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Insulin resistance in human adipocytes occurs downstream of IRS1 after surgical cell isolation but at the level of phosphorylation of IRS1 in type 2 diabetes

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Insulin resistance is a cardinal feature of type 2 diabetes and also a consequence of trauma such as surgery. Directly after surgery and cell isolation, adipocytes were insulin resistant, but this was reversed after overnight incubation in 10% CO_2 at 37 °C. Tyrosine phosphorylation of the insulin receptor and insulin receptor substrate (IRS)1 was insulin sensitive, but protein kinase B (PKB) and downstream metabolic effects exhibited insulin resistance that was reversed by overnight incubation. MAP-kinases ERK1/2 and p38 were strongly phosphorylated after surgery, but was dephosphorylated during reversal of insulin resistance. Phosphorylation of MAP-kinase was not caused by collagenase treatment during cell isolation and was present also in tissue pieces that were not subjected to cell isolation procedures. The insulin resistance directly after surgery and cell isolation was different from insulin resistance of type 2 diabetes; adipocytes from patients with type 2 diabetes remained insulin resistant after overnight incubation. IRS1, PKB, and downstream metabolic effects, but not insulin-stimulated tyrosine phosphorylation of insulin receptor, exhibited insulin resistance. These findings suggest a new approach in the study of surgery-induced insulin resistance and indicate that human adipocytes should recover after surgical procedures for analysis of insulin signalling. Moreover, we pinpoint the signalling dysregulation in type 2 diabetes to be the insulin-stimulated phosphorylation of IRS1 in human adipocytes.

Insulin controls cell metabolism via metabolic signal transduction pathways and cell proliferation via mitogenic signal pathways. Metabolic signalling occurs through receptor-activated phosphorylation of insulin receptor substrate (IRS) proteins that subsequently activate phosphatidylinositide 3-kinase (PI3-kinase) to generate second messengers that produce increased phosphorylation and activation of protein kinase B/Akt (PKB). PKB appears to be central to downstream control of both glucose uptake and glycogen synthesis by insulin [1,2]. Although adipocytes are terminally differentiated cells that do not divide further, insulin has the potential for genomic control via a mitogenic signalling pathway. This may also be mediated by IRS; insulin activation of the G-protein Ras leads to phosphorylation and activation of mitogen-activated protein (MAP) kinases – extracellular signal-related kinase (ERK) 1 and 2 [3], and p38 [4,5] – protein kinases that phosphorylate and control the activity of other downstream protein kinases and

Abbreviations
ERK, extracellular signal-related kinase; GLUT4, insulin-sensitive glucose transporter-4; IRS, insulin receptor substrate; MAP, mitogen-activated protein; PKB, protein kinase B; PI3-kinase, phosphatidylinositide 3-kinase.

transcription factors. However, the MAP-kinase p38 together with the c-Jun NH_2-terminal kinases (JNK) are primarily activated in response to stress and cytokines [6].

Failure to properly respond to insulin – insulin resistance – is a prime characteristic of type 2 diabetes, but also of other related conditions such as obesity. Trauma, including surgical trauma, is also known to cause insulin resistance in man [7–10], which in turn may cause or aggravate tissue wasting following surgery. Even relatively uncomplicated abdominal surgery causes postoperative peripheral insulin resistance in both man and animals [8]. Attempts to examine this at cellular and molecular levels have yielded conflicting results. In isolated human fat cells obtained after, as compared to before, abdominal surgery (cholecystectomy) a reduction of insulin-stimulated glucose uptake and lipogenesis, by 35 and 50%, respectively, has been found [11]. The sensitivity to insulin – but not the maximal response – for glucose uptake in rat skeletal muscle was reduced when the tissue was obtained and analyzed after, as compared to before, abdominal (intestinal resection) surgery [12]. However, IRS1, PI3-kinase, and PKB were reported to be even more responsive to insulin after surgery [12]. Using the same animal model, these authors did not find any effect on insulin stimulation of glucose uptake in adipocytes by surgical trauma [13].

The insulin resistance in type 2 diabetes has been the subject of intensive research for many years. Yet, we don't know the details of the molecular dysregulation in the target cells of the hormone. Studies of cells from patients with the disease and nondiabetic subjects have demonstrated that mutations in the insulin receptor cannot explain the vast majority of cases of type 2 diabetes. Downstream defects in insulin receptor signalling to tyrosine phosphorylation of IRS1 has been reported for skeletal muscle [14–17]. Corresponding effects in human adipose tissue has not been reported, but lowered serine phosphorylation and impaired translocation of PKB to the plasma membrane has been described in adipocytes from type 2 diabetic patients [18]. A lowered expression of adipocyte IRS1 has, however, been described in some obese individuals and relatives of patients with diabetes [19]. Animal studies have also indicated a role for IRS1 in insulin resistance in adipose tissue (reviewed in [20,21]).

We aimed to compare the insulin resistance of surgical trauma with that in type 2 diabetes and to define, in some detail, the dysfunction in insulin signal transduction in these conditions. We demonstrate that adipocytes were insulin resistant when isolated from normal subjects, but that this insulin resistance could be reversed. The insulin resistance in cells from patients with type 2 diabetes, on the other hand, was not reversible.

Results

Non-diabetic control subjects

In adipocytes analyzed directly (within 4 h) after their excision during open abdominal surgery, MAP-kinases ERK1/2 and p38 proteins were highly phosphorylated and addition of insulin had no, or very little, effect on their extent of phosphorylation (Fig. 1A,B).

