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## **Insulin resistance in obese patients with type 2 diabetes mellitus : effects of a very low calorie diet**

Jazet, I.M.

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# CHAPTER 6

## **Effect of a 2-day very low energy diet on skeletal muscle insulin sensitivity in obese type 2 diabetic patients on insulin therapy.**

Ingrid M. Jazet<sup>1</sup>, D. Margriet Ouwens<sup>2</sup>, Gert Schaart<sup>3</sup>, Hanno Pijl<sup>1</sup>, Hans Keizer<sup>3</sup>, J. Antonie Maassen<sup>2</sup>, A. Edo Meinders<sup>1</sup>.

Departments of <sup>1</sup>General Internal Medicine and <sup>2</sup>Molecular Cell Biology, Leiden University Medical Centre, Leiden, The Netherlands. <sup>3</sup>Department of Movement Sciences, Maastricht University, Maastricht, The Netherlands.

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**ABSTRACT**

This study investigates the molecular mechanisms underlying the blood glucose-lowering effect of a 2-day very low-energy diet (VLED, 1890 kJ/d = very low calorie diet [VLCD, 450 kCal/day]) in 12 obese (body mass index  $36.3 \pm 1.0$  kg/m<sup>2</sup> [mean  $\pm$  SEM]) type 2 diabetic (HbA<sub>1c</sub>  $7.3 \pm 0.4\%$ ) patients simultaneously taken off all glucose-lowering therapy, including insulin.

Endogenous glucose production (EGP) and glucose disposal ([6,6 <sup>2</sup>H<sub>2</sub>]-glucose) were measured before and after the VLED in basal and hyperinsulinaemic (insulin infusion rate 40 mU/m<sup>2</sup>/min) euglycaemic conditions. Insulin signalling and expression of GLUT4, FAT/CD36 and triglycerides were assessed in muscle biopsies, obtained before the clamp and after 30 minutes of hyperinsulinaemia.

Fasting plasma glucose decreased from  $11.3 \pm 1.3$  to  $10.3 \pm 1.0$  mmol/L because of a decreased basal EGP ( $14.2 \pm 1.0$  to  $11.9 \pm 0.7$   $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ,  $p = 0.009$ ). Insulin-stimulated glucose disposal did not change. No diet effect was found on the expression of the insulin receptor and insulin receptor substrate-1 or on phosphatidylinositol 3'-kinase activity, or on FAT/CD36 expression pattern, GLUT4-translocation or triglyceride distribution in either the basal or insulin-stimulated situation. Unexpectedly, basal PKB/Akt-phosphorylation on T308 and S473 increased after the diet, at equal protein expression.

In conclusion, a 2-day VLED lowers fasting plasma glucose *via* a decreased basal EGP without an effect on glucose disposal. Accordingly, no changes in activation of phosphatidylinositol 3'-kinase, triglyceride distribution, FAT/CD36 expression and GLUT-4 translocation were found in skeletal muscle biopsies.

## INTRODUCTION

Energy restriction (ER) and weight loss<sup>1,2</sup> improve the insulin resistance (IR) seen in obese type 2 diabetic patients<sup>3</sup>. Because skeletal muscle is the primary site of insulin-stimulated glucose disposal<sup>4</sup> with glucose transport over the membrane as rate limiting step<sup>5</sup>, skeletal muscle IR might play an important role in obese type 2 diabetic patients.

Intramyocellular lipid (IMCL) accumulation is strongly associated with IR<sup>6</sup>. The cause for IMCL accumulation might include an increased sarcolemmal expression of the fatty acid transporter FAT/CD36 in obese and non-obese type 2 diabetic patients<sup>7</sup>, leading to an increased rate of fatty acid transport<sup>7,8</sup>.

Intramyocellular lipids, in turn, can impair insulin signal transduction<sup>5</sup>. It has been proposed that fatty acid metabolites induce a sustained activation of serine/threonine kinases, such as protein kinase C isoforms, I $\kappa$ B kinase- $\beta$  and Jun N-terminal kinase, which phosphorylate the insulin receptor substrates (IRS) IRS-1 and IRS-2 on serine and threonine sites<sup>5</sup>. Serine-phosphorylated forms of IRS1/2 cannot associate with and activate phosphatidylinositol 3'-kinase (PI3K), resulting in a decreased activation of GLUT-4-regulated glucose transport.

Energy restriction improves blood glucose values and insulin-stimulated glucose disposal in humans with type 2 diabetes as early as 7 days after the initiation of a 3347 kJ/d [800 kCal/day] diet<sup>1</sup>. The molecular mechanism underlying this improvement in insulin sensitivity is largely unknown. In rat skeletal muscle, 20 days of ER enhanced insulin-stimulated GLUT-4 translocation<sup>9</sup>. However, this effect occurred independent of activation of PI3K, indicating that ER ameliorates insulin-stimulated GLUT-4 translocation *via* other mechanisms, possibly down-stream of PI3K. In this regard, PKB/Akt is an attractive candidate given its putative role in insulin-stimulated glucose transport<sup>10,11</sup> and the observation that 20 days of ER led to an increased activation of this protein in rat skeletal muscle<sup>12</sup>.

