Diabetologia (2002) 45:1128–1135
DOI 10.1007/s00125-002-0875-9

Diabetologia

Insulin signal transduction and glucose transport in human adipocytes: effects of obesity and low calorie diet

M. Björnholm[1, 2], **L. Al-Khalili**[1], **A. Dicker**[3], **E. Näslund**[4], **S. Rössner**[5], **J. R. Zierath**[1, 2], **P. Arner**[3]

[1] Department of Clinical Physiology, Karolinska Hospital, Stockholm, Sweden
[2] Section of Integrative Physiology, Karolinska Institutet, Stockholm, Sweden
[3] Medicine and Clinical Research Centre at Huddinge University Hospital, Stockholm, Sweden
[4] Division of Surgery, Karolinska Institutet Danderyd Hospital, Stockholm, Sweden
[5] Obesity Unit at Huddinge University Hospital, Stockholm, Sweden

Abstract

Aim/hypothesis. We examined insulin signal transduction at the level of insulin receptor substrates (IRS) 1 and 2, phosphatidylinositol (PI) 3-kinase and glucose transport in isolated subcutaneous adipocytes from obese and lean women.
Methods. Glucose transport and insulin signalling were investigated in isolated adipocytes from six obese women (BMI 36–43 kg/m^2) (before and after 11 days of very low calorie diet) and from six lean women (BMI 22–26 kg/m^2).
Results. Insulin sensitivity of glucose transport was reduced in adipocytes from obese women ($p<0.05$), with further reductions in basal and maximal insulin-stimulated glucose transport after a very low calorie diet ($p<0.05$). In obese women, IRS-1 associated PI 3-kinase activity was markedly impaired ($p<0.05$), whereas, IRS-2 associated PI 3-kinase activity was normal. IRS-1 associated PI 3-kinase activity remained blunted after a very low calorie diet, whereas IRS-2 associated PI 3-kinase activity was increased. GLUT4 protein was reduced by 37% in obese versus lean subjects ($p<0.05$), and decreased further after a very low calorie diet (from 19±4 to 14±4 arbitrary units; $p<0.05$).
Conclusion/interpretation. IRS-1 signalling to PI 3-kinase is a site of insulin resistance in adipocytes from obese women, whereas insulin action on IRS-2 is normal. Thus, IRS-1 and IRS-2 undergo differential regulation in adipocytes from obese insulin resistant subjects. Finally, a very low calorie diet is associated with a further impairment in glucose transport in adipose tissue. The defect in glucose transport after a very low calorie diet occurs independent of further defects in insulin signalling at the level of the PI 3-kinase. [Diabetologia (2002) 45:1128–1135]

Keywords Insulin receptor, insulin receptor substrates, PI 3-kinase, glucose transport, GLUT4.

Obesity is associated with insulin resistance in peripheral tissues and the development of Type II (non-insulin-dependent) diabetes mellitus [1]. The molecular mechanisms responsible, for peripheral insulin resistance in humans are not known, but are likely to result from polygenetic lesions expressed in central and peripheral tissues [2]. Since many of the currently available insulin resistant and diabetic animal models arise from monogenetic lesions, molecular mechanisms leading to insulin resistance in humans are likely to be different. In obese humans, insulin resistance at the level of glucose transport is a characteristic defect in adipose tissue [3] and skeletal muscle [4, 5, 6]. Exercise and low calorie diet constitute the main components of therapeutic strategies to reduce obesity [7]. In adipose tissue, insulin resistance is further aggravated during a very low calorie diet (VLCD) [8]. The molecular mechanism for this finding is not known.

Received: 8 February 2002 / Revised: 5 April 2002
Published online: 22 June 2002

Corresponding author: J. R. Zierath, MD, Karolinska Institutet, Integrative Physiology, von Eulers väg 4, II, S-171 77 Stockholm, Sweden. E-mail: Juleen.Zierath@fyfa.Ki.se
Abbreviations: IR, Insulin receptor; IRS, insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; VLCD, very low calorie diet; PMSF, phenylmethylsulfonyl fluoride; ECL, enhanced chemiluminescence; TBST, Tris-buffered saline with tween-20.

Thus, a greater understanding of molecular defects contributing to impaired insulin signal transduction and glucose transport in insulin responsive cells, such as adipocytes, is of importance to identify relevant targets for therapeutic strategies to enhance insulin action.

Phosphorylation of a family of insulin receptor substrates (IRS) constitutes one of the earliest detectable post-receptor events mediating signal transduction to metabolic and mitogenic pathways in insulin sensitive tissues. IRS-1 plays a prominent role in insulin signalling to PI 3-kinase [9] and defects have been noted in this target in skeletal muscle [10, 11] and adipocytes [12] from Type II diabetic patients. PI 3-kinase plays an essential role in mediating insulin-stimulated glucose transport in skeletal muscle and adipose tissue [13]. Thus, glucose transport defects could be due to impaired signal transduction. Defects in IRS-1 signalling to PI 3-kinase have been noted in skeletal muscle from insulin resistant obese humans [14] and are likely to be present in adipose tissue; however, the latter has yet to be ascertained. In adipose tissue [12], but not skeletal muscle [11, 15], IRS-2 seems to partially compensate for impaired insulin action on IRS-1, as shown by normal IRS-2-associated PI 3-kinase. Little is known of the role of IRS proteins in the regulation of PI 3-kinase activity in adipocytes from obese human subjects.

