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# Primary defects in lipolysis and insulin action in skeletal muscle cells from type 2 diabetic individuals

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ABSTRACT

*Background and objective:* A decrease in skeletal muscle lipolysis and hormone sensitive-lipase (HSL) expression has been linked to insulin resistance in obesity. The purpose of this study was to identify potential intrinsic defects in lipid turnover and lipolysis in myotubes established from obese and type 2 diabetic subjects.
*Methods:* Lipid trafficking and lipolysis were measured by pulse–chase assay with radiolabeled substrates in myotubes from non-obese/non-diabetic (lean), obese/non-diabetic (obese) and obese/diabetic (T2D) subjects. Lipolytic protein content and level of Akt phosphorylation were measured by Western blot. HSL was overexpressed by adenovirus-mediated gene delivery.
*Results:* Myotubes established from obese and T2D subjects had lower lipolysis (−30–40%) when compared to lean, using oleic acid as labelled precursor. Incorporation of oleic acid into diacylglycerol (DAG) and free fatty acid (FFA) level was lower in T2D myotubes, and acetate incorporation into FFA and complex lipids was also lower in obese and T2D subjects. Both protein expression of HSL (but not ATGL) and changes in DAG during lipolysis were markedly lower in cells from obese and T2D when compared to lean subjects. Insulin-stimulated glycogen synthesis (−60%) and Akt phosphorylation (−90%) were lower in myotubes from T2D, however, overexpression of HSL in T2D myotubes did not rescue the diabetic phenotype.
*Conclusions:* Intrinsic defects in lipolysis and HSL expression co-exist with reduced insulin action in myotubes from obese T2D subjects. Despite reductions in intramyocellular lipolysis and HSL expression, overexpression of HSL did not rescue defects in insulin action in skeletal myotubes from obese T2D subjects.


## 1. Introduction

Type 2 diabetes (T2D) is a metabolic disorder characterized with chronic hyperglycemia that affects the way the body utilizes energy. It is initiated by a combination of factors, including defects in regulation of glucose homeostasis and insulin resistance, a condition in which the body's skeletal muscle, adipose and liver tissue do not respond effectively to insulin [1]. Insulin resistance is possibly partly induced by chronic lipid overload in skeletal muscle, especially caused by long-chain acyl-CoAs, diacylglycerols (DAG) and ceramides [2–4].

Lipids are stored as triacylglycerols (TAG) in lipid droplets within skeletal muscle, called intramuscular triacylglycerol (IMTG), and upon energy demand e.g. during exercise, IMTG is used as energy source by healthy subjects [5,6]. There are evidences that increased IMTG is associated with higher levels of lipotoxic intermediates such as DAG and ceramides that might inhibit insulin signalling [7]. However, the mechanism by which IMTGs might contribute to lipotoxicity in obese, insulin resistant, or T2D subjects, remains poorly understood. Recent data suggest that intramyocellular dynamics, like lipid influx and altered rate of lipid turnover, may play an important role in developing insulin resistance [8]. Lipid turnover has a significant impact on insulin sensitivity and glucose homeostasis. Skeletal muscle tissue lipid oxidation and fatty acid (FA) incorporation into TAG are altered in obese individuals with T2D compared to BMI-matched controls, but only the disturbances in TAG incorporation are conserved in cultured myotubes [9–11].

TAG breakdown is mediated by lipases. The first step in hydrolysis of TAGs in skeletal muscle is catalysed by adipose triglyceride lipase (ATGL) [12]. Monoacylglycerol (MAG) lipase (MGL) and hormone-

*Abbreviations:* ACSL, acyl-CoA synthetase; ATGL, adipose triglyceride lipase; CE, cholesteryl esters; DAG, diacylglycerol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GIR, glucose infusion rate; HSL, hormone sensitive lipase; IMTG, intramuscular triacylglycerol; MGL, monoacylglycerol (MAG) lipase; OA, oleic acid; PL, phospholipids; T2D, type 2 diabetes; TAG, triacylglycerols

