol enrichment was determined to investigate when steady-state concentrations were achieved. Glycerol enrichment was measured in arterialized blood and venous blood draining the forearm during primed (3 μmol. kg^{-1}) constant infusion of [^{2}H$_5$]-glycerol (0.20 μmol. kg^{-1}. min^{-1}) for 6h. Blood samples were taken simultaneously from the two sites, at baseline for background enrichment (t0) and at ten time points during [^{2}H$_5$]-glycerol infusion (t60, t90, t120, t150, t180, t210, t240, t330, t345, t360).

During the actual muscle lipolysis experiment, glycerol enrichment and exchange across the forearm were investigated during primed (3 μmol. kg^{-1}) constant infusion of [^{2}H$_5$]-glycerol (0.20

μmol. kg^{-1}. min^{-1}) for 3h. Following a 120 min baseline period, isoprenaline (ISO) was infused at a rate of 20 ng. kg FFM^{-1}. min^{-1} for 60 min. At this infusion rate plasma ISO concentrations are comparable in lean and obese subjects (17). At the beginning of the experiment an arterialized blood sample was taken for measurement of background enrichment. Furthermore, arterialized and deep venous blood samples were taken simultaneously at three baseline time points (t90, t105 and t120) and at three time points during the last 30 min of ISO infusion (t150, t165 and t180). In both the pilot and muscle lipolysis experiment, forearm blood flow (FBF) was measured just before blood sampling to calculate substrate fluxes across the forearm (see *forearm blood flow*). Skeletal muscle biopsies were taken from the vastus lateralis muscle under local anesthesia of skin and fascia (Xylocaine®, AstraZeneca BV, Zoetermeer, The Netherlands) immediately before the baseline period (t0) and just before the end of ISO infusion (t180). Muscle biopsies were immediately frozen in liquid nitrogen and stored at −80°C until further analysis. During the experiment, heart rate was recorded continuously by means of a three-lead electrocardiogram (ECG). When heart rate increased more than 30 beats/min or in case of ECG irregularities, ISO infusion was stopped (n=2, 1 lean/1 obese).

Clinical methods. All subjects were asked to refrain from drinking alcohol and to perform no strenuous exercise for a period of 24h before the experiment. Subjects came to the laboratory by car or bus at 8 a.m. after an overnight fast. Before initiating the experiment a catheter was inserted retrogradely into a superficial dorsal hand vein to obtain arterialized venous blood. The hand was warmed in a hotbox, which was maintained at 60 ºC to achieve adequate arterialization (18). In the same arm, a second catheter was inserted in a forearm antecubital vein for the infusion of [^{2}H$_5$]-glycerol tracer

and ISO. In the contralateral arm, a third catheter was introduced retrogradely in an antecubital vein of the forearm for sampling of deep venous blood draining forearm skeletal muscle. The subjects rested in a supine position for the entire duration of the study.

Forearm blood flow (FBF). FBF was measured by venous occlusion plethysmography (EC5R plethysmograph, Hokanson, Bellevue, USA) using mercury-in-silastic strain gauges applied to the widest part of the forearm (19). During measurement periods, the hand circulation was occluded by rapid inflation of a sphygmomanometer cuff (E20 rapid cuff inflator, Hokanson, Bellevue, USA) placed around the wrist to a pressure of 200 mmHg. In this way, FBF can be assessed without interference of the hand circulation. A second cuff, placed just above the anticubital fossa, was inflated to 45 mmHg (which was lower than the diastolic blood pressure, which was > 70mmHg in all subjects) to achieve venous occlusion and obtain plethysmographic recordings. During venous occlusion, the plethysmographic recordings reflect the rate of arterial inflow, indicating FBF.

Muscle lysates. Muscle tissue was freeze-dried, dissected free of all visible adipose tissue, connective tissue and blood under a microscope and was subsequently homogenized (1:80 w/v) in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM L-glycerophosphate, 10 mM NaF, 2 mM sodium orthovanadate, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 2mM PMSF, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 3 mM benzamidine. Homogenates were rotated end over end for 1h at 4°C and then cleared by centrifugation for 1h at 17500g, 4°C. Protein content in the supernatant was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Illinois, USA).

Western blotting. Expression of HSL protein as well as phosphorylation of HSL Ser[563], Ser[565] and Ser[659] was detected by Western blotting on the muscle lysates. The lysates were boiled in Laemmli buffer before being subjected to SDS-PAGE and immunoblotting. Primary antibodies were rabbit anti-HSL (kindly donated by Prof. dr. Cecilia Holm, Dept. of Cell and Molecular Biology, Lund University, Sweden) and sheep anti-phospho HSL Ser[563], sheep anti-phosho HSL Ser[565] (11) and sheep anti-phosho HSL Ser[659] (13). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit (cat. no. P0448 DAKO, Glostrup, Denmark), and anti-sheep (cat. no. 81-8620; Zymed, CA, USA). Antigen/antibody complexes were visualized using enhanced chemiluminescence (ECL+, Amersham Biosciences, UK) and quantified by a Kodak Image Station E440CF (Kodak, Glostrup, Denmark).