We assess whether defects in insulin signal transduction contribute to the reduced glucose transport observed in isolated adipocytes from obese humans. A very low calorie diet is associated with further deterioration of insulin action on glucose transport in adipose tissue from obese subjects [8, 16]. Effects of obesity and VLCD on insulin signal transduction at the level of IRS-1, IRS-2, PI 3-kinase and glucose incorporation into lipid in isolated subcutaneous adipocytes from obese women was studied. Finally, we ascertained whether changes in expression of key proteins involved in insulin signal transduction to glucose transport account for aberrant phosphorylation and activity of key targets involved in the insulin-signalling pathway in human adipocytes.

Subjects and methods

Subject characteristics and dietary intervention. The study groups consisted of six obese (29–56 years; BMI 36.2–43.1 kg/m^2) and six lean (26–50 years; BMI 21.7–25.7 kg/m^2) women. All subjects were Caucasian and born in Sweden. None of the women were on regular medication or had any known metabolic disorder (besides obesity). Subjects had maintained diet and exercise habits during the six previous months. The subjects gave their informed consent and the Ethics Committee at Huddinge University Hospital approved the study.

After an overnight fast, the subjects underwent a biopsy of subcutaneous paraumbilical fat (~4 g) under local anaesthesia [8]. The obese women were investigated twice and thus adipose tissue was obtained from the left and right side in a random manner. Before removing the biopsy, a venous blood sample was obtained for analysis of plasma glucose (by the routine chemistry laboratory at Huddinge Hospital) and insulin by a radioimmuno-assay kit (Amersham Pharmacia, Uppsala, Sweden).

Each obese patient was studied twice, before and after a hypocaloric protein-rich diet (Nutrilett, Nycomed Pharma, Oslo, Norway) (2.2 MJ; 8 g fat, 60 g protein, and 50 g carbohydrate per day). The VLCD period was scheduled for 10 to 13 days (mean duration 11 days). Patients rendered daily urine samples for confirmation of ketonuria, as assessed by a dipslide method (N. Labstic, Bayer Sweden, Gothenburg, Sweden). Subjects were admitted to the Research Centre in the morning (8:00) after an overnight fast, and were instructed to drink only small amounts of water from 22:00 the day before the study.

Isolation of adipocytes and assessment of cell size and number. Adipocytes were isolated and cell size and number assessed as described [8, 17]. Adipose tissue was transported immediately to the laboratory and isolated adipocytes were prepared by collagenase treatment. Direct microscopic assessment of adipocyte diameter was carried out using 200 cells and adipocyte volume and weight were calculated. Total lipid content was assessed gravimetrically after organic extraction. The number of adipocytes was calculated by dividing total lipid weight by mean cell weight.

Methodological validation of protein concentration in adipocyte extracts. Samples of isolated subcutaneous adipocytes from eight obese patients were selected from a large-cohort of women participating in a study of the genetic control of human adipocyte metabolism. Adipose tissue was obtained and prepared exactly as for the previous study group described above. Age ranged from 39 to 47 years and BMI from 30 to 40 kg/m^2 in this sub-group. The women were selected on the basis of either large adipocytes (range 1017–1088 pl) or small adipocytes (range 555–887 pl). There were four patients in each group. Isolated packed adipocytes (200 µl) were sonicated on ice in 300 µl of extraction buffer [1% triton X-100, 50 mmol/l tris, pH 7.4, 1 mmol/l pervanadate (50:1 molar mixture of orthovanadate:H_2O_2), 1 mmol/l PMSF, 25 mmol/l benzamidine and 0.15 mol/l NaCl] and thereafter, centrifuged to remove the fat cake. The infranatant was used to assess the protein concentration using a commercial kit (Pierce, Rockford, Ill., USA). Results were expressed either as protein concentration in cell extracts or as the amount of extracted protein per 1000 cells.

Glucose transport (measured as lipogenesis). Direct assessment of glucose transport can be made using radiolabelled glucose analogues such as 3-*O*-methylglucose that cannot be metabolised once transported across the cell membrane [18]. We used an indirect method [16] to assess glucose transport that measures the incorporation of (3-^{3}H)glucose into adipocyte lipids [19]. This method requires less tissue than the direct method and results are comparable with studies using 3-*O*-methylglucose in human adipocytes [16, 20]. Isolated adipocytes were incubated at a concentration of 2% (vol/vol) in Krebs Ringer phosphate buffer (pH 7.4) containing albumin (40 mg/ml), (3-^{3}H)glucose (5×10^6 dpm/ml), unlabelled glucose (1 µmol/l), and human insulin in different concentrations (0–70 nmol/l). Incubations were conducted for 2 h at 37°C using air as the gas phase. Incubations were stopped by rapidly chilling the incubation vials to 4°C. Incorporation of radiolabelled glucose into adipocyte lipids was assessed [16]. Incorporation of radiolabelled glucose into lipids (i.e., lipogenesis) reflects glucose transport because at micromolar glucose con-

centrations, glucose transport is the rate-limiting step for lipogenesis in human adiocytes [16]. Lipogenesis was expressed as the amount of glucose incorporated per adipocyte number [16]. Insulin concentration-response curves were analysed as follows: lipogenesis at maximum effective concentration was assessed (responsiveness) and half maximum effective concentration (EC_{50}) was calculated by log-logit transformation of concentration-response curves. EC_{50} was converted to the negative logarithm of mol/l (=pD_2), which reflects insulin sensitivity.