We found that a very low energy (calorie) diet (VLED = very low calorie diet [VLCD], Modifast<sup>®</sup>, Novartis Consumer Health, Breda, The Netherlands, 1883 kJ/d [450 kCal/day]) improves fasting plasma glucose (FPG) levels as early as 2 days after the initiation of the diet in obese type 2 diabetic patients simultaneously taken off all blood glucose-lowering medication, including insulin<sup>13</sup>. The present study was conducted to elucidate the mechanism underlying this effect. At the whole-body level, the blood glucose-lowering effect of a 2-day VLED appeared to be due to a decrease in basal endogenous glucose production (EGP) with no effect on whole-body insulin-stimulated glucose disposal, as assessed with the hyperinsulinaemic euglycaemic clamp technique with stable isotopes<sup>14</sup>. However, because no effect on whole-body insulin-stimulated glucose disposal does not preclude any effect (or a beginning effect) on skeletal muscle at the molecular level, we also took muscle biopsies. In fact, beforehand we assumed a beginning effect of ER on insulin signal transduction that might become apparent at the whole-body level after 7 to 10 days.

We therefore examined IRS-1-associated PI3K-activity and PKB/Akt phosphorylation in skeletal muscle biopsies taken before and after 2 days of a VLED, both in the basal and in the insulin-stimulated situation. In addition, we determined the expression and translocation of the fuel transporters GLUT-4 and FAT/CD36. Finally, we examined intramyocellular triglyceride content with an oil red O staining.

## RESEARCH DESIGNS AND METHODS

### Subjects

Twelve obese type 2 diabetic patients, 5 male and 7 female (age  $55 \pm 4$  years [mean  $\pm$  SEM], body mass index [BMI]  $36.3 \pm 1.0$  kg/m<sup>2</sup>) participated in this study, which was approved by the Medical Ethical Committee of Leiden University Medical Centre. Written informed consent was obtained from all patients after the study was explained.

Patients used at least 30 units of exogenous insulin with or without oral blood glucose-lowering medication. Only subjects with remaining insulin secretion, defined as a fasting plasma C-peptide level of more than 0.8 ng/mL or a 2 times increase of the basal C-peptide level after 1 mg glucagon iv<sup>15</sup>, were included.

Patients had to have stable body weight for at least 3 months and were instructed not to alter life style habits (eating, drinking, exercise) from screening until the start of the study. None of the patients were smokers, and the use of medication known to alter glucose or lipid metabolism was prohibited.

### Diet and protocol outline

Three weeks before the start of the study, all oral blood glucose-lowering medication was discontinued. At day -1 and day 4, only short-acting insulin was given. On day 0, baseline investigations (day 0) were performed as outlined below. Insulin therapy was restarted after this study day until the start of the 2-day VLED on day 5 (to ensure complete washout of stable isotopes) and remained stopped during the 2-day VLED. On day 7 the second study day (day 2) took place. The VLED consisted of 3 sachets of Modifast<sup>®</sup> per day, amounting approximately 1883 kJ/d [450 kcal/day]. Patients were provided with muesli, shakes, and potage, from which they could chose freely. The exact amount of carbohydrates, protein, and fat in the Modifast<sup>®</sup> sachets varies a little between the different substances; but with 3 sachets of Modifast<sup>®</sup> per day, patients receive about 50 g protein, 50 to 60 g carbohydrates, 7 to 9 g lipids, and 10 g of dietary fibres. Patients followed the VLED at home and were only admitted to the research ward for study days.

### Study days

All studies started at 7:00 AM after an overnight fast. Length (m), weight (kg) and BMI (BMI = length [kg] / length<sup>2</sup> [m]) were measured according to WHO recommendations<sup>16</sup>.

Metabolic studies were performed as described previously<sup>14</sup>. In short, basal rates of glucose and glycerol turnover were assessed after 3 hours of an adjusted primed (17.6  $\mu\text{mol/kg} \times$  actual plasma glucose concentration [mmol/L]/5 (normal plasma glucose)<sup>17</sup> continuous (0.33  $\mu\text{mol/kg}$  per min) infusion of [6,6-<sup>2</sup>H<sub>2</sub>]-glucose (Enrichment 99.9%, Cambridge Isotopes, MA, USA) and 1.5 hours of a primed (1.6  $\mu\text{mol/kg}$ ) continuous (0.11  $\mu\text{mol/kg}$  per min) infusion of [<sup>2</sup>H<sub>3</sub>]-glycerol (Cambridge Isotopes). Insulin-stimulated rates of glucose and glycerol turnover were assessed after 4.5 hours of a hyperinsulinaemic-euglycaemic clamp (Actrapid®, Novo Nordisk Pharma, Alphen aan de Rijn, The Netherlands, rate 40 mU/m<sup>2</sup>/min<sup>18</sup>). Glucose values were clamped at 5 mmol/L by the infusion of a variable rate of 20% glucose enriched with 3% [6,6-<sup>2</sup>H<sub>2</sub>]-glucose.

### Blood chemistry

Serum insulin was measured by an ultrasensitive Human Insulin assay (Linco Research, St Charles, MO, USA) with a detection limit of 0.1 mU/L. The interassay coefficient of variation was below 6%. Serum C-peptide was measured with a radioimmunoassay from Linco Research. Serum triglycerides were determined with a fully automated Hitachi 747 system (Hitachi, Tokyo, Japan).

Serum glucose and [6,6-<sup>2</sup>H<sub>2</sub>]-glucose were determined in a single analytical run, using gas chromatography coupled to mass spectrometry as described previously<sup>19,20</sup>.