Analytical methods . A small portion of blood was used for the measurement of oxygen saturation ($\%HbO_2$) to ensure adequate arterialization (ABL510, Radiometer, Copenhagen, Denmark). Blood was collected in tubes containing EDTA and centrifuged for 10 min at 1000g, 4°C. The supernatant (plasma) was used for the enzymatic colorimetric quantification of fatty acids (NEFA C kit; Wako Chemicals, Neuss, Germany), free glycerol (Boehringer, Mannheim, Germany) and triacylglycerol (TAG, Sigma, St Louis, USA) on a COBAS FARA centrifugal spectrophotometer (Roche Diagnostica, Basel, Switzerland). Plasma glucose concentration (ABX Diagnostics, Montpellier, France) and lactate (ABX diagnostics) were measured enzymatically on a COBAS MIRA automated spectrophotometer (Roche Diagnostica). Plasma insulin was measured with a double antibody radioimmunoassay (Linco Research Inc., St. Charles, Missouri, USA). Insulin sensitivity was assessed by the homeostasis model assessment index for insulin resistance

(HOMA$_{IR}$), calculated from baseline glucose and insulin (20). Hematocrit was measured using a microcapillary system (Hirschmann Laborgeräte GmbH & Co.KG, Eberstadt, Germany).

Isotope enrichment. To determine isotopic enrichment of glycerol, samples were first derivatized. 1 ml acetone was added to 150 µL plasma and each tube was vortexed for 2 min and centrifuged for 20 min at 17500g, 4°C. The supernatant was transferred to a clean tube, dried under nitrogen at 37°C and derivatized using 80 µL ethyl acetate (cat. no. 45765, Sigma-Aldrich GmbH, Seelze, Germany) and 80 µL heptafluorobutyric acid anhydride (HFAA; cat. no. 63164, Pierce Biotechnology, Rockford, IL, USA). The tubes were vortexed for 2 min and incubated for 1h at 70°C. Samples were then rotated end over end for 5 min, 25°C and evaporated under nitrogen at room temperature. 70 µL of ethyl acetate was added before injection into the GC-MS (Finnigan MAT 252, Bremen, Germany) for measurement of glycerol enrichment by selectively monitoring the mass-to-charge ratio (m/z) of molecular ions 253 and 257 for glycerol (21).

Calculations. The exchange of metabolites across the forearm was calculated by multiplying the arterio-venous plasma concentration difference of metabolites by forearm plasma flow. Plasma flow was calculated as FBF × (1-hematocrit), with hematocrit expressed as a fraction. A positive net exchange indicates net uptake. Whereas a negative net exchange indicates net release.

The expected deep venous glycerol enrichment, in case of no glycerol uptake, was calculated as arterialized enrichment multiplied by arterialized glycerol concentration and subsequently divided by deep venous glycerol concentration.

The rate of appearance (Ra) of glycerol was calculated according to the following steady-state equation:

$$Ra\ (\mu mol.\ kg^{-1}.\ min^{-1}) = TTR^{-1} \times F$$

TTR is tracer/tracee ratio and F is the isotope infusion rate ($\mu mol.\ kg^{-1}.\ min^{-1}$).

The fractional extraction (fract) of glycerol across the forearm was calculated by dividing the arterio-venous concentration difference of [$^2H_5$]-glycerol by the arterialized [$^2H_5$]-glycerol concentration. Total glycerol uptake across the forearm was then calculated as follows:

$$Total\ glycerol\ uptake\ =\ fract\ \times [glycerol_{art}] \times FBF$$

where the unit is nmol. 100ml tissue^{-1}. min^{-1}; [glycerol$_{art}$] is arterialized glycerol concentration ($\mu mol/l$); and FBF is forearm skeletal muscle blood flow (ml. 100ml tissue^{-1}. min^{-1}). Forearm total glycerol release was calculated from the formula:

$$Total\ glycerol\ release\ =\ net\ glycerol\ balance\ -\ total\ glycerol\ uptake$$

net glycerol balance (exchange) was calculated as explained above.