IRS-1 tyrosine phosphorylation. Adipocytes were incubated [21]. An aliquot (2 ml) of adipocytes (15% cell suspension) was incubated for 10 min at 37°C in the absence or presence of 3 or 1000 nmol/l insulin. The incubation was terminated by rapid centrifugation of cells and removal of the infranatant below the fat cake. Extraction buffer was added to the fat sample. After vigorous mixing, fat was removed by centrifugation. Protein content was assessed in the supernatant. Cell extracts (800 µg) were immunoprecipitated with IRS-1 antibody. The immune complex was washed as indicated [14]. Samples were re-suspended in Laemmli buffer with β-mercaptoethanol and heated (95°C) for 5 min. Proteins were separated by SDS-PAGE (6% resolving gel), transferred to nitrocellulose membrane and blocked in 5% milk in tris-buffered saline with 0.1% tween-20 (TBST). Membranes were incubated with phosphotyrosine antibody. Phosphorylated proteins were visualised by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, Ill., USA) and quantified by densitometry using Molecular Analyst software (Bio-Rad, Richmond, Calif., USA).

PI 3-kinase activity. An aliquot of the cell extract was immunoprecipitated overnight (4°C) with either IRS-1 or IRS-2 antibody coupled to protein A-sepharose (Amersham Pharmacia). PI 3-kinase activity was assessed [22]. Reaction products were resolved by thin layer chromatography and quantified using a PhosphorImager (Bio-Rad).

Western blot analysis. For protein expression of IR, IRS-1, p85α and GLUT4 an aliquot (40 µg) of cell extract was re-suspended in Laemmli buffer with β-mercaptoethanol and heated (95°C) for 5 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and blocked in 5% milk. Membranes were incubated with appropriate primary antibodies. Membranes were washed and incubated with appropriate secondary antibodies as recommended by the supplier (Bio-Rad). Proteins were visualised by ECL and quantified by densitometry as indicated above.

Materials and antibodies. PI was from Avanti Polar Lipids (Alabaster, Ala., USA), and aluminium backed Silica Gel 60 thin-layer chromatography plats were from EM Separations (Gibbstown, N.J., USA). All other chemicals were purchased from Sigma (St. Louis, Mo., USA) or Merck (Rahway, N.J., USA). The insulin receptor antibody (CT-3) was from Dr. K. Siddle (Cambridge University, UK). The phosphotyrosine antibody (RC20) was from Transduction Laboratories (Lexington, Ky., USA). IRS-1 (JD159) and IRS-2 (JD110) antibodies were generously provided by Dr. M. G. Myers Jr. and Dr. M. F. White (Joslin Diabetes Center, Boston, Mass., USA). The p85α antibody was a generous gift from Dr. J. M. Backer (Albert Einstein College of Medicine, Bronx, N.Y., USA). The GLUT4 polyclonal antibody was from Biogenesis (Poole, UK).

Statistics. Data are shown as mean ± SE. Statistical difference was ascertained by a paired (obese women before vs after VLCD) or an unpaired (obese before VLCD vs lean women) Student's *t* test. Statistical significance was accepted at a value of less than 0.05.

Results

Subject characteristics. Subject characteristics are shown (Table 1). Obese women were studied before and after 11 days of VLCD. Weight loss ranged from 2.4 to 7.2 kg ($p<0.01$). All women had ketonuria at the second investigation. Lean women on an isocaloric diet served as control subjects. Obese women were normoglycaemic, but hyperinsulinaemic compared to lean subjects ($p<0.05$). A very low calorie diet normalised insulin concentration in obese women ($p<0.01$). Glucose concentration was also improved after VLCD ($p<0.05$). Adipocyte volume was two-fold ($p<0.01$) greater in obese women and was not altered by VLCD.

Protein concentration in adipocyte extracts. As a methodological consideration, we ascertained whether there was a difference in large versus small adipocytes with regard to (i) protein concentration in the cell extract and (ii) protein content per cell. For this purpose we selected obese women who either had a large or a small adipocyte volume. Adipocyte volume was greater in patients with large versus small cells (1055±17 vs 688±81 pl, respectively; $p=0.005$). Thus, there was a 35% difference in adipocyte volume between the two groups. Protein per cell was greater in large versus small adipocytes (1.28±0.07 vs 0.87±0.006 µg protein per 1000 cells, respectively; $p=0.006$). However, total protein concentration in extracted adipocytes did not differ between large versus small cells