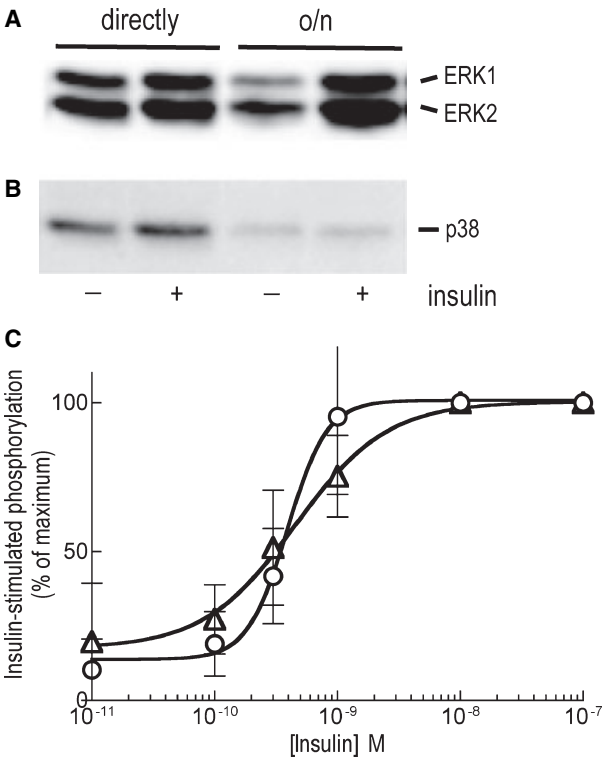

Fig. 1. Phosphorylation of MAP-kinases before and after overnight recovery; effects of insulin. (A, B) Human adipocytes, from control subjects, were incubated with 100 nM insulin for 10 min, directly or after overnight (o/n) recovery. Whole-cell lysates were subjected to SDS/PAGE and immunoblotting against phospho-ERK1/2 (A) or phospho-p38 (B). (C) Dose–response relationship for insulin stimulation of phosphorylation of ERK1 (○) and 2 (△). After overnight recovery cells were incubated with indicated concentration of insulin for 10 min. Mean ± SE, $n = 5$ subjects. In this and the following figures, the insulin-stimulated effect was obtained by setting the value with no insulin to 0% and that of 100 nM insulin to 100% effect. Dose–response curves were fitted to experimental data using the sigmoidal dose–response algorithm in GRAPHPAD Prism 4 software.

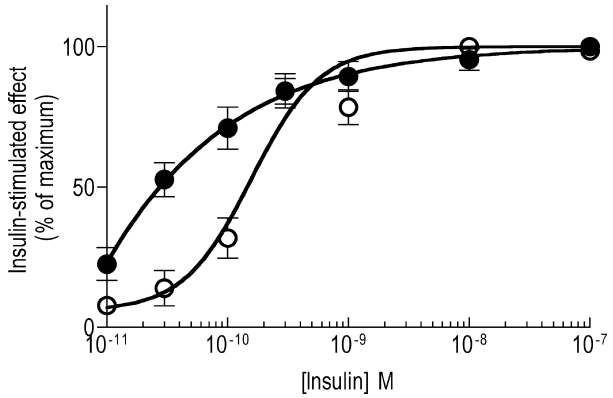

Fig. 2. Dose–response effect of insulin on glucose uptake by adipocytes before (○) and after (●) overnight recovery. Incubation of adipocytes, from control subjects, with insulin at indicated concentrations for 10 min. Glucose transport was determined as uptake of 2-deoxy-D-[1-^{3}H]glucose by the cells. Mean ± SE, $n = 8$ subjects. The dose–response curves were significantly different, $P < 0.05$.

When we analyzed the cells after overnight incubation (20 to 24 h), all three MAP-kinases exhibited lowered levels of phosphorylation (Fig. 1A,B). Insulin treatment now caused a significant increase in the phosphorylation of ERK 1 and 2 (Fig. 1A), but had no effect on the phosphorylation of p38 MAP-kinase (Fig. 1B). Half-maximal effects (EC_{50}) on the phosphorylation of both ERK 1 and 2 were at 0.3 nM insulin (Fig. 1C).

Directly after their isolation, adipocytes responded to insulin by increasing the uptake of 2-deoxyglucose. Neither the maximal effect of insulin on glucose uptake and hence the amount of GLUT4 (M. Karlsson, H. Wallberg-Henriksson, P. Strålfors, unpublished observations), nor basal glucose uptake was substantially affected by overnight incubation of the cells prior to analysis (not shown). Insulin stimulated, however, glucose transport at markedly lower concentrations after overnight incubation; EC_{50} was 0.1 to 0.2 nM insulin when analyzed directly and 0.02 to 0.03 nM after overnight recovery (Fig. 2 and Table 1). This increased sensitivity to insulin was similar in the subjects, irrespective of the maximal effect of insulin on the rate of glucose uptake, which in contrast was highly variable among the subjects and ranged from 19 to 214 nmol 2-deoxyglucose·min^{-1}·L^{-1} packed cell volume (126 ± 32, mean ± SE, $n = 8$) and was not affected by overnight incubation of the cells. Incubation for 48 h did not further increase (or decrease) the insulin sensitivity.