Statistical analysis. Differences within groups (i.e. between baseline and ISO) were tested using the paired Student's t-test (two-sided). Comparisons between groups (at baseline and during ISO infusion, and changes from baseline to ISO respectively) were made using Student's two-sample t-test (equal variance assumed). Statistical calculations were performed using SPSS for Macintosh (version11.0; SPSS inc., Chicago, IL, USA). Data are presented as mean ± standard error of the mean (SEM) if not otherwise stated. P<0.05 was considered statistically significant.

RESULTS

Pilot experiment. In Figure 1 we show that arterialized and deep venous TTR, obtained with a 6h [$^2H_5$]-glycerol infusion, reached a steady-state after 1h of infusion. The measured deep venous enrichment was consistently lower than the expected enrichment, implying uptake of glycerol across the forearm (Figure 1). In the actual muscle lipolysis experiment (3h [$^2H_5$]-

glycerol infusion) TTR also reached a steady-state after 1h and remained stable during ISO infusion (data not shown). Thus, our data support the use of a relatively short infusion time ($\geq$ 1h) to accurately study glycerol metabolism.

Circulating metabolites. Baseline plasma arterialized TAG concentration was twice as high for the obese than the lean subjects (P<0.001, Table 2). Beta-adrenergic stimulation increased plasma arterialized TAG concentrations in obese (P=0.047, table 2) subjects, whilst in lean subjects TAG concentrations decreased during ISO (P=0.08, Table 2). Thus, the change in plasma arterialized TAG concentrations from baseline to ISO was different between lean and obese subjects (P=0.004, Table 2). Due to an irregular ECG or failure of the cannulation there are 3 persons less in each group in the ISO experiment. Therefore, delta values for the remaining10 lean and 7 obese subjects are included in Table 2. Baseline values for this subgroup did not differ from those of the whole group.

Baseline plasma arterialized FFA and glycerol concentrations did not differ significantly between lean and obese subjects. Beta-adrenergic stimulation increased FFA and glycerol in lean (P<0.001 and P=0.015, respectively) and obese (P=0.001 and P<0.001, respectively) subjects (Table 2). Moreover, the beta-adrenergic mediated increase in arterialized FFA and glycerol was more pronounced in obese subjects (P=0.037 and P=0.008, Table 2), suggesting a higher whole-body lipolytic response in the obese. Likewise, beta-adrenergic stimulation increased whole-body glycerol Ra in lean and obese subjects (P<0.001, Table 2), and this increase tended to be higher in the obese (P=0.067; Table 2). Expressed per unit fat free mass, baseline glycerol Ra was not significantly different between groups. Beta-adrenergic stimulation increased the glycerol

Ra per unit fat free mass in lean and obese subjects (P<0.001, Table 2), but this increase in glycerol Ra per unit fat free mass was not significantly different between groups.

Plasma arterialized insulin and lactate concentrations were higher in obese than in lean subjects during baseline and ISO (P=0.002, Table 2), while glucose did not differ significantly between lean and obese subjects in both conditions. Beta-adrenergic stimulation increased circulating insulin concentrations in lean and obese (P<0.001, Table 2). This increase in circulating insulin concentrations was significantly higher in obese than in lean subjects (P<0.001, Table 2). Circulating glucose and lactate concentrations were unchanged during beta-adrenergic stimulation

Regional forearm metabolism Baseline FBF was not different between lean and obese subjects (P=0.15, Table 3). FBF was significantly elevated during beta-adrenergic stimulation in both lean and obese subjects (P<0.001), but the increase in FBF during beta-adrenergic stimulation was not significantly different between groups.

Fractional extraction of $[^2H_5]$-glycerol from the circulation (lean vs. obese: 40.2±3.4 vs. 40.5±6.1%) was not significantly different between groups. Significant glycerol uptake across the forearm was observed in both obese and lean subjects (P<0.001 compared to zero, Figure 2A). The increase in total glycerol uptake during beta-adrenergic stimulation was not significantly different between groups.

Baseline net glycerol efflux across the forearm was significantly lower in the obese than in the lean subjects (P=0.025; Table 3). Accordingly, obese subjects showed significantly less total glycerol release across the forearm at baseline compared with lean subjects (P=0.045, Figure 2B). These data indicate a blunted glycerol release during baseline in obese subjects. Total glycerol uptake expressed relative to total glycerol

release at baseline was not significantly different between lean and obese subjects (lean vs. obese: 92.7±13.5 vs. 91.7±23.9% of total release). Furthermore, obese subjects had higher net fatty acid uptake across the forearm at baseline (P=0.064, Table 3). Beta-adrenergic stimulation increased total glycerol release in lean and obese subjects (P=0.037 and P=0.042, Figure 2B), but this increase was not significantly different between groups. Finally, the increase in net lactate efflux during beta-adrenergic stimulation tended to be higher in obese than in lean subjects (P=0.06, Table 3).