Serum non-esterified fatty acids (NEFA) were measured using the enzymatic colorimetric acyl-CoA synthase, acyl-CoA oxidase assay (Wako Chemicals, Neuss, Germany) with a detection limit of 0.03 mmol/L. The interassay coefficient of variation was below 3%.

### Muscle biopsies

Muscle biopsies were taken from the vastus lateralis muscle, after localised anaesthesia with 1% lidocaine, with a modified Bergström needle (Maastricht Instruments, Maastricht, The Netherlands) using applied suction<sup>21</sup>. The muscle biopsies were taken in the basal situation (8:00 AM, i.e., 1 hour after patients came in and were in a semirecumbent position) and 30 minutes after the start of the insulin infusion (10 minute prime followed by a constant rate of 40 mU/m<sup>2</sup>/min<sup>18</sup>), while blood glucose levels were kept at initial values during these first 30 minutes *via* the infusion of 20% glucose at a variable rate. Muscle samples were snap-frozen in isopentane chilled on dry ice and stored at -80°C until further analysis.

### Insulin Signalling

Muscle biopsies were homogenised in PI3K lysis buffer using an ultraturrax mixer and centrifuged (15 minutes, 14.000 rpm, 4°C), then protein content was determined using a BCA-kit

(Pierce, Rockford, IL)<sup>22</sup>. Insulin receptor substrate-1 (IRS-1) was immunoprecipitated overnight (4°C) from 1.5 mg protein using IRS-1 antibody K6, and PI3K-activity was determined as described previously<sup>22</sup>.

To determine expression and phosphorylation of other components of the insulin signaling system, proteins (25 µg/lane) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and blotted on polyvinylidene difluoride membranes (Millipore, Bedford, MA). Filters were incubated overnight (4°C) with phospho-specific PKB/Akt-Thr308, PKB/Akt-Ser473 (Cell Signalling Technology, Beverly, MA), IRS1 K6 and Akt-1 antibody (Upstate, Lake Placid, USA). Bound antibodies were detected using appropriate horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI) in a 1:10.000 dilution, followed by visualization by enhanced chemiluminescence. Blots were quantitated by densitometric analysis of the films using Scion Image beta 4.02 software.

#### **Immunofluorescence assay for FAT/CD36 and GLUT-4 and Oil Red O staining**

Routine indirect (double) immunofluorescence assays were performed as described previously<sup>23</sup>. Serial cryosections were fixed and incubated overnight at 4°C with the following primary antibodies: MO25, a monoclonal antibody directed against human FAT/CD36<sup>23</sup>; sc-7309 (Santa Cruz, TeBu-Bio, Heerhugowaard, the Netherlands), a mouse IgM monoclonal antibody reactive to FAT/CD36 of human origin; GLUT-4-BW, a polyclonal rabbit antibody directed against the final 12 amino acids of the C-terminus of the human GLUT-4 protein<sup>24</sup>; a polyclonal laminin antibody (L-9393, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands); a monoclonal caveolin-3 antibody (clone 26; BD Biosciences Pharmingen, Alphen aan de Rijn, The Netherlands); and a mouse monoclonal antibody directed against adult human slow myosin heavy chain (A4.840; developed by Dr. Blau<sup>25</sup>).

After washing the slides with phosphate-buffered saline (PBS), sections were incubated with the appropriate secondary fluorescent-labelled antibodies and thereafter mounted with Mowiol.

According to Koopman *et al.*<sup>26</sup>, tissue sections were stained with oil red O combined with an immunofluorescence assay. Oil red O epifluorescence signal was quantified for each muscle cell of each cross section as described before<sup>27</sup>. Lipid droplet density was calculated by dividing the total numbers of droplets by the total (IMCL) area measured. Statistical significance of differences between trials was assessed by paired *t*-tests.

Images were examined in a Nikon E800 microscope (Uvikon, Bunnik, the Netherlands) and were digitally captured using a 1.3 Megapixel Basler A101C progressive scan colour CCD colour camera, driven by LUCIA laboratory image processing and analysis software (Laboratory Imaging, Prague, Czech Republic).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting for FAT/CD36 and GLUT-4**

Western blotting analyses were performed as described before for GLUT-4<sup>24</sup> and FAT/CD36<sup>23</sup>. Briefly, forty 20- $\mu$ m-thick cryosections of muscle biopsies were sampled and homogenised. After centrifugation, the membrane fraction (pellet) and cytosol fraction (supernatant) were separated and both suspended in PBS.

For SDS-polyacrylamide gel electrophoresis and Western blotting, 1 part of the samples was boiled for 4 min in 2 parts of SDS-sample buffer (Bio-Rad Laboratories, Veenendaal, The Netherlands). Equal amounts of proteins were loaded on 10% polyacrylamide SDS-gels (Bio-Rad Laboratories). After electrophoretic separation, the proteins were transferred to nitrocellulose in Western blotting, then the blots were preincubated for 20 min with 5% non-fat dry milk in 0.05% Tween 20 (Sigma-Aldrich Chemicals) in PBS and incubated overnight at room temperature with the polyclonal GLUT-4-BW antibody<sup>24</sup> or the MO25 monoclonal antibody specific for FAT/CD36<sup>23</sup>. Chemiluminescence detection was performed after incubation with the appropriate horseradish-conjugated secondary antibodies. Proteins bands were analysed by densitometry using Image Master (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

**Calculations**

The rate of appearance ( $R_a$ ) and rate of disappearance ( $R_d$ ) for glucose were calculated using the steady state equation by Steele as adapted for stable isotopes using a single-compartment kinetic model<sup>28</sup>.