The insulin receptor, the immediate downstream signal mediator IRS1, and the further downstream PKB were not significantly phosphorylated under basal conditions in cells analyzed directly (Fig. 3), in contrast to the MAP-kinases (Fig. 1). A maximal insulin concentration (100 nM) caused an increased phosphorylation of all three proteins (Fig. 3). This pattern was not significantly changed by overnight incubation of the cells (Fig. 3). Maximal insulin-stimulated increase in tyrosine phosphorylation of the insulin receptor was 10.6 ± 2.3 and 9.6 ± 4.2-fold ($n = 5$) directly and after overnight incubation, respectively; of IRS1 10.3 ± 3.2 and 14.7 ± 7.3 -fold, respectively, and glucose uptake 3.9 ± 0.9 and 3.8 ± 0.8-fold, respectively. There was no significant difference when analyzed directly compared with after overnight incubation.

When the insulin-responsiveness of the cells was examined at different concentrations of insulin, we found that insulin enhanced the phosphorylation of PKB at lower concentrations after overnight recovery when compared to analysis the same day as the surgery (Fig. 4C and Table 1). The EC_{50} was reduced from about 1 nM to 0.4 nM. Moreover, after overnight recovery, the increased phosphorylation of PKB occurred over a more narrow range of insulin concentrations (Fig. 4C). In contrast, the sensitivity to insulin for insulin receptor or IRS1 phosphorylation was not affected by overnight incubation; EC_{50} was 1.4 nM and 0.6 nM insulin, respectively (Fig. 4A,B and Table 1).

Table 1. EC_{50} for insulin effects in human adipocytes. Adipocytes from nondiabetic subjects or patients with type 2 diabetes were analyzed directly or after an overnight (o/n) recovery period. The EC_{50} values, given in nM, were obtained from the dose–response curves in Figs 2,4, and 7.

	Subjects					
	Normal		Female diabetic		Male diabetic	
Analysis	Directly	o/n	Directly	o/n	Directly	o/n
Insulin receptor	1.1–1.8	1.1–1.8	1.1–1.8	1.1–1.8	1.1–1.8	1.1–1.8
IRS1	0.6–0.7	0.6–0.7	1.8–2.0	1.8–2.0	1.8–2.0	1.8–2.0
PKB	0.9–1.1	0.3–0.4	0.6–0.7	0.6–0.7	0.6–0.7	0.6–0.7
Glucose transport	0.1–0.2	0.02–0.03	0.1–0.2	0.1–0.2	0.1–0.2	0.1–0.2

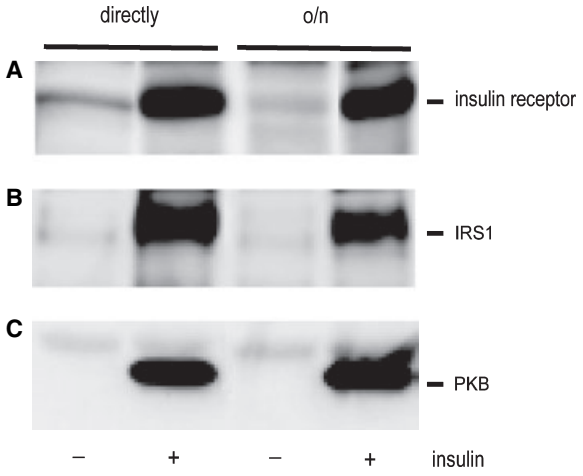

Fig. 3. Phosphorylation of insulin receptor, IRS1, and PKB before and after overnight recovery; maximal effects of insulin. Adipocytes from control subjects were incubated with 100 nM insulin for 10 min, either directly or after overnight (o/n) recovery. Whole-cell lysates were subjected to SDS/PAGE and immunoblotting against phospho-tyrosine (A,B), or phospho-PKB (C).

The overnight incubation could have selected for small and sturdy cells that might be more insulin-responsive. We found, however, that the mean fat cell diameter was similar before and after overnight incubation: 94 ± 2.0 μm and 93 ± 1.4 μm (mean ± SE, $n = 3$ subjects), respectively.

The effect of insulin on the insulin receptor and downstream effectors IRS1 and PKB, eventually leading to enhanced glucose transport, appeared at successively lower concentrations of insulin, when the cells were analyzed after overnight recovery (Fig. 5). It was striking that the phosphorylation of PKB occurred over a very narrow range of insulin concentrations compared with the effect of insulin on the insulin receptor, IRS1, or glucose transport, which were all affected over a similar range of insulin concentrations (Fig. 5).

The fat tissues in these experiments were obtained during surgery and general anaesthesia. We therefore compared these with subcutaneous adipocytes from tissue obtained by a small incision in the abdominal skin under local anaesthesia. Also, in these cases ERK1/2 were phosphorylated and insulin had no further effect when analyzed directly (Fig. 6A), but when analyzed after overnight incubation ERK1/2 were dephosphorylated and now responded to insulin stimulation (2.3/2.3-fold increased phosphorylation of ERK1/2, respectively) (Fig. 6A). This was similar to the effect of insulin on ERK1/2 in cells obtained during surgery and general anaesthesia from normal controls and from