Table 1. Subject characteristics

	Obese before VLCD	Obese after VLCD	Lean
n	6	6	6
Age (years)	42.3±5.5		41.0±3.6
BMI (kg/m^2)	39.3±1.2	37.6±1.1[d]	24.0±0.5
f-Glucose (mmol/l)	5.6±0.6	4.2±0.3[c]	4.8±0.1
f-Insulin (pmol/l)	88.2±17.4[a]	28.2±6.0[d]	34.8±10.2
Fat cell volume (pl)	917±63[b]	864±52	542±75
Lipogenesis (nmol×2 h^{-1}×10^7 cells^{-1})			
Basal	1.9±0.5	0.6± 0.2[c]	2.1±0.6
Maximal insulin	4.0±1.1	1.6±0.3[c]	4.7±0.9
Insulin pD_2	12.5±0.3[a]	12.3 ±0.6	13.8±0.3

[a] $p<0.05$, [b] $p<0.01$ for comparisons between lean and obese subjects before VLCD and [c] $p<0.05$, [d] $p<0.01$ for obese subjects before versus after VLCD

Values are means ± SE. f refers to fasting values. Lean were compared with obese before VLCD and obese were compared before and after VLCD

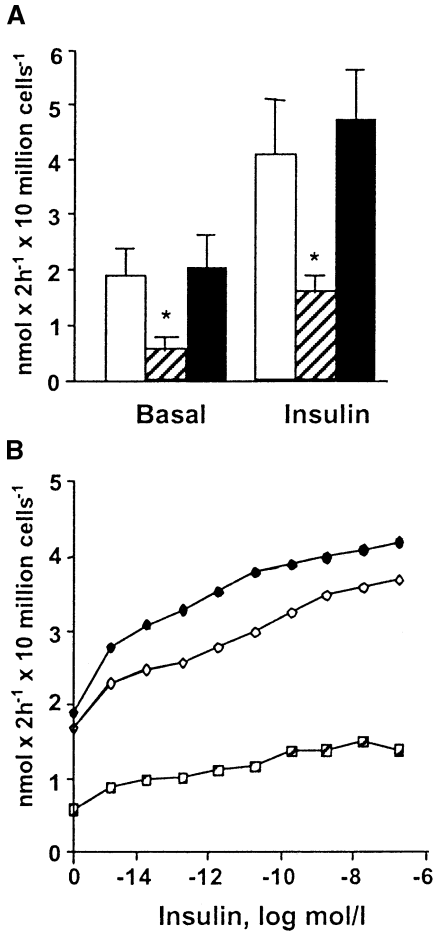

Fig. 1A, B. A Glucose transport (measured as lipogenesis) under basal and insulin-stimulated conditions measured in lean subjects (*closed bars*) and obese patients before (*open bars*) and after (*hatched bars*) VLCD. Data are means ± SE for n=6. *p<0.05, significantly different from obese patients before diet. **B** Mean insulin concentration-response curves for glucose transport lean subjects (*closed circles*) and obese patients before (*open circles*) and after (*hatched rectangles*) VLCD

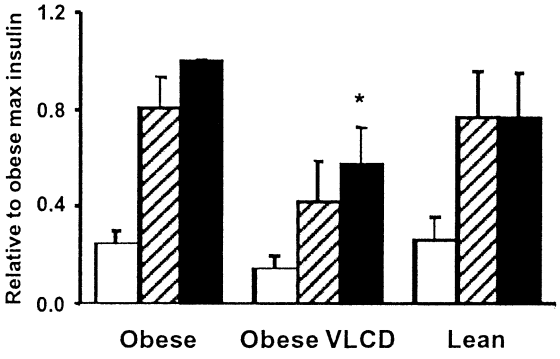

Fig. 2. IRS-1 tyrosine phosphorylation. Isolated adipocytes were incubated in the absence (*open bars*) or presence of 3 (*hatched bars*) or 1000 nmol/l (*closed bars*) insulin for 10 min. Tyrosine phosphorylation was assessed in anti-IRS-1 immunoprecipitates. Data are expressed as means ± SE for n=3–6 subjects, relative to obese maximal insulin-stimulation. *p<0.05, significantly different from insulin-stimulated (1000 nmol/l) in obese patients before diet

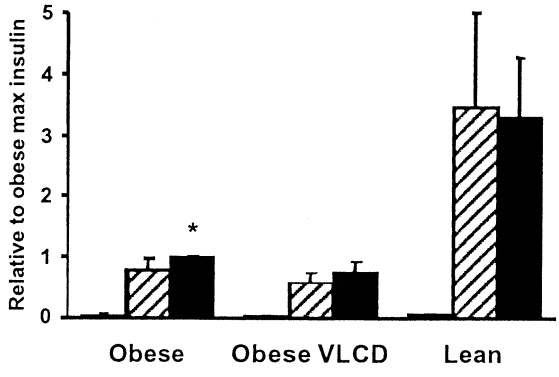

Fig. 3. IRS-1 associated PI 3-kinase activity. Isolated adipocytes were incubated as described in Fig. 2. PI 3-kinase activity was assessed after immunoprecipitation with IRS-1. Data are expressed as means ± SE for n=4–6 subjects, relative to obese maximal insulin-stimulation. *p<0.05, significantly different from insulin-stimulated activity (1000 nmol/l) in lean subjects

(0.81±0.05 vs 0.86±0.05 µg protein per µl extract for large vs small cells; p=0.52).