Total HSL protein expression and serine phosphorylation in the vastus lateralis muscle. Muscle HSL protein expression was significantly lower in obese compared with lean subjects (P=0.004) and did not change during beta-adrenergic stimulation (Figure 3A). Baseline HSL phosphorylation on Ser^{563} (P=0.041), Ser^{565} (P=0.007) and Ser^{659} (P=0.09) was reduced in obese compared with lean subjects (Figures 3B-D). When corrected for total HSL protein, HSL Ser^{563}, Ser^{565} and Ser^{659} phosphorylation was comparable between lean and obese subjects (data not shown). No effect of beta-adrenergic stimulation was observed on HSL Ser^{563} or HSL Ser^{565} phosphorylation in both lean and obese subjects (Figures 3B and C). Obese subjects showed an increased HSL Ser^{659} phosphorylation (P=0.008, Figure 3D), whilst in lean subjects HSL Ser^{659} phosphorylation was unchanged after ISO infusion.

DISCUSSION

The present study revealed a blunted fasting muscle glycerol release in obese compared to lean men (Fig. 2B). This blunted skeletal muscle glycerol release was accompanied by a lower total HSL protein expression and phosphorylation of HSL Ser^{563}, Ser^{565} and Ser^{659} (Fig. 3), suggesting a blunted fasting muscle lipolysis in obesity. The beta-adrenergic mediated muscle

lipolytic response was not significantly different between lean and obese subjects. In contrast to lean subjects, an increased HSL Ser^{659} phosphorylation was observed in skeletal muscle of obese subjects during beta-adrenergic stimulation compared with baseline.

Whole-body lipolysis. There seemed to be a directionally opposite effect of beta-adrenergic stimulation on circulating TAG. Circulating TAG concentrations during beta-adrenergic stimulation increased in obese subjects, while lean subjects showed slightly decreased circulating TAG concentration during beta-adrenergic stimulation. This could indicate a greater TAG clearance in lean than in obese subject following beta-adrenergic stimulation. In the present study, skeletal muscle TAG clearance was not significantly different between groups. Adipose tissue lipoprotein lipase (LPL) mediated TAG hydrolysis might be the cause of an increased net TAG extraction during beta-adrenergic stimulation. An increased rate of action of LPL has been shown during epinephrine infusion in lean subjects (22). Furthermore, it has been shown that obese subjects have a diminished adipose tissue LPL activity during postprandial conditions (23). However, it remains to be elucidated whether an impaired adipose tissue LPL activity during beta-adrenergic stimulation in obese compared with lean subjects can explain the difference in circulating TAG concentration.

Baseline muscle glycerol uptake. The present observation of significant uptake of glycerol across the forearm (Fig. 2A) is in agreement with previous reports (24). The first indications for significant metabolism of glycerol in muscle came from Elia et al. (25) showing 50% loss of enriched glycerol across the forearm. More recently, studies confirmed the finding of significant uptake of glycerol by forearm muscle (26) and vastus lateralis muscle (27). The enzymatic machinery for utilization of glycerol seems to be present in

skeletal muscle. Glycerol dehydrogenase, the enzyme that could initiate glycerol oxidation by skeletal muscle, has been demonstrated in humans (28), and oxidation of glycerol by skeletal muscle has been shown to occur in humans (28). Furthermore, glycerol kinase expression has been demonstrated in human muscle cells (29). Thus, in humans glycerol taken up from the circulation might be oxidized or incorporated into IMTG as shown in rats (30).