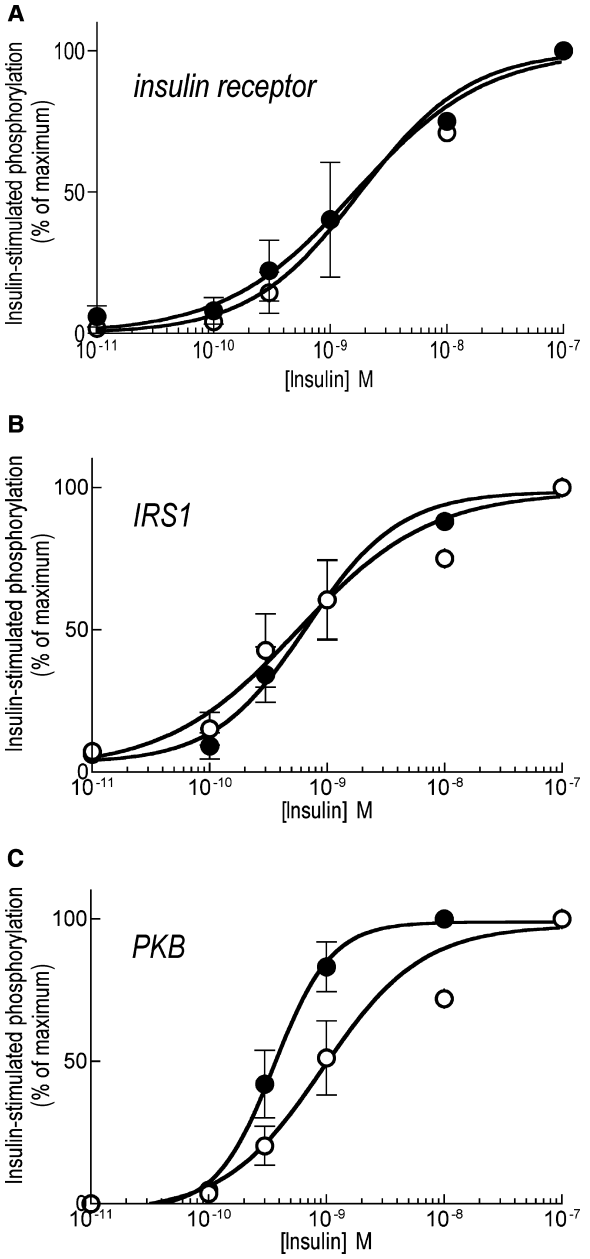

Fig. 4. Dose–response effect of insulin on phosphorylation of insulin receptor, IRS1, and PKB before (○) and after (●) overnight recovery. Whole cell lysates, of adipocytes form control subjects, were subjected to SDS/PAGE and immunoblotting against phospho-tyrosine [insulin receptor (A), IRS1 (B)]. (C) phospho-PKB. Mean ± SE, $n = 4$ subjects. The dose–response curves in C, but not in A,B were significantly different, $P < 0.05$.

patients with diabetes (Table 2). As these analyses don't distinguish between effects of the surgery per se and the postsurgical isolation of adipocytes, we subjected isolated adipocytes, which had been incubated overnight, to a second round of collagenase treatment. As shown in

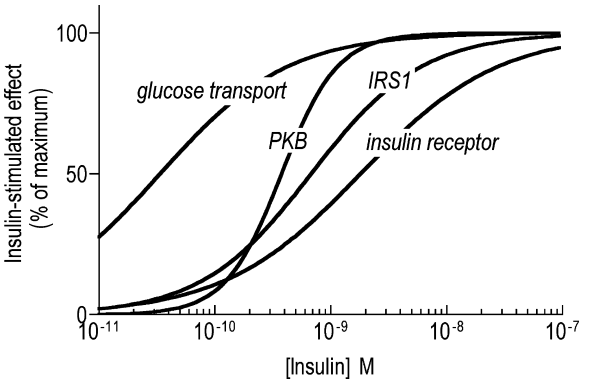

Fig. 5. Dose–response relationship for insulin control of the metabolic signalling pathway (data from Figs 3 and 4, after overnight recovery). Following overnight recovery, EC_{50} for insulin was found at decreasing concentrations, from the signal generator (the insulin receptor) to the target effect (glucose uptake). Note that MAP-kinases ERK1 and 2 of insulin's mitogenic signalling pathway exhibited a similar sensitivity (EC_{50}) to insulin as PKB (Fig. 1C).