Glucose transport. Insulin stimulated lipogenesis (under present conditions equivalent to glucose transport) in a concentration dependent fashion in all three groups (Fig. 1). In obese women, the concentration-response curve was shifted markedly to the right as compared with the lean subjects. After a VLCD, basal and maximal insulin-stimulated lipogenesis was markedly blunted. Individual values for insulin pD_2 and basal or maximal insulin-stimulated lipogenesis are shown (Table 1). In obese compared with lean subjects, rates of basal and maximal insulin-stimulated lipogenesis were similar, but pD_2 was slightly reduced (p<0.05). After a VLCD, basal and maximal insulin-stimulated lipogenesis in obese patients was reduced 68 and 60% respectively (p<0.05). However, pD_2 was similar before and after VLCD, indicating no change in insulin sensitivity.

IRS-1 tyrosine phosphorylation. Tyrosine phosphorylation of IRS-1 was ascertained by western blot analysis of IRS-1 immunoprecipitates, using a phosphotyrosine antibody. Isolated adipocytes were incubated for 10 min in the absence or presence of 3 or 1000 nmol/l insulin. Insulin-mediated IRS-1 tyrosine phosphorylation in isolated adipocytes was similar between lean and obese women (Fig. 2). In adipocytes from obese women, a decreased (43%) maximal (1000 nmol/l) insulin-stimulated IRS-1 tyrosine phosphorylation was observed after VLCD (p<0.05, before vs after VLCD).

IRS-1 associated PI 3-kinase activity. In adipocytes from lean women, insulin (1000 nmol/l) increased IRS-1 associated PI 3-kinase activity (p<0.001), with similar effects noted in the presence of 3 and 1000 nmol/l insulin (Fig. 3). However, insulin-stimulated IRS-1 associated PI 3-kinase activity was blunt-

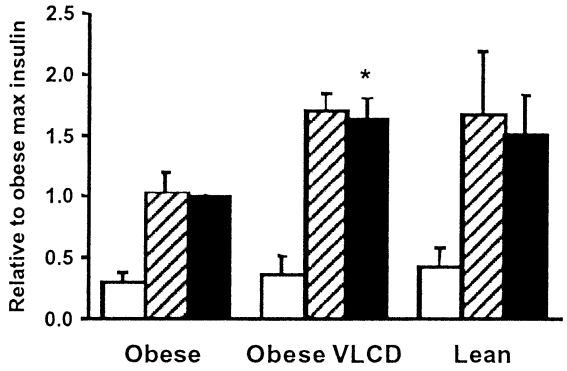

Fig. 4. IRS-2 associated PI 3-kinase activity. Isolated adipocytes were incubated as described in Fig 3. PI 3-kinase activity was assessed after immunoprecipitation with IRS-2. Data are expressed as means ± SE for n=3–6 subjects, relative to obese maximal insulin-stimulation. *p<0.05, significantly different from insulin-stimulated (1000 nmol/l) adipocytes from obese patients before diet

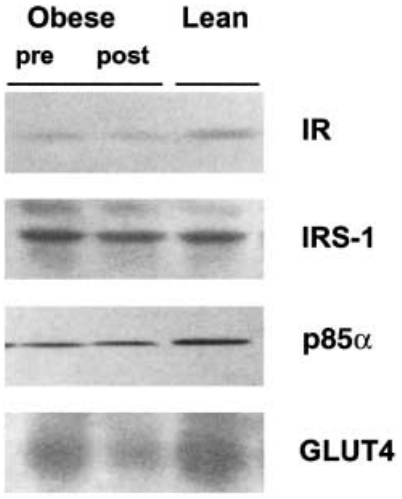

Fig. 5. Representative western blots showing insulin signalling protein expression in adipocytes from obese patients (pre and post VLCD) and lean subjects

ed (70%) in isolated adipocytes from obese women (p<0.05). IRS-1 associated PI 3-kinase was reduced in obese women, despite an apparently normal insulin-stimulated IRS-1 tyrosine phosphorylation. Thus, a clear divergence between insulin action on IRS-1 tyrosine phosphorylation and IRS-1 associated PI 3-kinase activity was noted in obese women. Insulin-stimulated IRS-1 associated PI 3-kinase was not altered after VLCD in obese women. Since large fat cells from the obese patients contained more protein per cell than the small fat cells from the lean subjects, theoretically the extent of the insulin signalling defect noted in adipocytes from obese patients could be slightly overestimated if compared on a per cell basis. However, per gram of total protein, the results suggest that there is a defect in insulin signalling in adipocytes from obese patients.

IRS-2 associated PI 3-kinase activity. Insulin (1000 nmol/l) increased IRS-2 associated PI 3-kinase activity by 3.5-fold in isolated adipocytes from lean subjects (p<0.01) (Fig. 4). In adipocytes from obese women, insulin action on IRS-2 associated PI 3-kinase activity was normal, despite impaired IRS-1 associated PI 3-kinase activity. After a VLCD, insulin-stimulated (1000 nmol/l) IRS-2 associated PI 3-kinase activity was increased (p<0.05, vs before VLCD).