Endogenous glucose production during the basal steady state is equal to the  $R_a$  of [6,6-<sup>2</sup>H<sub>2</sub>]-glucose, whereas EGP during the clamp was calculated as the difference between  $R_a$  and the glucose infusion rate.

**Statistical analysis**

Data are presented as mean  $\pm$  SEM. Differences before (day 0) and after (day 2) the VLED were analysed by the Student's *t*-test for paired samples. Correlation analysis was carried out using Pearson's correlation. All analyses were performed using SPSS for Windows version 11.0 (SPSS Inc., Chicago, IL, USA). Significance was accepted at  $p < 0.05$ .

**RESULTS****Clinical and metabolic characteristics**

Patient characteristics can be found in Table 1.

After 2 days of a VLED, FPG levels decreased ( $11.3 \pm 1.3$  to  $10.3 \pm 1.0$  mmol/L), despite the cessation of all blood glucose-lowering medication. At that moment, weight loss amounted  $2.9 \pm 0.4$  kg ( $p = 0.001$ ). The decrease in FPG was accompanied by a significant decrease in basal EGP (Table 2) although basal insulin levels had also significantly decreased.



**Table 1.** Patient characteristics.

Sex (male/female)	5 : 7
Age (years)	55 ± 4
BMI (kg/m <sup>2</sup> )	36.3 ± 1.0
Waist circumference (cm)	120 ± 3
Waist-hip ratio	1.02 ± 0.03
Fasting plasma glucose (mmol/L)	11.3 ± 1.3
HbA <sub>1c</sub> (%)	7.3 ± 0.4
Fasting serum insulin (mU/L)	20.7 ± 2.1
Fasting serum C-peptide (ng/mL)	1.0 ± 0.1
Duration of type 2 diabetes (years)	7.9 ± 1.3
Units of insulin injected per day	78 ± 9
Additional use of oral glucose-lowering medication	6 metformin 1 rosiglitazone

Data are presented as mean ± SEM.

**Table 2.** Metabolic parameters on day 0 and after 2 days of a VLED in obese type 2 diabetic patients.

	Day 0			Day 2		
	Basal	Clamp	P	Basal	Clamp	P
Glucose (mmol/L)	11.3 ± 1.3	5.0 ± 0.4	0.0001	10.3 ± 1.0	4.9 ± 0.4	0.0001
Insulin (mU/L)	20.7 ± 2.3 <sup>*</sup>	88.1 ± 5.9	0.0001	15.9 ± 1.8 <sup>*</sup>	83.7 ± 4.8	0.0001
NEFA (mmol/L)	1.1 ± 0.1	0.39 ± 0.07	0.001	1.5 ± 0.1	0.35 ± 0.04	0.0001
Triglycerides (mmol/L)	1.8 ± 0.2	2.1 ± 0.2	0.028	2.0 ± 0.2	2.0 ± 0.2	NS
Glucose R <sub>d</sub> <sup>Δ</sup>	14.2 ± 1.0 <sup>†</sup>	12.1 ± 0.7	NS	11.9 ± 0.7 <sup>†</sup>	11.3 ± 1.0	NS
EGP <sup>Δ</sup>	14.2 ± 1.0 <sup>†</sup>	5.5 ± 0.8	0.0001	11.9 ± 0.7 <sup>†</sup>	5.2 ± 0.5	0.0001
Glycerol R <sub>a</sub> <sup>Δ</sup>	5.2 ± 1.0	1.9 ± 0.2	0.008	4.0 ± 0.6	1.8 ± 0.2	0.008

Basal R<sub>d</sub> = R<sub>a</sub> = EGP. During insulin stimulation, the amount of 20% glucose has to be subtracted from the R<sub>d</sub> to get EGP.

The data in this table have already been published<sup>14</sup>.

<sup>Δ</sup> data in μmol.kg<sup>-1</sup>.min<sup>-1</sup>

Basal day 0 versus day 2: \* p = 0.033, † p = 0.008

On both study days, we achieved comparable clamp serum glucose and insulin values (Table 2, these data have already been published<sup>14</sup>). Neither insulin suppressibility of EGP nor insulin stimulation of whole-body glucose disposal differed significantly after 2 days of a VLED (Table 2). Serum NEFA levels were more, but not significantly (p = 0.057), suppressed during hyperinsulinaemia on day 2. In line with this finding, the capacity of insulin to suppress whole-body lipolysis as measured by R<sub>a</sub> of glycerol, also did not change after 2 days of a VLED (data not shown).