Baseline muscle glycerol release. Our data show a blunted baseline total glycerol release per unit muscle mass in obese subjects (Fig. 2B). This blunted baseline total glycerol release was accompanied by a lower total HSL protein expression in skeletal muscle of obese subjects (Fig. 3A), suggesting a blunted baseline muscle lipolysis. However, it can be argued that glycerol tracer release does not only reflect lipolysis. Thus, a blunted glycerol release might reflect an increased intramuscular glycerol use (i.e. oxidation or TAG synthesis). To our knowledge our data provide the first indication of a reduced muscle HSL protein expression in obese compared with lean subjects. It is well known that expression of HSL is markedly decreased in subcutaneous adipocytes and differentiated adipocytes from obese subjects. This suggests that at least in adipose tissue a decreased HSL expression is a primary defect in obesity (31; 32). However, we cannot exclude that the blunted muscle lipolysis in obese subjects was a secondary phenomenon caused by a higher degree of hyperinsulinemia. Still, it has been suggested that muscle lipolysis is primarily regulated by substrate supply and to a lesser degree is under hormonal control (33). This seems to be supported by studies showing no apparent suppression of *in vivo* skeletal muscle lipolysis by insulin (34; 35). Furthermore, our data suggest that phosphorylation of HSL on the PKA target sites Ser[563] and Ser[659], and on the AMPK target site Ser[565] was lower in obese than in lean subjects. It should be recognized, however, that when corrected for total HSL protein, HSL Ser[563], Ser[565] and Ser[659] phosphorylation was comparable between lean and obese subjects, suggesting that a similar percentage of HSL molecules were phosphorylated on these three serine sites in lean and obese subjects. Nevertheless, the reduced absolute number of HSL molecules phosphorylated on Ser[659] may at least partly explain the blunted baseline glycerol release in obese compared to lean subjects. On the other hand, HSL Ser[563] and Ser[565] phosphorylation have been suggested not to be major regulators of HSL activity in human skeletal muscle (11; 13). Thus, the reduced phosphorylation of HSL on these two sites may not have been important in determining the blunted baseline lipolysis in obese subjects. For practical reasons the arterio-venous differences were measured across the forearm muscle and biopsies were taken from the vastus lateralis muscle. Since, there may be heterogeneity in lipolysis between different muscle groups (36), the combination of forearm substrate fluxes with lipolytic enzymes in muscle biopsies from the thigh has to be interpreted with caution. Finally, it should be mentioned that also other lipases might contribute to the blunted baseline muscle glycerol release observed in obese subjects. Recently, we identified adipose triglyceride lipase (ATGL) expression in human skeletal muscle (37). More research is needed to elucidate the potential role of ATGL in human skeletal muscle lipolysis.

Baseline net muscle fatty acid uptake. An increased basal net fatty acid uptake was observed across the forearm of obese subjects. An impaired fatty acid uptake and oxidation both by the leg (5) and the arm (21; 38) have been observed previously in obese type 2 diabetic subjects as compared to healthy individuals. Disturbances in fatty acid handling and an impaired muscle lipolysis may contribute to the increased IMTG storage

in obese subjects. However, since this also depends on lipid turnover, both TAG synthesis and breakdown have to be known to draw final conclusions with respect to the mechanisms underlying increased IMTG content in obesity. For practical reasons and since our primary objective was to study glycerol metabolism, in the present study no carbon labeled long chain fatty acid tracer was used in order to measure fluxes and oxidation rates across the forearm.

Muscle glycerol release during beta-adrenergic stimulation. The present results showed equal forearm glycerol release during systemic infusion of the non-selective beta-adrenergic agonist isoprenaline in lean and obese subjects, suggesting a comparable lipolytic response. Previously, *in situ* microdialysis using a beta-2 agonist, salbutamol, showed a blunted lipolysis in the gastrocnemius muscle of obese insulin resistant subjects compared with lean subjects (8). Differences in systemic *versus* local infusion of beta-adrenergic agonists might partly explain this discrepancy. Secondly, in microdialysis studies, interstitial glycerol is used as a measure of lipolysis. As mentioned previously, glycerol is taken up by skeletal muscle, suggesting that interstitial glycerol may not reflect the overall rate of lipolysis, but may instead be the net result of TAG and glycerol metabolism in muscle thus reflecting net glycerol turnover (39). Finally, there may be marked heterogeneity in lipolysis between different muscle groups, possibly correlated to composition of fibre types (36). Accordingly, in rats it was shown that muscles with a majority of type 1 fibres had greater HSL activity compared with muscles with a majority of type 2 fibre (10). The higher content of type 1 fibres in the gastrocnemius muscle compared with forearm muscle may not only cause a generally higher lipolytic sensitivity to beta-adrenergic stimulation but may also influence the difference in beta-adrenergically stimulated lipolysis between lean and obese subjects.

It is known from studies with purified bovine adipocyte HSL (15) and in different cell lines transfected with wildtype and mutant forms of HSL (14) that beta-adrenergic stimulation increases HSL activity through phosphorylation on several serine residues. In the present study HSL Ser[659] phosphorylation significantly increased during beta-adrenergic stimulation in skeletal muscle of obese subjects, whilst no effect was seen in lean subjects. A previous study in men and women during exercise has shown that muscle Ser[659] phosphorylation and muscle HSL activity show a very similar pattern with respect to exercise response and dependency on gender, indicating that Ser[659] serves an important role in the regulation of HSL activity in human skeletal muscle (13), as has been demonstrated in adipocytes (14). It can be speculated that obese subjects increase HSL Ser[659] phosphorylation during beta-adrenergic stimulation to deal with a reduced total HSL protein expression, increasing muscle HSL activity to a level comparable with lean subjects. In addition, HSL Ser[660] appears to be a major PKA target site and HSL activity-controlling site (14). In the present study, HSL Ser[660] phosphorylation was not measured. Finally, phosphorylation of the PKA target site Ser[563] on HSL did not increase significantly during beta-adrenergic stimulation. This is in accordance with previous studies, where HSL Ser[563] phosphorylation was not increased during exercise despite an increase in circulating epinephrine (11; 13). Maybe HSL Ser[563] is already maximally phosphorylated in the basal, resting state. Moreover, it has been argued that HSL Ser[563] may not be an important regulator of HSL activity in human skeletal muscle (11).