Protein expression. Protein expression of IR, IRS-1, p85α subunit of PI 3-kinase and GLUT4 was ascertained and representative immunoblots (Fig. 5) and arbitrary densitometric units are shown (Table 2). Protein expression of the IR β-subunit was similar between lean and obese subjects, and was reduced 19% (p<0.01) in obese patients after VLCD. IRS-1 protein expression was similar between lean and obese women, and tended to be decreased in obese patients after VLCD (p=0.09). In contrast to our findings for IR and IRS-1, protein expression of the p85α-subunit of PI 3-kinase was reduced by 55% in obese women (p<0.05), with no further change after VLCD. GLUT4 protein expression was also reduced (37%) in obese as compared with lean women (p<0.05), with a further reduction noted after VLCD (26% reduction vs before VLCD, p<0.05).

Table 2. Effect of obesity or VLCD on insulin signalling protein expression in isolated adipocytes

	Obese before VLCD	Obese after VLCD	Lean
IRβ	4.3±0.9	3.5±0.8[c]	5.3±0.8
IRS-1	4.8±1.3	3.7±1.0	3.8±0.9
p85α	5.3±1.6[a]	4.6±1.3	11.8±2.1
GLUT4	19.1±4.3[a]	14.2±3.6[b]	30.3±2.7

[a] p<0.05 for comparisons between lean and obese subjects before VLCD and [b]p<0.05, [c]p<0.01 for obese subjects before versus after VLCD

Data are expressed as arbitrary units (mean ± SE). n=6 in all groups. Lean were compared with obese before VLCD and obese were compared before and after VLCD. Representative western blot are shown in Fig. 5

Discussion

The molecular defects leading to impaired insulin action in isolated adipocytes from obese insulin resistant humans have not been fully elucidated. Understanding the regulation of pathways that mediate insulin action could lead to the identification of molecular targets for therapy to improve insulin sensitivity. Previous studies provide evidence that initial signalling events related to insulin receptor tyrosine kinase activity are

normal in adipocytes from obese insulin-resistant patients [23]. Only a fraction of total insulin receptors expressed in the cell are required to be occupied to achieve full activation of glucose transport in adipocytes. Thus, a decrease in insulin sensitivity is likely to be due to alterations in early post-receptor signalling events, whereas a decrease in insulin responsiveness can also be attributed to downstream signalling events at or near GLUT4 [24].

We ascertained whether early and intermediate post-receptor signal events to glucose transport are altered in isolated adipocytes from obese women. We confirm earlier findings of a decrease in insulin sensitivity of glucose transport and reduced GLUT4 expression in isolated adipocytes from obese humans [3, 24]. However, the maximal effect of insulin on glucose transport in isolated adipocytes was not different between lean and obese subjects. This finding is physiologically relevant and suggests that a higher insulin concentration is needed to obtain a similar rate of glucose transport in adipocytes from obese patients as compared to lean subjects. However, when the insulin concentration is sufficiently high, the hormone sensitivity defect is overcome and equal glucose transport rates are achieved. Future studies whereby glucose transport rather than lipogenesis (as measured in our study) is directly assessed in vivo could definitively address whether changes in insulin responsiveness contribute to the glucose transport defects in human adipose tissue. Insulin signalling was determined in isolated adipocytes from lean and obese women. While insulin-stimulated IRS-1 tyrosine phosphorylation was normal, IRS-1 associated PI 3-kinase activity was impaired in isolated adipocytes from obese women. Impaired signalling to PI 3-kinase was selective for IRS-1, since IRS-2 associated PI 3-kinase activity was normal. Thus, glucose transport defects in isolated adipocytes from obese women occur from impaired insulin signal transduction from IRS-1 to PI 3-kinase, as well as from decreased GLUT4 protein expression.

One of several proteins that dock to phosphorylated IRS's is the p85α regulatory subunit of PI 3-kinase, which through this association, increases the activity of PI 3-kinase [9]. The p85α (*grb-1*) gene encodes three splice variants, p85α, p55α and p50α, of which only p85α and p50α are reported to be expressed in human adipose tissue [25]. Mice with a targeted disruption of the gene encoding the p85α regulatory subunit of PI 3-kinase show increased insulin sensitivity and hypoglycaemia, due to increased glucose uptake in skeletal muscle and adipocytes via signalling through the p50α alternative splice variant of the p85α gene [26]. The mRNA expression of p85α has been reported to be similar in adipose tissue from lean versus obese humans [27]. However in our study, protein expression of the p85α regulatory subunit of PI 3-kinase was reduced by 55% in adipocytes from obese women. Based on the findings in p85α knockout mice [26] and p85α heterozygous mice [28], increased insulin action on glucose transport or increased whole-body insulin sensitivity could be predicted when p85α expression is reduced. However we noted reduced insulin-stimulated glucose transport (measured as lipogenesis) in isolated adipocytes from obese women. Thus, defects in insulin signalling to PI 3-kinase in insulin resistant adipocytes from obese women are likely to be related to a weaker association of IRS-1 to PI 3-kinase, rather than to changes in IRS-1 protein expression or from changes in protein expression of the p85α regulatory subunit or splice variants of PI 3-kinase. This could arise from increased serine/threonine phosphorylation of IRS-1; however this has yet to be addressed in human adipocytes. Importantly, after a VLCD a greater impairment in glucose transport was observed, occurring independent of additional insulin signalling defects at the level of PI 3-kinase, suggesting that other factors downstream from PI 3-kinase contribute to the further impaired insulin action on glucose transport after VLCD.