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sensitive lipase (HSL) were the first lipases identified, and both are highly expressed in skeletal muscle. HSL displays a 10-fold higher specificity for DAG compared to TAG, MAG and cholesteryl esters (CE) [13–15]. Recently, we and others have observed that the expression of ATGL and HSL seems to be altered in myotubes from obese and obese type 2 diabetic individuals when compared to lean controls, however the results are inconsistent. In short, protein expression of ATGL and HSL has been reported to be unaltered or reduced in myotubes from obese and T2D subjects [12,16–21]. Of interest, a selective pharmacological inhibition of lipolysis in myotubes from lean healthy donors was sufficient to inhibit insulin action [16]. The molecular mechanism involves at least in part DAG-mediated protein kinase C (PKC) activation [16]. We therefore hypothesized that reduced muscle HSL protein content could contribute to obesity-related insulin resistance. Because primary human muscle cells retain some of the phenotypic characteristic of their donors [10,22,23], we aimed to identify potential intrinsic defects in lipolysis and HSL expression in myotubes established from obese and obese T2D compared to lean subjects. We further determined whether overexpression of HSL could rescue the insulin resistant phenotype of myotubes from T2D subjects.

## 2. Material and methods

### 2.1. Materials

Dulbecco's modified Eagles medium (DMEM–Glutamax™), DMEM without phenol red, heat-inactivated foetal calf serum (FCS), αMEM, human epithelial growth factor (hEGF), fetuin, gentamycin, and penicillin–streptomycin and amphotericin B were purchased from Gibco Invitrogen (Gibco, Life Technologies, Paisley, UK). Ultroser G was purchased from PALL Life Science (Port Washington, NY, US), insulin (Actrapid®) from NovoNordisk (Bagsvaerd, Denmark), BSA (bovine serum albumin) (essentially fatty acid-free), L-carnitine, Dulbecco's phosphate-buffered saline (DPBS with $Mg^{2+}$ and $Ca^{2+}$), oleic acid (OA, 18:1, n-9), glycerol, triacsin C, HEPES, extracellular matrix (ECM) gel, glycogen, dexamethasone, protease inhibitor and phosphatase I and II inhibitors, were all obtained from Sigma-Aldrich (St Louis, MO, US). [1-$^{14}$C]oleic acid (58.2 mCi/mmol), [1-$^{14}$C]acetate (56.0 mCi/mmol), [$^{14}$C(U)]glycerol (142 mCi/mmol) and D[$^{14}$C(U)]glucose (2.9 mCi/mmol) were from PerkinElmer NEN® (Boston, MA, US). Corning CellBIND® tissue culture plates (96- and 12-well plates) were obtained from Corning Life-Sciences (Schiphol-Rijk, The Netherlands). Isoplate® scintillation plates and OptiPhase Supermix, and all liquid scintillations were performed by the 1450 MicroBeta TriLux scintillation or Packard Tri-Carb 1600 counters, were obtained from PerkinElmer (Shelton, CT, US). Thin layer chromatography plates were purchased from Merck (Darmstadt, Germany), nitrocellulose membrane from Hybond ECL (Amersham Biosciences, Boston, MA, US) and chemiluminescence reagent and hyperfilm ECL from GE Healthcare. Antibodies for pAkt Ser473 (#4060), Akt (#4691), ATGL (#2138), HSL (#4107) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, #2118) was purchased from Cell Signalling Technology (Beverly, MA, US). Protein assay reagent was purchased from BioRad (Copenhagen, Denmark) or Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, IL). Human HSL cDNA was cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) and obtained from Vector Biolabs (Philadelphia, PA). All other chemicals used were of standard commercial high-purity quality.

### 2.2. Human study subjects

Eight non-obese/non-diabetic (lean) control subjects, nine obese/non-diabetic (obese) subjects and eight obese/diabetic (T2D) subjects participated in the study (Table 1) [24]. Only sedentary subjects were recruited. The diagnosis of type 2 diabetes was based on fasting plasma glucose ≥7.0 mmol/L, HbA1c ≥ 6.5% and/or use of one or more antidiabetic drug. Diabetic patients were treated either with diet alone or in combination with sulfonylurea, metformin or insulin, which was withdrawn 1 week before the study. The patients had no diabetic complications apart from simplex retinopathy that was self-reported based on previous diagnosis by an ophthalmologist. The control subjects had normal fasting glucose concentrations and HbA1c levels and no family history for type 2 diabetes. The groups were matched with respect to age, but differed by BMI, fasting plasma glucose concentrations, fasting serum insulin levels, HbA1c and glucose infusion rate by hyperinsulinemic euglycemic clamp (HEC, 40 mU/m$^2$ per min of insulin, after overnight fasting for 10 h). Muscle biopsies were obtained from *m. vastus lateralis* in the fasted state by needle biopsy under local anaesthesia. All subjects gave written informed consent, and the local ethics committee of Funen and Vejle County approved the study. Cell cultures were established from proliferated satellite cells as previously described [25].