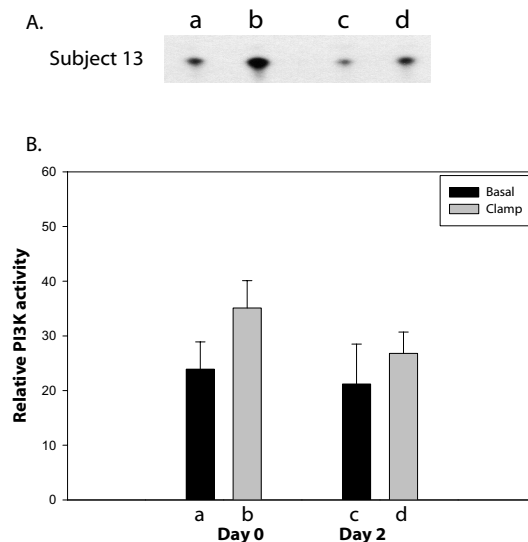
#### Effect of a 2-day VLCD on insulin signalling in skeletal muscle

To study the effect of 2 days of a VLED on insulin signalling, we examined IRS-1 associated PI3K activity in skeletal muscle biopsies obtained before and 30 minutes after the initiation

of a hyperinsulinaemic euglycaemic clamp. Of the 12 patients, 4 showed a higher basal PI3K activity after 2 days of a VLED, which was not associated with an increase in insulin-stimulated PI3K activity nor with an increase in insulin-stimulated glucose disposal both before and after the VLED. Only in 5 out of 12 subjects, insulin increased IRS-1-associated PI3K activity, and a 2-day VLED did not improve the magnitude of this insulin response. Collectively, IRS-1-associated PI3K activity did not change after 2 days of a VLED, neither in the basal nor in the insulin-stimulated situation (Fig. 1). In addition, there was no effect of the VLED on the protein expression of the insulin receptor and IRS-1 (data not shown).

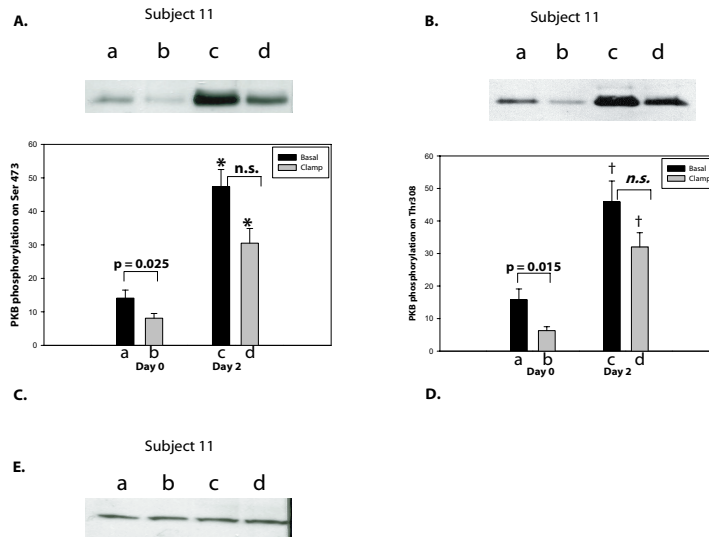
Basal PKB/Akt phosphorylation (both on T308 and S473) was significantly higher after 2 days of a VLED (Fig. 2), whereas the capacity of insulin to stimulate PKB/Akt activation was not significantly different between study days. When we looked at the individual data, none of the patients showed an increase in PKB/Akt phosphorylation during hyperinsulinaemia before the diet, whereas after the 2-day VLED, 3 of the 12 patients showed a 2-fold increase with hyperinsulinaemia. Protein expression of PKB/Akt (Fig. 2E) did not differ between study days, neither in the basal nor in the insulin-stimulated situation.

In line with the finding that insulin-stimulated whole-body glucose disposal did not change, we also found no change in the total amount of GLUT-4 expression (Fig. 3A) nor in translocation of GLUT-4 from the cytoplasm to the sarcolemma (Fig. 3B-E) as assessed by immunofluorescence staining (Fig. 3B-E) and Western blotting (Fig. 3A) in the skeletal muscle biopsies. Insulin-stimulated GLUT-4 translocation was monitored by a previously published

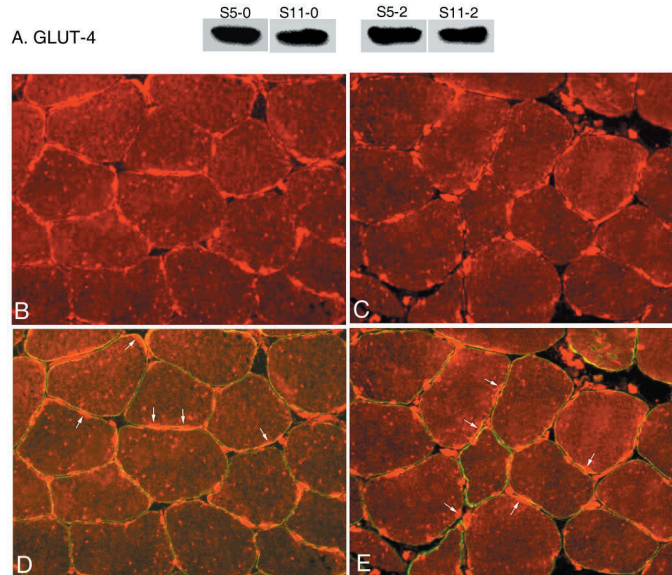


**Figure 1.**

Autoradiograph (A) and quantification (B) of IRS-1-associated PI3K activity in vastus lateralis muscle biopsies obtained before (a and b) and after a 2-day VLED (c and d) in basal (a and c) and hyperinsulinaemic euglycaemic conditions (b and d), changes are not significant. Data are expressed as mean ± SEM.