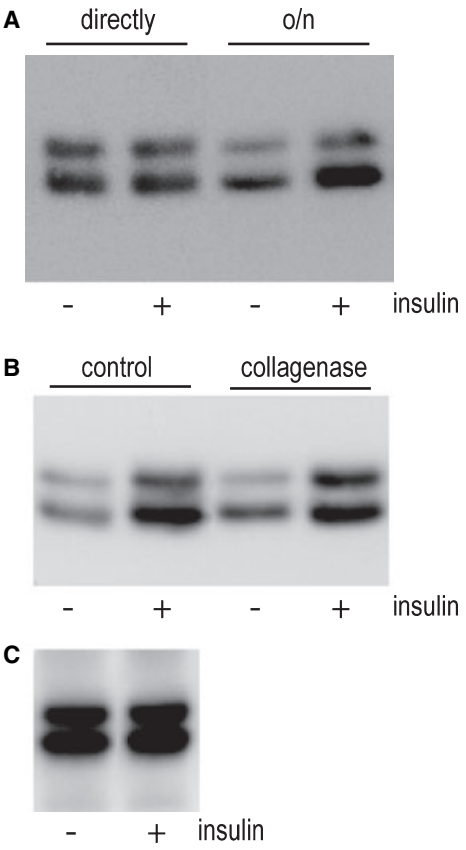

Fig. 6. Effects on ERK1/2 phosphorylation by alternative tissue and cell treatments. Tissue was obtained from female nondiabetic subjects. (A) Abdominal subcutaneous adipose tissue was obtained by a small incision under local anaesthesia and cells isolated. The cells were incubated with or without 100 nM insulin for 10 min, directly or after overnight incubation (o/n). Insulin stimulated the phosphorylation of Erk1/2 1.0/1.1-fold, respectively (directly) and 2.3/2.3-fold (o/n) (average of cells from two different subjects). (B) Cells obtained after surgery were incubated overnight, treated with or without collagenase for 15 min and then with or without 100 nM insulin for 10 min. Insulin stimulated the phosphorylation of Erk1/2 2.4/2.4-fold (nontreated control) and 4.2/3.5-fold (collagenase treated) (average of cells from two different subjects). (C) Adipose tissue obtained during surgery was cut into small pieces and directly incubated (without collagenase treatment) with or without 100 nM insulin for 20 min. Insulin did not affect the phosphorylation of Erk1/2 1.1/1.0-fold (average of tissue from two different subjects).

Fig. 6B, the collagenase treatment did not affect ERK1/2 phosphorylation and insulin retained the ability to increase the phosphorylation of ERK1/2, by 4.2/3.5-fold, respectively. When we analyzed small pieces of adipose tissue, which had not been subjected to collagenase treatment at all, without overnight incubation, insulin did not affect the phosphorylation of ERK1/2 (Fig. 6C) as they were most probably already fully phosphorylated. Using a different approach we analyzed rat adipocytes that were obtained without any surgical procedures (post mortem) following rapid cervical dislocation, and with the same cell isolation procedure as used for human adipocytes. Directly after isolation, ERK1/2 phosphorylation was low in the rat adipocytes and they responded to insulin with increased phosphorylation of ERK1/2 (not shown). When the rat adipocytes were analyzed directly, insulin stimulated glucose uptake 9.0-fold (mean of two separate cell preparations), but after overnight incubation of the cells, insulin stimulated glucose uptake only 2.3-fold.

Patients with type 2 diabetes

We next isolated adipocytes from a group of female and a group of male patients with type 2 diabetes and examined the insulin responsiveness of the cells after overnight incubation (to avoid interference from the insulin resistance that we found when cells were analyzed directly). In these cells, the insulin receptor autophosphorylation in response to insulin was similar to cells from nondiabetic subjects (Fig. 7A and Table 1). IRS1 phosphorylation, however, occurred at substantially higher concentrations of insulin, EC_{50} = 2.0 nM insulin, compared to 0.6 nM in nondiabetic subjects (Fig. 7B and Table 1). PKB phosphorylation similarly occurred at higher concentrations of insulin, EC_{50} = 0.7 nM insulin, compared to 0.4 nM in nondiabetic subjects (Fig. 7C and Table 1). Moreover, the dose–response curve for insulin activation of PKB did not exhibit the steep increase over a very small range

Table 2. Maximal insulin effects in human adipocytes. Adipocytes from nondiabetic subjects or patients with type 2 diabetes were analyzed after an overnight recovery period. The maximal insulin-stimulation is expressed as -fold over basal ± SE. Student's *t*-test for comparison of the indicated diabetic group with the normal nondiabetic group; ND, not determined as basal level of phosphorylation was close to zero; (n), number of subjects.

	Subjects		
Analysis	Normal	Female diabetic	Male diabetic
Insulin receptor	9.6 ± 4.2 (5)	5.4 ± 1.7 (5), $P = 0.4$	16.5 ± 4.5 (4), $P = 0.3$
IRS1	14.7 ± 7.3 (4)	4.6 ± 1.1 (5), $P = 0.2$	10.2 ± 1.7 (4), $P = 0.6$
PKB	ND	ND	ND
Glucose transport	3.8 ± 0.8 (6)	3.2 ± 1.3 (5), $P = 0.5$	6.1 ± 4.4 (3), $P = 0.5$
ERK1	2.0 ± 0.4 (4)	2.2 ± 0.8 (5), $P = 0.8$	1.8 ± 0.5 (4), $P = 0.8$
ERK2	2.3 ± 0.3 (4)	2.2 ± 0.6 (5), $P = 0.9$	1.5 ± 0.3 (4), $P = 0.1$