Defects in PI 3-kinase activity in isolated adipocytes from obese women are selective for IRS-1, since IRS-2 associated PI 3-kinase was normal. Due to insufficient sample size, we were not able to assess IRS-2 tyrosine phosphorylation. However, our results provide evidence that insulin action on IRS-2 in human adipocytes is not influenced by obesity. Discordance between insulin action on IRS-1 and IRS-2 associated PI 3-kinase activity has also been observed in isolated adipocytes from obese people with Type II diabetes, such that IRS-2, rather than IRS-1 associated PI 3-kinase is normal [12]. Thus in adipocytes [12], but not skeletal muscle [11, 15], IRS-2 seems to be the major docking protein for PI 3-kinase activation; however the increased IRS-2 associated PI 3-kinase does not rescue glucose transport as additional defects were noted after a VLCD. In contrast to our findings in obese non-diabetic subjects, in obese Type II diabetic patients, IRS-1 protein expression and insulin-stimulated IRS-1 tyrosine phosphorylation are also reduced [12]. Furthermore, low cellular IRS-1 protein expression in adipocytes is directly associated with insulin resistance and Type II diabetes, but not obesity [29]. These studies highlight important differences in the regulation of IRS-1 in obesity versus Type II diabetes.

VLCD, a common treatment of obesity, does not influence insulin-stimulated total body glucose uptake as measured by hyperinsulinaemic clamp studies in obese women [30]. However, since this approach largely shows glucose uptake in skeletal muscle, the influence of a VLCD on insulin action in adipocytes cannot be ascertained. We have shown [8] that a VLCD is associated with a marked reduction in basal and maximal insulin-stimulated glucose transport (measured as lipogenesis), with no change in insulin sensitivity in isolated adipocytes from obese women.

These findings suggest that there is a spare signalling capacity leading to the activation of glucose transport, and further indicate the defects distal to PI 3-kinase could have a greater contribution to the impaired insulin action in adipocytes after a VLCD. This hypothesis is further supported by the observation that IRS-1 and IRS-2 associated PI 3-kinase activity were not further impaired after a VLCD, whereas glucose transport defects were more profound.

Evidence to support a defect distal to PI 3-kinase comes from our observation of a further reduction in GLUT4 protein expression after a VLCD. The importance of total GLUT4 expression in the regulation of glucose transport has been shown using knock-out mice. Adipose selective targeting of the GLUT4 gene markedly impaired insulin-stimulated glucose uptake compared to wild-type adipocytes, providing definitive evidence that adipocyte-specific depletion of GLUT4 causes insulin resistance in adipocytes [31]. Thus, reduced GLUT4 protein expression, as well as impaired insulin signalling, contributes to glucose transport defects in adipocytes from obese women.

In summary, insulin sensitivity of glucose transport (measured as lipogenesis) in adipocytes is decreased in obese women, partly due to impaired IRS-1, rather than IRS-2 signalling to PI 3-kinase. Thus, IRS-2 seems to be an important mediator of insulin signalling to PI 3-kinase in insulin resistant adipocytes, but it does not rescue glucose transport. These defects in insulin action are not limited to impaired insulin signalling, since changes in GLUT4 protein were also observed. Importantly, VLCD in obese women leads to a further decrease in GLUT4 protein expression and a decrease in insulin-stimulated glucose transport. This suggests defects distal to PI 3-kinase, including reductions in GLUT4 protein expression, contribute to the impaired insulin action in adipocytes after VLCD.

Acknowledgements. This study was supported by grants from the Swedish Medical Research Council, Novo Nordisk Foundation, Swedish Diabetes Association, Marcus and Amelia Wallenberg Foundation, a Junior Individual Grant and a Project Grant from the Foundation for Strategic Research Council.