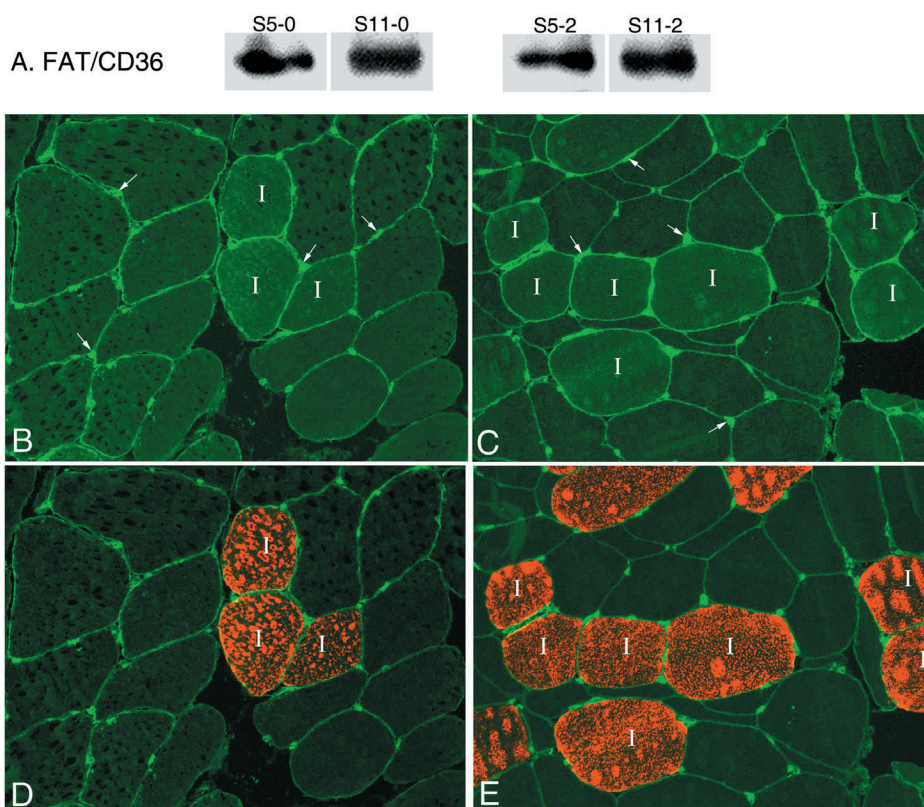
**Figure 2.**

Immunoblot and quantification of Akt/PKB phosphorylation at Ser 473 (A and C) and Thr 308 (B and D) in vastus lateralis muscle biopsies obtained before (a and b) and after a 2-day VLED (c and d) in basal (a and c) and hyperinsulinaemic (b and d) conditions. An immunoblot of PKB protein expression is given in E. Data are expressed as mean  $\pm$  SEM. Note the increase in basal PKB/Akt phosphorylation, at SER 473 as well as Thr 308, after 2 days of a VLED. \*  $p < 0.001$ , †  $P < 0.005$ , day 2 compared to day 0.

**Figure 3.**

Immunoblotting (A) of total muscle fractions of two subjects (S5 and S11) before (0) and after a 2-day (2) VLED. Double-immunofluorescence staining (B-E) of GLUT-4 (red) and caveolin-3 (green) in insulin-stimulated cryosections of human vastus lateralis muscle before (B, D) and after a 2-day VLED (C, E).

B and C, GLUT4. D and E, GLUT4 and caveolin-3. Note the GLUT-4 accumulations near the plasmalemma both before and after the 2-day VLED (arrows).



**Figure 4.**

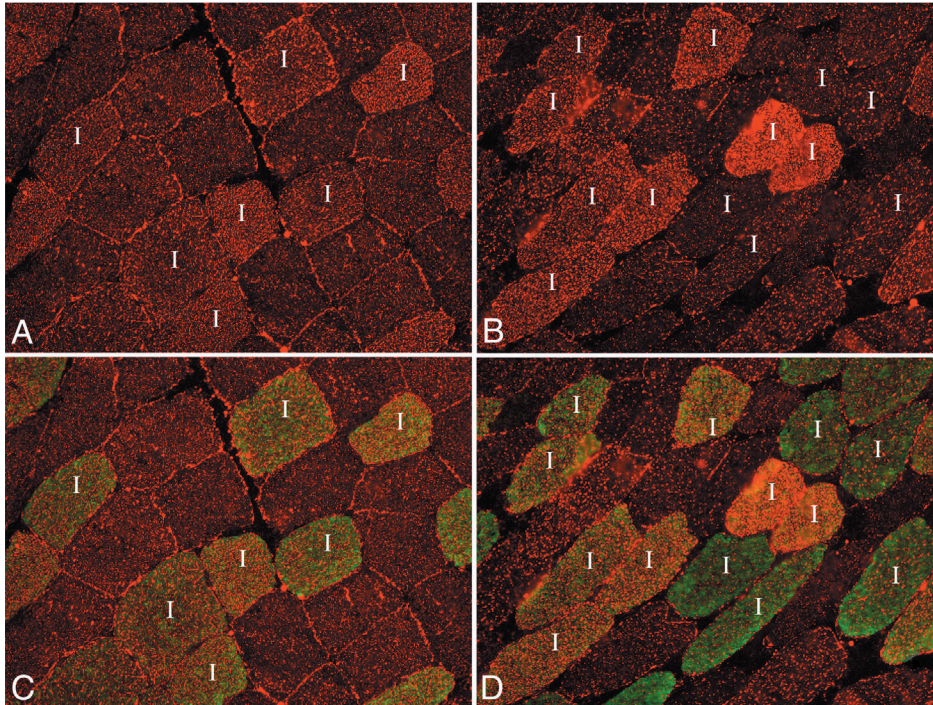
Immunoblotting (A) of the muscle cell membrane fraction. Shown are two subjects (S5 and S11) before (0) and after a 2-day (2) VLCD. Double-immunofluorescence staining of FAT/CD36 (green) and myosin heavy chain type 1 (MHC-1) (red) in insulin-stimulated cryosections of vastus lateralis muscle before (A, C) and after 2-days of diet intervention (B, D). A and B, FAT/CD36. C and D, FAT/CD36 and MHC-1.