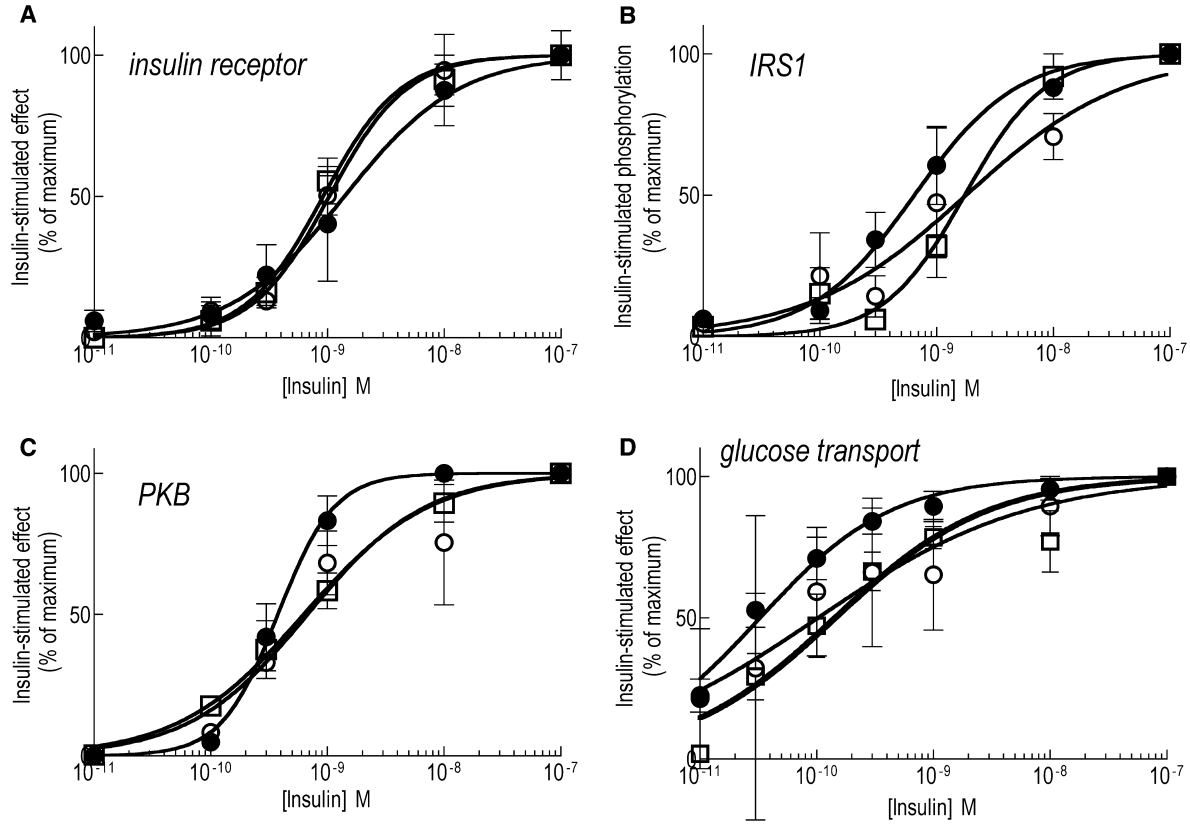

Fig. 7. Dose–response effect of insulin in adipocytes from controls subjects and type 2 diabetic patients after overnight incubation. Cells were incubated overnight and then with the indicated concentration of insulin for 10 min before whole-cell lysates were subjected to SDS/PAGE and immunoblotting against phospho-tyrosine [insulin receptor (A), IRS1 (B)]; (C), phospho-PKB; (D) glucose transport, determined as uptake of 2-deoxy-D-[1-^{3}H]glucose by the cells. ●, control subjects, mean ± SE, $n = 4$ (glucose transport, $n = 8$); ○, male diabetic patients, mean ± SE, $n = 4$; □, female diabetic patients, mean ± SE, $n = 5$. The dose–response curves for control vs. the diabetic group were significantly different in B,C,D, $P < 0.05$, but they were not significantly different in A.

of insulin concentration that characterized the response to insulin in cells from control subjects.

As a result of the resistance to insulin, activation of IRS1 and the downstream PKB, the EC_{50} for glucose uptake was at 0.1 to 0.2 nM insulin in adipocytes from the diabetic patients, compared to an $EC_{50} = 0.02$ to 0.03 nM in cells from nondiabetic subjects (Fig. 7D and Table 1). The maximal rate of glucose uptake in the fat cells from the female patients with type 2 diabetes, 199 ± 26 nmol 2-deoxyglucose·min^{-1}·L^{-1} packed

cell volume (mean ± SE, $n = 5$), varied (118 to 255 nmol·min^{-1}·L^{-1}) from individual to individual. The maximal rate of glucose uptake in the fat cells from the group of male patients with type 2 diabetes, 74 ± 32 nmol 2-deoxyglucose·min^{-1}·L^{-1} packed cell volume (mean ± SE, $n = 4$), varied considerably (12 to 152 nmol·min^{-1}·L^{-1}) from individual to individual. Maximal insulin-stimulated rate of glucose uptake in cells from the diabetic patients was not different from cells from the nondiabetic control subjects. Similarly, the maximal effects of insulin on the state of tyrosine-phosphorylation of the insulin receptor or of IRS1, or of phosphorylation of ERK1/2, was not significantly different in either group of diabetics compared with the nondiabetic controls (Table 2).

The dose–response curves for insulin effects on the insulin receptor, IRS1, PKB, and glucose transport analyzed directly after surgery were identical and with the same EC_{50} values (Table 1) as when analyzed after overnight recovery. The insulin resistance in the cells from patients with diabetes was thus not reversible.

The average size of the adipocytes from diabetic patients (92 ± 2.4 μm diameter) did not differ from those of nondiabetic control subjects (94 μm, see above).