**Table 1** Q1

| Clinical variables | Lean | Obese | T2D |
|---|---|---|---|
| Age (years) | 51 ± 3.5 | 47 ± 4.5 | 50 ± 4.8 |
| Body mass index (kg/m$^2$) | 24 ± 1.8 | 34 ± 5.0* | 33 ± 3.8* |
| Fasting plasma glucose (mmol/L) | 5.7 ± 0.4 | 5.7 ± 0.6 | 10 ± 2.1*# |
| Fasting serum insulin (pmol/L) | 25 ± 20 | 57 ± 16* | 97 ± 33*# |
| HbA1c (%) | 5.6 ± 0.2 | 5.4 ± 0.3 | 7.6 ± 1.5*# |
| Glucose infusion rate (mg/min/m$^2$) | 392 ± 64 | 235 ± 64* | 121 ± 61*# |

Values represent means ± SD (n = 8–9 per group).
* $p < 0.05$ vs lean.
\# $p < 0.05$ vs obese. T2D, type 2 diabetes (Bonferroni adjusted).

### 2.3. Cell culture

Myoblasts from control, obese and T2D subjects were cultured on multi-well plates or 25 cm$^2$ flasks in DMEM–Glutamax™ (5.5 mmol/L glucose), 2% FCS, 2% Ultroser G, 25 IU penicillin, 25 μg/mL streptomycin, and 1.25 μg/mL amphotericin B or in DMEM–Glutamax™ (5.5 mmol/L glucose) supplemented with 10% FCS, 10 ng/mL hEGF, 0.39 μg/mL dexamethasone, 0.05% BSA, 0.5 mg/mL fetuin, 50 ng/mL gentamycin and 50 ng/mL amphotericin B. At 70–80% confluence, the growth medium was replaced by DMEM–Glutamax™ supplemented with 2% FCS, 25 IU penicillin, 25 μg/mL streptomycin, 1.25 μg/mL amphotericin B, and 25 pmol/L insulin or αMEM supplemented with 2% FCS, 0.5 mg/mL fetuin, 25 IU penicillin and 25 μg/mL streptomycin to induce differentiation. The cells were cultured in humidified 5% $CO_2$ atmosphere at 37 °C, and the media were changed every 2–3 days. Human myotubes were allowed to differentiate at a physiological concentration of insulin (25 pmol/L) and glucose (5.5 mmol/L).

### 2.4. Pulse–chase assay and lipid distribution from oleic acid

Myotubes were cultured on 96-well or 12-well plates. On day six of differentiation the myotubes were pulsed with [1-$^{14}$C]oleic acid (OA, 100 μmol/L, 0.5 μCi/mL) or [$^{14}$C(U)]glycerol (10 μmol/L, 0.5 μCi/mL) for 24 h in differentiation medium. After pre-labelling, the cells were washed twice with 0.5% fatty acid-free BSA in DPBS at 37 °C. Some of the OA-labelled cells were harvested at the end of the pulse period (T0) with two additions of 125 μL distilled water to determine OA incorporation into TAG, DAG, FFA (free fatty acid), CE (cholesteryl esters) and PL (phospholipids). Following the pulse, myotubes were chased for 3 h with DPBS-medium containing 10 mmol/L HEPES, 100 μmol/L glucose and 0.5% fatty acid-free BSA. After 3 h cell-associated radioactivity for [$^{14}$C(U)]glycerol was determined, and lipid distribution of [1-$^{14}$C]OA was measured. Lipolysis was measured as [1-$^{14}$C]OA and [$^{14}$C(U)]glycerol released from the cells. Re-esterification of OA was calculated as total lipolysis (w/triacsin C, 10 μmol/L) minus basal lipolysis (wo/triacsin C) after 3 h [26]. Triacsin



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