Muscle lactate release during beta-adrenergic stimulation. Net lactate release across the forearm increased during beta-

adrenergic stimulation. This increase was higher in obese compared with lean subjects, suggesting that the glycolytic flux was stimulated to a greater extent by isoprenaline in obese than in lean subjects. This seems in line with previous findings showing an increased lactate release during beta-adrenergic stimulation in obese subjects (17) which persisted after weight reduction (40), indicating that this disturbance might be an early factor in the etiology of obesity.

Limitations of the study. Considering the increased discomfort and risk associated with arterial catheters, we used arterialized hand vein blood as substitute for arterial blood. Arterialized blood has been shown to be an acceptable alternative to arterial sampling. (41; 42). Arterialization was achieved by heating the hand in a warm air box at 60°C for at least 30 min, which has been previously validated as the appropriate procedure for obtaining arterialized blood (43). In the present study mean oxygen saturation was 94.5% in both lean and obese subjects, indicating comparable and successful arterialization in both groups.

In conclusion, the obese insulin resistant state is characterized by a reduced muscle glycerol release during baseline fasting conditions, which was accompanied by a lower HSL protein expression and phosphorylation on the PKA target sites Ser[563] and Ser[659], and on the AMPK target site Ser[565]. This suggests a blunted fasting skeletal muscle lipolysis in obesity, which may be an important factor contributing to the increased lipid storage in skeletal muscle of obese insulin resistant subjects. Further studies are necessary to address in more detail whether these disturbances are primary factors or adaptation responses to the obese insulin resistant state.

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Tables & Figures

Table 1.

Subjects' characteristics

	Lean (n=13)	**Obese (n=10)**
Age (yr)	49±9	54±8
Weight (kg)	75±6	102±10 [1]
Height (m)	1.81±0.07	1.79±0.07
BMI (kg/m^2)	23.0±1.8	31.9±1.9 [1]
BF% (kg)	20.2±3.5	31.7±1.5 [1]
FFM (kg)	60.1±5.4	69.7±6.7 [1]
WHR	0.91±0.04	1.01±0.03 [1]
SBP (mmHg)	126±11	137±13 [1]
DBP (mmHg)	77±7	85±9 [1]
HOMA$_{IR}$	1.8±0.7	3.4±0.9 [1]

All subjects are men. BMI, body mass index (kg/m^2); BF%, body fat percentage; FFM, fat free mass; WHR, waist to hip ratio; SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg); HOMA$_{IR}$, homeostasis model assessment for insulin resistance.

[1]P<0.05 obese vs. lean.

Values are mean±SD.

Table 2.

Circulating arterialized metabolite concentrations during baseline and isoprenaline infusion in lean and obese subjects

	Lean			Obese		
	Baseline (n=13)	ISO (n=10)	Delta (n=10)	Baseline (n=10)	ISO (n=7)	Delta (n=7)
TAG (µmol/l)	701±66	648±64 [3]	-46±23	1464±190 [2]	1667±217 [2,3]	112±44 [1]
FFA (µmol/l)	661±41	942±53 [3]	271±46	638±42	1124±82 [3]	469±82 [1]
Glycerol (µmol/l)	102±5	118±7 [3]	15±5	106±4	147±10 [2,3]	44±9 [1]
Glycerol Ra (µmol/min)	199±12	311±28 [3]	109±13	220±15	391±30 [3]	172±19
Glycerol Ra/FFM (µmol. kg FFM^{-1}. min^{-1})	3.4±0.2	5.5±0.5 [3]	2.0±0.5	3.3±0.3	5.8±0.6 [3]	2.5±0.5
Glucose (mmol/l)	5.3±0.1	5.4±0.1	0.14±0.06	5.5±0.2	5.4±0.1	-0.12±0.10
Insulin (mU/l)	7.2±0.6	10.7±0.9 [3]	3.4±0.6	13.6±1.0 [2]	24.0±2.3 [2,3]	10.6±1.5 [1]
Lactate (mmol/l)	0.61±0.04	0.72±0.03	0.09±0.04	0.98±0.11 [2]	0.99±0.06 [2]	0.05±0.07

ISO, Isoprenaline; TAG, Triacylglycerol; FFA, Free Fatty Acids; Ra, rate of appearance (µmol. min^{-1});

Ra/FFM, rate of appearance per kg fat free mass (µmol. kg FFM^{-1}. min^{-1}).