Discussion

Insulin resistance resulting from surgical procedures

The findings herein demonstrate that MAP-kinases ERK 1 and 2, and p38, are phosphorylated and hence activated *in situ* in normal human adipose tissue obtained during surgery. This phosphorylation was reversed after overnight recovery and stimulation with insulin then increased the phosphorylation of ERK1/2 while it had no effect on the phosphorylation of p38 MAP-kinase in human adipocytes. This was similar to what has been shown in rat skeletal muscle [25] but is in contrast to reports that insulin activates p38 in 3T3-L1 adipocytes and L6 myotubes [4,5]. The insulin receptor and its metabolic downstream signal mediators (IRS1 and PKB) were largely unphosphorylated in fresh adipocytes and unaffected by overnight recovery. We therefore exclude insulin as causing the basal activation of MAP-kinases; especially as we found that a substantial degree of phosphorylation of the insulin receptor and IRS1 was required to increase the phosphorylation of ERK1/2 (Figs 1C and 5).

Our findings indicate that the collagenase treatment to isolate adipocytes from the tissue was not the cause of the basal ERK1/2 phosphorylation that we detected directly after surgery. It is probable that the insulin resistance we found directly after surgery was the result of the surgical procedures and not of post surgical isolation of the cells. Similar to the whole-body insulin resistance that results from minor and major surgical procedures, a small incision during local anaesthesia had a similar effect to abdominal surgery under general anaesthesia on ERK1/2 in the adipocytes. In contrast to the human adipocytes, rat adipocytes did not fare well during overnight incubation as demonstrated by impaired glucose uptake in response to insulin. Evidently human adipocytes are not affected by cell isolation procedures and prolonged incubations in the same way as rat and mouse [26] cells.

The insulin-sensitivity for phosphorylation of the insulin receptor and the immediate downstream mediator IRS1 was not measurably affected by the surgical cell isolation procedures and overnight recovery. However, the downstream mediator PKB as well as the crucial metabolic effect – glucose transport – exhibited insulin resistance directly after surgery, which was reversed after overnight recovery of the cells. It is notable that the maximal effect of insulin on PKB and glucose transport was not significantly affected by the overnight recovery period, while the sensitivity to insulin was invariably improved. The fact that even minor surgery produces insulin resistance [8] indicates that it is difficult to obtain control tissue to study trauma-induced insulin resistance, which may explain the conflicting results reported earlier [11–13]. Obtaining the insulin resistant cells directly and the control cells after overnight recovery, as described herein, is a new approach to further investigate trauma-induced insulin resistance on a cellular and molecular level.

It should be noted that the analyses of insulin effects on glucose transport and the different signal mediators of the hormone were performed on the same cell sample from the same individual. Responses for the different signal mediators are therefore directly comparable. The results demonstrate increasing insulin sensitivity downstream of the insulin receptor, probably resulting from the inherent signal amplification in the succeeding enzymatic signalling steps. This is clearly compatible with and explains the fact that only a small percentage of insulin receptors need to be activated to produce a substantial downstream response [27]. It is interesting that the effects of insulin on PKB phosphorylation occurred over a much narrower concentration range than on the insulin receptor, IRS1, or glucose transport (Fig. 5). The steep dose–response curve indicates a cooperative effect of insulin on PKB phosphorylation. This could be explained by the

complicated translocation and activation processes involved in control of PKB, in response to insulin, which involves dual phosphorylation of PKB by insulin-activation of the phosphoinositide-dependent protein kinase-1 (PDK1) [28] and the yet unidentified PDK2 [29,30]. Our findings, furthermore, suggest that insulin resistance due to the surgical cell isolation procedures or to type 2 diabetes may involve loss of the cooperative effect on PKB, which is compatible with earlier findings that serine and threonine phosphorylation of PKB is differently affected in type 2 diabetes [18].

MAP-kinases, particularly p38, but also ERK 1 and 2, have been shown to be phosphorylated/activated when cells are exposed to various types of stress [6,31,32]. Stress hormones such as adrenaline [33] and glucocorticoids [34] have been shown to inhibit insulin-stimulated glucose disposal in man. It is therefore possible that a stress response due to the surgical procedure has caused the extensive phosphorylation/activation of the MAP-kinases reported here. Similar results with human adipocytes were reported recently, but overnight recovery was not used and the highly phosphorylated ERK1/2 and p38 was attributed to type 2 diabetes [35] rather than to the surgical procedures as indicated herein.

We can conclude that a node of cross-talk between the stress-generated signal and insulin signalling is located at the level of IRS1 or between IRS1 and PKB. The effect and ultimate function of stress signalling in adipose tissue is not known. Discovering how a stress signal is translated into a reduced sensitivity to insulin for phosphorylation of PKB and for glucose transport control may ultimately allow improved surgical procedures to avoid or reduce postoperative insulin resistance.

Insulin resistance in type 2 diabetes

Tyrosine phosphorylation of the insulin receptor increased over the same concentration range of insulin in cells from patients with type 2 diabetes as from nondiabetic subjects, when assayed directly as well as after overnight incubation. Phosphorylation of IRS1 required, however, significantly higher concentrations of insulin in the cells from patients with diabetes than from nondiabetic subjects, both when assayed directly and after overnight incubation. It thus appears that IRS1 is the first step in insulin signalling that contributes to diabetic insulin resistance in human adipocytes, similar to that found earlier in human skeletal muscle in diabetes [14–16] and obesity [17]. This may be the result of, e.g. enhanced serine/threonine phosphorylation of IRS1, making it a worse substrate for the insulin receptor as described in various *in vitro* systems and models of insulin resistance [36–40]. Lowered expression of IRS1 in adipocytes has been described in some obese individuals or relatives of diabetes patients [19]. Naturally occurring mutations in IRS1 have been identified in subjects with type 2 diabetes and also reported to impair insulin action [41–45]. Our findings indicate that insulin resistance is not different in adipocytes from female and male patients with type 2 diabetes.