I, indicates type-1 muscle fibers. No changes were observed between study days.

immunofluorescence method, albeit in a different model (increased GLUT-4 translocation upon 36 hours of pharmacologic blocking of fat oxidation using CPT1<sup>29</sup>). Using this methodology, we also were able to detect, in a semi-quantitative manner, insulin-induced GLUT-4 translocation after 2 hours of a hyperinsulinaemic euglycaemic clamp in healthy human subjects. Given these data (reflecting a positive control) we are also confident that the method used is of sufficient sensitivity to detect insulin-mediated changes in GLUT-4 localization.

Immunofluorescence staining showed that FAT/CD 36 was expressed at the sarcolemma as well in the cytoplasm of muscle cells (Fig. 4B-E) and that FAT/CD 36 staining was more intense in type 1 muscle fibres. Neither the VLCD nor hyperinsulinaemia affected the FAT/CD 36 staining pattern. A Western blot analysis confirmed the findings of the immunofluorescence staining (Fig. 4A).





**Figure 5.**

Oil red O staining (A, B) in combination with myosin heavy chain type 1 (MHC-1) immunofluorescence assay (C, D) in cryosections of vastus lateralis muscle before (A, C) and after 2-days of diet intervention (B, D). No changes were observed between study days.

Triglyceride content in skeletal muscle cells, as assessed with oil red O staining, did not change between study days, neither in the basal nor in the insulin-stimulated situation (Fig. 5).

## DISCUSSION

This study was performed to elucidate the molecular mechanism underlying the blood glucose-lowering effect of a 2-day VLED in insulin-treated obese type 2 diabetic patients. In line with our previous observations<sup>13</sup>, this study again shows that 2 days of a VLED, in combination with the cessation of all blood glucose-lowering medication in obese type 2 diabetic patients lowers FPG levels. At the whole-body level this decrease in FPG could be explained by a decrease in basal EGP without an improvement in insulin-stimulated glucose disposal. These results are described elsewhere<sup>14</sup>.

Although we did not find any improvement in insulin-stimulated glucose disposal at the whole-body level, we did analyse the muscle biopsies we took during this study because we still expected a beginning effect of the VLED at the molecular level in skeletal muscle

biopsies. However, we did not find a significant diet effect either in GLUT-4 content or in GLUT-4 translocation from the cytoplasm to the plasma membrane (Fig. 3) in skeletal muscle biopsies. In addition, no diet effect was found on the protein expression of IRS-1 and on IRS-1-associated PI3K activation. Of the 12 patients, 4 showed a higher basal PI3K activity after 2 days of a VLED, which was not associated with an increase in insulin-stimulated PI3K activity nor with an increase in insulin-stimulated glucose disposal both before and after the VLED. Remarkably, 7 of 12 patients lacked an increase in insulin-stimulated PI3K activity. This is in accordance with several other studies in which a decreased insulin-stimulated tyrosine phosphorylation of IRS-1 and PI3K activity was found in skeletal muscle of type 2 diabetic patients compared to control subjects<sup>30-32</sup>. The fact that we did not find any stimulation of PI3K activation during hyperinsulinaemia in most of our patients probably reflects their severely insulin-resistant state with a grossly disturbed insulin signal transduction. A 2-day VLED does not (yet) improve this.

With regard to PKB/Akt we, unexpectedly, found a markedly enhanced phosphorylation on T308 and S473 after 2 days of a VLED in the basal situation, whereas we failed to observe insulin-stimulated PKB/Akt phosphorylation under our experimental conditions. Other studies found both decreased<sup>33</sup> and normal<sup>34</sup> insulin-stimulated PKB/Akt activity in patients with type 2 diabetes as compared with controls. In the latter study, supraphysiological doses of insulin have been used however (infusion rate of 120-300 mU/m<sup>2</sup>/min). Another problem with the comparison of our results with those of others is that some studies, like we did, used biopsies taken during *in vivo* physiological hyperinsulinaemia, whereas others take muscle biopsies and incubate the muscle strips *in vitro*<sup>33</sup> with varying insulin concentrations. With regard to the increase in basal PKB/Akt phosphorylation, another study<sup>35</sup> showed that obese patients presenting with atypical diabetes had impaired Akt-2 expression and activation that increased after normalisation of glycaemia with intensive insulin therapy. There are 3 Akt isoforms (insulin action in muscle predominantly involves Akt-1 and Akt-2 stimulation) with Akt-2 knockout mice having impaired glucose homeostasis<sup>11</sup>. We did not measure Akt isoforms, and the interventions (VLED *versus* insulin therapy) are different but both are aimed at lowering blood glucose levels, and it might have been interesting to see whether 2 days of caloric deprivation would have the same results on PKB/Akt phosphorylation in these newly diagnosed type 2 diabetic patients.