[1]P<0.05 change (delta) from baseline obese vs. lean,

[2]P<0.05 obese vs. lean,

[3]P<0.05 ISO vs. baseline.

. Values are mean±SEM.

Table 3.

Regional forearm blood flow and net metabolite flux during baseline and isoprenaline infusion in lean and obese subjects

	Lean		Obese	
	Baseline (n=13)	ISO (n=10)	Baseline (n=10)	ISO (n=7)
Forearm blood flow **(ml. 100ml tissue^{-1}. min^{-1})**	2.9±0.2	4.6±0.4 [3]	2.5±0.3	3.5±0.3 [3]
Forearm net flux (nmol.100ml tissue^{-1}.min^{-1})				
TAG	17±5	24±19 [0]	-2±9 [0]	46±31 [0]
FFA	6±59 [0]	-53±143 [0]	156±42	230±88
Glycerol	-21±11 [0]	-39±33 [0]	11±5 [0, 2]	6±21 [0]
Glucose	142±51	213±117 [0]	226±81	289±223 [0]
Lactate	-90±27	-171±73	8±45	-327±78

Positive fux = net uptake; negative flux = net release

[0] Exchange not different from zero

[1] $P<0.05$ change (delta) from baseline obese vs. lean,

[2] $P<0.05$ obese vs. lean,

[3] $P<0.05$ ISO vs. baseline.

Values are mean±SEM.
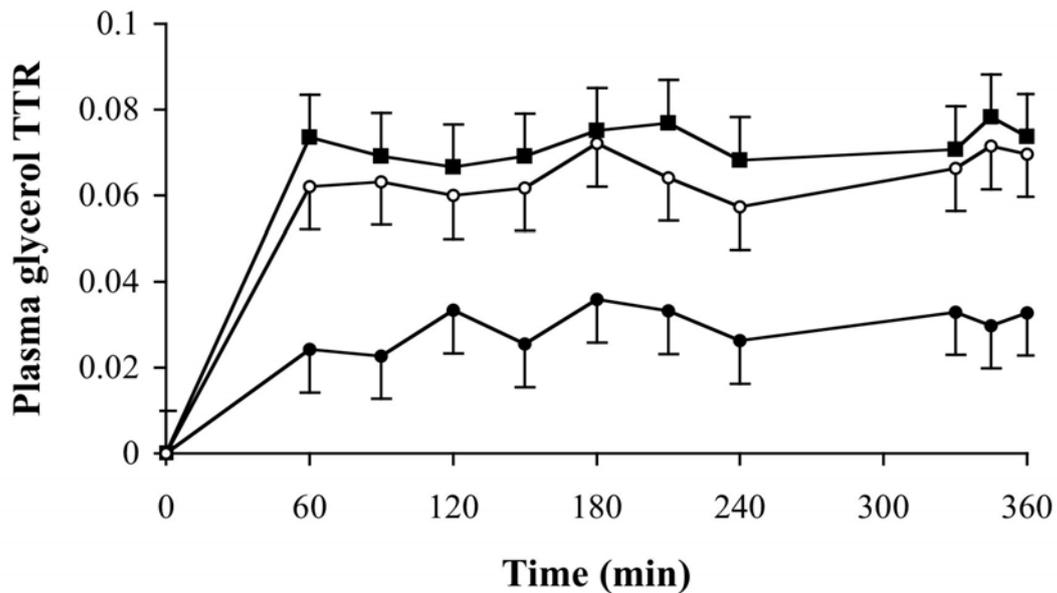
Figure 1.