References

1. Kahn CR (1994) Banting lecture. Insulin action, diabetogenes, and the cause of type II diabetes. Diabetes 43: 1066–1084
2. Froguel P, Velho G (2001) Genetic determinants of type 2 diabetes. Recent Prog Horm Res 56:91–105
3. Caro JF, Dohm LG, Pories WJ, Sinha MK (1989) Cellular alterations in liver, skeletal muscle, and adipose tissue responsible for insulin resistance in obesity and type II diabetes. Diabetes Metab Rev 5:665–689
4. Andréasson K, Galuska D, Thörne A, Sonnenfeld T, Wallberg-Henriksson H (1991) Decreased insulin-stimulated 3-O-methylglucose transport in in vitro incubated muscle strips from type II diabetic subjects. Acta Physiol Scand 142:255–260
5. Dohm GL, Tapscott EB, Pories WJ et al. (1988) An in vitro human skeletal muscle preparation suitable for metabolic studies. Decreased insulin stimulation of glucose transport in muscle from morbidly obese and diabetic subjects. J Clin Invest 82:486–494
6. Galuska D, Nolte L, Zierath JR, Wallberg-Henriksson H (1994) Effect of metformin on glucose transport in isolated skeletal muscle obtained from Type II diabetic patients and healthy individuals. Diabetologia 37:872–879
7. Zierath JR, Wallberg-Henriksson H (1992) Exercise training in obese diabetic patients. Sports Med 14:171–189
8. Hellström L, Reynisdottir S, Langin D, Rössner S, Arner P (1996) Regulation of lipolysis in fat cells of obese women during long-term hypocaloric diet. Int J Obes Relat Metab Disord 20:745–752
9. White MF (1998) The insulin signalling system: A network of docking protein that mediate insulin action. Mol Cell Biochem 182:3–11
10. Björnholm M, Kawano Y, Lehtihet M, Zierath JR (1997) Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation. Diabetes 46:524–527
11. Krook A, Björnholm M, Galuska D et al. (2000) Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. Diabetes 49:284–292
12. Rondinone CM, Wang LM, Lönnroth P, Wesslau C, Pierce JH, Smith U (1997) Insulin receptor substrate (IRS) is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. Proc Natl Acad Sci USA 94:4171–4175
13. Virkamäki A, Ueki K, Kahn CR (1999) Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. J Clin Invest 103:931–943
14. Goodyear LJ, Giorgino F, Sherman LA, Carey J, Smith RJ, Dohm GL (1995) Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. J Clin Invest 95:2195–2204
15. Kim YB, Nikoulina SE, Ciaraldi TP, Henry RR, Kahn BB (1999) Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. J Clin Invest 104:733–741
16. Arner P, Engfeldt P (1987) Fasting mediated alteration studies of insulin action on lipolysis and lipogenesis in obese women. Am J Physiol 253:193–201
17. Rodbell M (1964) Metabolism of isolated cells. I. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem 239:375–380
18. Whitesell RR, Glieman J (1979) Kinetic parameters of transport of 3-O-methyl glucose in adipocytes. J Biol Chem 254:5276–5283
19. Moody AJ, Stan MA, Stan M, Glieman J (1974) A simple free fat cell bioassay for insulin. Horm Metab Res 6:12–16
20. Nordenström J, Sonnenfeld T, Arner P (1989) Characterization of insulin resistance after surgery. Surgery 1:28–35
21. Zierath JR, Livingston JN, Thörne A et al. (1998) Regional differences in insulin inhibition of non-esterified fatty acid release from human adipocytes: Relation to insulin receptor phosphorylation and intracellular signaling through the insulin receptor substrate-1 pathway. Diabetologia 4:1343–1354

22. Krook A, Whitehead JP, Dobson SP et al. (1997) Two naturally occurring insulin receptor tyrosine kinase domain mutants provide evidence that phophatidylinositol 3-kinase activation alone is not sufficient for the mediation of insulin's metabolic and mitogenic effects. J Biol Chem 272:30208–30214
23. Freidenberg GR, Reichart D, Olefsky JM, Henry RR (1988) Reversibility of defective adipocyte insulin receptor kinase activity in non-insulin-dependent diabetes mellitus. Effect of weight loss. J Clin Invest 82:1398–1406
24. Garvey WT, Maianu L, Hueckstead TP, Birnbaum MJ, Molina J, Ciaraldi TP (1991) Pretranslational suppression of a glucose transporter protein causes insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity. J Clin Invest 87:1072–1081
25. Lefai E, Roques M, Vega N, Laville M, Vidal H (2001) Expression of the splice variants of the p85α regulatory subunit of phosphoinositide 3-kinase in muscle and adipose tissue of healthy subjects and type 2 diabetic patients. Biochem J 360:117–125
26. Terauchi Y, Tsuji Y, Satoh S et al. (1999) Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 alpha subunit of phosphoinositide 3-kinase. Nat Genet 21:230–235
27. Andreelli F, Laville M, Ducluzeau P-H et al. (1999) Defective regulation of phosphoinositide 3-kinase gene expression in skeletal muscle and adipose tissue of non-insulin-dependent diabetes mellitus patients. Diabetologia 42:358–364
28. Mauvais-Jarvis F, Ueki K, Fruman DA et al. (2002) Reduced expression of the murine p85α subunit of phosphoinositide 3-kinase improves insulin signaling and ameliorates diabetes. J Clin Invest 109:141–149
29. Carvalho E, Jansson PA, Axelsen M et al. (1999) Low cellular IRS 1 gene and protein expression predict insulin resistance and NIDDM. FASEB J 13:2173–2178
30. Hagström-Toft E, Thörne A, Reynisdottir S et al. (2001) Evidence for a major role of skeletal muscle lipolysis in the regulation of lipid oxidation during caloric restriction in vivo. Diabetes 50:1604–1611
31. Abel ED, Peroni O, Kim JK et al. (2001) Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. Nature 409:729–733