In conclusion, our findings demonstrate a physiologically relevant cell model for analyses, at the cell and molecular levels, of how surgical cell isolation procedures may interfere with insulin's control of metabolism. We demonstrated that reversible insulin resistance directly after isolation of the cell exhibits fundamental differences from the chronic insulin resistance in type 2 diabetes. In particular, signalling dysregulation in adipocytes from patients with type 2 diabetes was demonstrated at the level of insulin-stimulated phosphorylation of IRS1.

Experimental procedures

Subjects

Samples of subcutaneous abdominal fat were obtained from patients at the University Hospital of Linköping. Pieces of adipose tissue were excised during elective abdominal surgery and general anaesthesia at the beginning of the operation [eight nondiabetic control subjects (females: age 32–89 years; BMI 17–27) and five diabetic patients (females; age 44–72 years; BMI 28–48; HbA1c 5.7 to 9.7%]. Subcutaneous adipose tissue was excised by incision under local anaesthesia from four volunteers with type 2 diabetes (males: age 41–70 years; BMI 31–39; HbA1c 3.9–6.8%). Patients with diabetes were treated with sulfonylurea, sulfonylurea in combination with metformin, or with insulin. The study was approved by the Local Ethics Committee and participants gave their informed approval.

Materials

Rabbit anti-insulin receptor β-chain polyclonal and mouse anti-phosphotyrosine (PY20) monoclonal Igs were from Transduction Laboratories (Lexington, KY, USA). Rabbit anti-phospho(Thr308)-PKB/Akt polyclonal Igs were from Upstate Biotech. (Charlottesville, VA, USA). Rabbit polyclonal antibodies against phospho-ERK1/2 and phospho-p38 MAP-kinase were from Cell Signaling Techn. (Beverly, MA, USA). Rabbit anti-IRS1 polyclonal Igs were from Santa Cruz Biotech. (Santa Cruz, CA, USA). 2-Deoxy-D-[1-^{3}H]glucose was from Amersham Biotech (Uppsala, Sweden). Insulin and other chemicals were from Sigma–Aldrich

(St. Louis, MO, USA) or as indicated in the text. Harlan Sprague–Dawley rats (160–200 g) were from B & K Universal (Sollentuna, Sweden). The animals were treated according to Swedish Animal Care regulations.

Isolation and incubation of adipocytes

Adipocytes were isolated by collagenase (type 1, Worthington, NJ, USA) digestion as described [22]. At a final concentration of 100 μL packed cell volume per ml, cells were incubated in Krebs/Ringer solution (0.12 M NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4) containing 20 mM Hepes, pH 7.40, 1% (w/v) fatty acid-free bovine serum albumin, 100 nM phenylisopropyladenosine, 0.5 $U{\cdot}mL^{-1}$ adenosine deaminase with 2 mM glucose, at 37 °C on a shaking water bath for immediate analysis. For analysis after 20 to 24 h incubation, cells were incubated at 37 °C, 10% (v/v) CO_2 in the same solution mixed with an equal volume of DMEM containing 7% (w/v) albumin, 200 nM adenosine, 20 mM Hepes, 50 $UI{\cdot}mL^{-1}$ penicillin, 50 $\mu g{\cdot}mL^{-1}$ streptomycin, pH 7.40. Before analysis, cells were washed and transferred to the Krebs/Ringer solution. Average cell diameter was determined from microscopy photo enlargements using a ruler (≈ 200 cells from each subject were analyzed).

SDS/PAGE and immunoblotting

Cell incubations were terminated by separating cells from medium by centrifugation through dinonylphtalate (5000 ***g*** for 3 s at room temperature). The cells were dissolved immediately in SDS and 2-mercaptoethanol with protease and protein phosphatase inhibitors, frozen within 10 s, and thawed in boiling water to minimize postincubation signalling modifications in the cells and protein modifications during immunoprecipitation [22]. Equal amounts of cells (i.e. total cell volume), as determined by lipocrit, was subjected to SDS/PAGE and immunoblotting. After SDS/PAGE and electrotransfer, membranes were incubated with the appropriate antibodies detected using enhanced chemiluminescence (ECL+ Amersham Biosciences) with horseradish peroxidase-conjugated anti-IgG as secondary antibody, and evaluated by chemiluminescence imaging (Las1000, Image-Gauge, Fuji, Tokyo, Japan).

Using two-dimensional electrofocusing (pH 3–10), SDS/PAGE analysis [23] and immunoblotting against phosphotyrosine and IRS1, > 95% of the tyrosine phosphorylated 180-kDa band was determined to represent IRS1.

Determination of glucose transport

Glucose transport was determined as uptake of 2-deoxy-D-[1-^{3}H]glucose [24] after transfer of cells to medium without glucose. 2-Deoxy-D-[1-^{3}H]glucose was added to a final concentration of 50 μM (10 $\mu Ci{\cdot}mL^{-1}$) and the cells were incubated for 30 min. It was verified that uptake was linear for at least 30 min.

Statistics

Dose–response curves were compared using *F*-test with the sigmoidal curve-fitting algorithm in GRAPHPAD Prism 4 (GraphPad Software, Inc., San Diego, CA, USA). The null hypothesis was rejected if $P < 0.05$.

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