Plasma glycerol tracer/tracee ratio (TTR) during 6h primed constant infusion of $[^2H_5]$-glycerol (n=3) in arterialized blood (■), forearm venous blood (●) and expected forearm venous enrichment (○). The expected deep venous glycerol enrichment was calculated as arterialized enrichment multiplied by arterialized glycerol concentration divided by deep venous glycerol concentration. The measured venous enrichment was consistently lower than the expected deep venous enrichment (P<0.05), implying uptake of glycerol across the forearm. Values are mean±SEM.
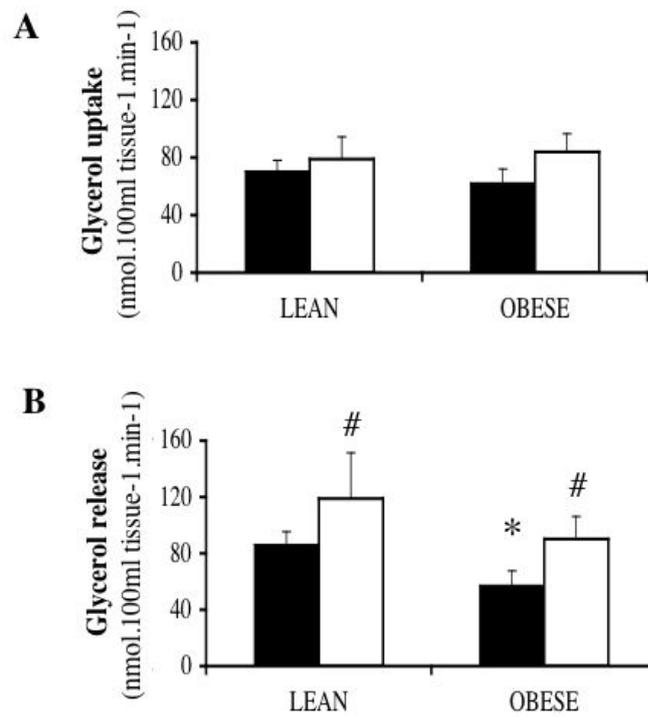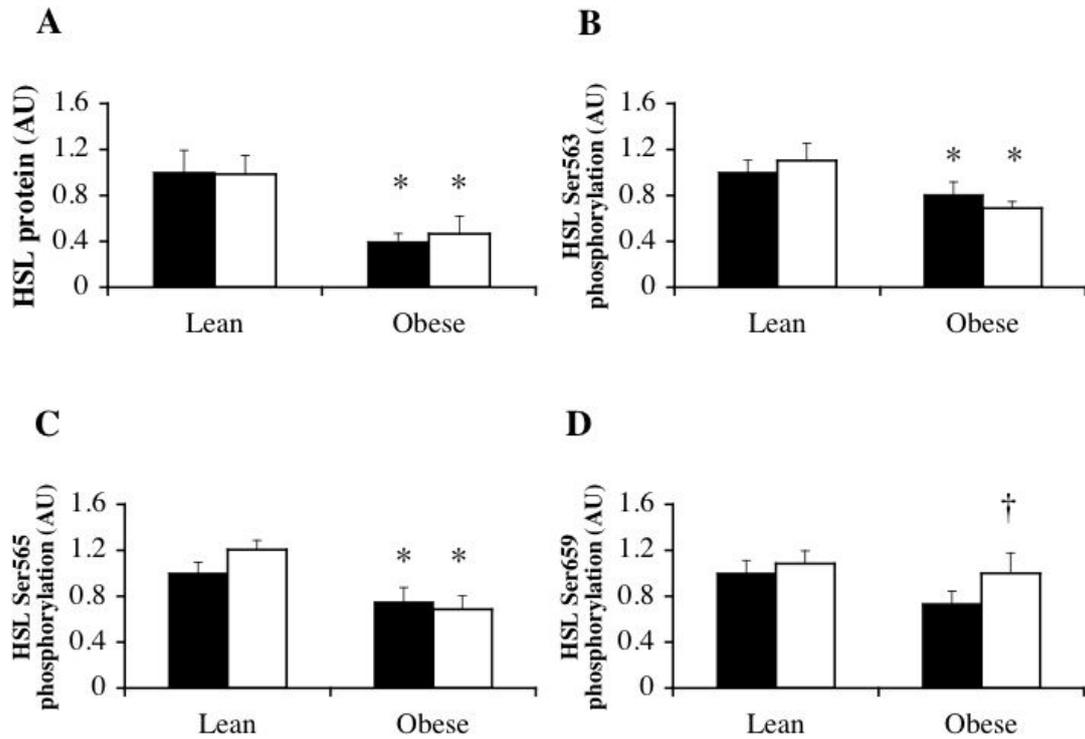
Figure 2.

Total glycerol uptake (**A**) and release (**B**) across the forearm during baseline (black

bars, ■) and isoprenaline infusion (white bars, □) using a $[^2H_5]$-labeled glycerol

tracer in lean and obese subjects**.**

(*) P<0.05 obese vs. lean;

(#) P<0.05 ISO vs. baseline

Values are mean ± SEM**.**

Figure 3.

HSL protein expression (**A**) and Ser[563] (**B**), Ser[565] (**C**) and Ser[659] (**D**) phosphorylation

during baseline (black bars, ■) and isoprenaline infusion (white bars, □) in lean and

obese subjects. Data are expressed as arbitrary units (AU).

(*) P<0.05 obese vs. lean;

(†) P<0.01 obese vs. lean in change between baseline and ISO.

Values are mean ± SEM**.**