Despite the fact that we found no changes in IRS-1 tyrosine phosphorylation and PI3K activity, basal PKB/Akt phosphorylation was increased after 2 days of a VLED, at equal PKB/Akt protein expression. This observation suggests that factors other than the IR-IRS-PI3K pathway also modulate the activity of PKB/Akt. In the liver, PKB/Akt has been shown to be involved in gluconeogenesis<sup>36</sup>. If the increased basal PKB/Akt activation we found in skeletal muscle also holds for the liver, this might explain the lower basal glucose production after 2 days of ER.

Studies regarding the expression pattern of FAT/CD 36 in humans are scarce<sup>37,38</sup>. Recently, 2 morphologic studies<sup>23,39</sup> using immunofluorescence microscopy showed that FAT/CD 36 is

indeed expressed at both the sarcolemma and in the cytoplasm in human skeletal muscle. In both studies it became apparent that FAT/CD 36 is more abundant in type 1 muscle fibres. In line with the study of Keizer *et al.*<sup>23</sup> we show, for the first time in obese, very insulin-resistant patients, a similar dual expression pattern of FAT/CD 36, which was also more prominent in type 1 muscle fibres. Unlike other studies, we did not find an effect of hyperinsulinaemia. This might be because many studies used the so-called giant vesicles method<sup>40,41</sup> or it might reflect the severely insulin-resistant state of our subjects. Recently, Bonen *et al.*<sup>7</sup>, found a 4-fold increase in long-chain fatty acid (LCFA) transport along with an increased intramuscular triacylglycerol content in giant sarcolemmal vesicles prepared from skeletal muscle of relatively lean (BMI  $25 \pm 1.1$  kg/m<sup>2</sup>) type 2 diabetic subjects (on diet or oral blood glucose-lowering agents only) compared with control subjects. This increased LCFA transport was associated with an increased expression of FAT/CD 36 at the sarcolemma at equal total FAT/CD 36 expression. This study supports the concept that augmented LCFA transport along with an imbalance between fatty acid reesterification and oxidation leads to an excess accumulation of triacylglycerols in the skeletal muscle cell, a marker for insulin resistance. It also shows that impaired trafficking of FAT/CD 36 between the sarcolemma and the cytosol (with an increased expression at the sarcolemma) might be the underlying pathogenetic mechanism. Because FAT/CD 36 can, at least partly, be stimulated *via* the insulin signal transduction pathway<sup>42</sup>, a possible link with the altered GLUT-4 trafficking (which in contrast has a decreased expression at the sarcolemma as a pathogenetic state) might be the cause of the impairment seen in both FAT/CD 36 and GLUT-4 trafficking in type 2 diabetic patients. We did not include control subjects and hence cannot confirm that our patients also had relatively more FAT/CD36 at the sarcolemma compared with control subjects.

One might argue that we studied patients while they were not normoglycaemic. Indeed, hyperglycaemia may have deleterious effects on insulin signalling<sup>43,44</sup>, but each patient was his/her own control, and we were only looking for changes in signal transduction after 2 days of a VLED. Moreover, although we discontinued all blood glucose-lowering agents, FPG tended to decline and certainly did not increase after 2 days of a VLED. Another criticism may be that the timing of the muscle biopsies might have been too soon after initiating hyperinsulinaemia. Serum samples showed that maximal insulin concentrations had already been achieved at the time of the biopsy (data not shown) although this does not mean that steady state insulin concentrations in the interstitium had been achieved. In addition, several studies have shown that the effect of hyperinsulinaemia on activation of insulin-signal transduction molecules such as IRS-1, PI3K and PKB/Akt occur as early as 15 minutes<sup>45-47</sup> and that over 50% of the maximal effect already occurred at this time although maximal activity was reached at 60 minutes<sup>47</sup>.

Kelley *et al.*<sup>1</sup> showed that peripheral glucose uptake increases and contributes to the blood glucose-lowering effect of a VLED already after 7 days. Because we had seen a decrease in FPG levels after only 2 days of a VLED<sup>13</sup>, we presumed a change in muscle glucose uptake or

at least already some changes at the molecular level in skeletal muscle biopsies. Our study shows that the very early (2 days) glucose-lowering, insulin-sparing effect of a VLED is predominantly due to a decreased EGP. Studies with a longer duration of the VLED have to be performed to detect the moment that an increased muscular glucose uptake contributes to the blood glucose-lowering effect, what the underlying molecular mechanisms are, and when these underlying molecular mechanisms become apparent.

In conclusion, this is one of the very few human studies investigating the short-term effect of ER on insulin-stimulated glucose disposal both at the whole-body and at the molecular level in obese type 2 diabetic patients in whom all blood glucose-lowering medication was discontinued. The participants in our study exhibit marked clinical insulin resistance. The clamp data indicate that two days of reduced food intake does not significantly affect basal and insulin-stimulated peripheral glucose disposal. This observation is in line with the inability of hyperinsulinaemia to activate PKB/Akt and the lack of an effect of the diet on other components of the insulin-signalling pathway such as PI3K activation and GLUT-4 expression and degree of GLUT-4 translocation. Remarkably, basal PKB/Akt phosphorylation is significantly increased after 2 days of reduced food intake indicating a link between the energy status and basal PKB/Akt activity. In the liver, PKB/Akt has been shown to be involved in regulating gluconeogenesis<sup>36</sup>. If this elevated basal PKB/Akt activation also holds for the liver, a situation difficult to test in the human situation, this could explain the observed significant decrease in EGP in the basal state after two days of reduced food intake.



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