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Insulin resistance induced by high glucose and high insulin precedes insulin receptor substrate 1 protein depletion in human adipocytes

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Abstract

The aim of this study was to investigate whether high glucose and/or high insulin produces cellular insulin resistance in human adipocytes and, if so, to evaluate the time course and content of key proteins in the insulin signaling pathway. Subcutaneous fat biopsies were taken from 27 nondiabetic subjects. Insulin action in vitro was studied by measurement of glucose uptake after incubation at a physiologic glucose level (6 mmol/L) for 24 hours or with the last 2, 6, or 24 hours at a high glucose level (20 mmol/L) with or without high insulin (10^4 μU/mL). High glucose alone for 24 hours produced a small but significant impairment (by ~20%, $P < .05$) of insulin's effect to stimulate glucose transport, whereas nonstimulated glucose uptake was left intact. In contrast, the combination of high glucose and high insulin for 6 hours or more reduced basal glucose uptake by ~40% ($P < .05$). In addition, insulin-stimulated glucose uptake capacity was reduced by ~40% already after 2 hours ($P < .05$) and reached a maximal decline (by ~50%, $P < .05$) after a 6-hour culture in high glucose and high insulin. Treatment with high glucose and high insulin in combination for at least 6 hours reduced cellular insulin receptor substrate (IRS)–1, but not IRS-2, protein content by ~45% or more ($P < .05$). Moreover, after 24 hours, the ability of insulin to activate protein kinase B (ie, the phosphorylated protein kinase B [pPKB]–protein kinase B ratio) was decreased by ~50% ($P < .05$). No significant effects were seen on insulin signaling proteins or glucose transporter 4 after a long-term high-glucose culture. Culture with high insulin alone (and low glucose, 6 mmol/L) decreased basal and insulin-stimulated glucose uptake in conformity with the high-glucose/high-insulin setting. However, IRS-1 protein content remained unchanged. We conclude that, in adipocytes from healthy humans, high insulin alone for 2 hours or more decrease glucose uptake capacity. Likewise, high glucose and high insulin in combination for 2 hours or more decrease glucose uptake to the same extent as when cells were cultured with high insulin alone but, in addition, with a diminishment in IRS-1 protein lagging behind. Thus, IRS-1 depletion appears to be a secondary phenomenon in this model of insulin resistance. High glucose alone induces only a minor insulin resistance in human fat cells.

1. Introduction

The cause of insulin resistance in type 2 diabetes mellitus is multifactorial and both genetic and acquired factors are involved [1]. Sustained hyperglycemia will contribute to the impairment of both insulin-stimulated glucose utilization in peripheral tissues (ie, muscle and fat) and the ability of pancreatic beta cells to respond properly to elevated glucose levels [2,3]. These observations led to the formulation of the "glucose toxicity" hypothesis [4].

Although there are consistent findings that hyperglycemia impairs glucose transport in both adipose tissue and muscle in vivo [5], there are conflicting data regarding the in vitro situation. Data from rat adipocytes and 3T3L1 preadipocytes cultured for 24 hours show a down-regulatory effect as well as no effect of glucose per se on glucose uptake capacity [6-9]. On the other hand, the glucose transport system in rat adipocytes cultured in the presence of high glucose combined with high insulin consistently exhibits insensitivity and unresponsiveness to insulin [6,7,10,11], that is, defects similar to those observed in human adipocytes from patients with type 2 diabetes mellitus [12].

The biochemical mechanism for cellular insulin resistance is not fully understood. After insulin binding to its receptor, intrinsic tyrosine kinases are activated that initiate a cascade of intracellular signaling events. Adapter proteins of the insulin receptor substrate (IRS) family are phosphorylated, and so are downstream effector proteins. Knockout mice lacking IRS-1 or IRS-2 develop insulin resistance or type 2 diabetes mellitus, respectively [13,14]. In vitro

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studies of the insulin signaling pathway have revealed the indispensable role of the IRSs in glucose transport. Furthermore, decreased IRS protein levels have been reported in multiple in vivo states of insulin resistance, including type 2 diabetes mellitus and obesity as well as in vitro models of insulin resistance indicating their possible involvement in the pathophysiology of insulin resistance and type 2 diabetes mellitus [15-20]. Our previous work in rat adipocytes however suggests otherwise because IRS-1/2 depletion is not detectable until after the establishment of an impaired glucose uptake capacity when cells are incubated with high glucose and high insulin [10].

One of the effector proteins downstream of IRSs suggested to play a key role regarding the metabolic effects of insulin signaling [21,22] is protein kinase B (PKB), although there has been some controversy on this [23]. The end point of the insulin signaling cascade toward glucose transport is mediated by the redistribution of glucose transporter 4 (GLUT4) from intracellular compartments to the cell surface. It has been shown that the cellular content of GLUT4 in adipose tissue is reduced in type 2 diabetes mellitus (by 85%) and obesity (by 40%) [24]. Glucose transporter 4 messenger RNA level in adipocytes from patients with impaired glucose tolerance, obesity, and type 2 diabetes mellitus are also decreased [25]. Interestingly, this is not observed in muscle from such individuals [26]. Whether these alterations in GLUT4 are seen in human adipocytes subjected to hyperglycemia, with or without hyperinsulinemia, in vitro is not known.

The purpose of this in vitro study was to (1) investigate whether high glucose and/or high insulin levels affect basal and insulin-stimulated glucose uptake capacity in human adipocytes, (2) explore the time course for such effects, and (3) quantify content of key proteins in the insulin signaling pathway.

2. Materials and methods

2.1. Subjects

Clinical and biochemical characteristics from 27 nondiabetic individuals are shown in Table 1. Their age was 43 ± 3 years (23-71 years) and their body mass index was 27.0 ± 0.7 (20.2-36.3). Of 16 women, 6 were postmenopausal. Twelve of the subjects had a first-degree relative with type 2 diabetes mellitus. One subject was treated with metoprolol and simvastatin because of hypertension and dyslipidemia, respectively, but no other participant had any regular medication and there were no other chronic diseases. There were 4 regular and 4 nonregular smokers. The Umeå University Ethics Committee approved the study protocol. All subjects gave their informed consent.

2.2. Protocol

After a 6-hour fast, subjects arrived at the metabolic unit at 2:00 PM. The timing was because of practical circumstances, to avoid experimental procedure on cultured biopsy material during nights. A venous blood sample was also taken for analysis of different blood parameters by routine methods at the Department of Clinical Chemistry, Umeå University Hospital (Umeå, Sweden), as previously described [27]. Body composition was determined by the bioelectrical impedance analysis technique (BIA-101 RJL-systems, Detroit, MI) [28]. A subcutaneous needle biopsy was taken from the lower part of the abdomen after dermal local anesthesia with lidocaine (Xylocain, AstraZeneca, Södertälje, Sweden). The fat biopsies were put in prewarmed medium 199 supplemented with 5.6 mmol/L glucose and 40 mg/mL bovine serum albumin (BSA) and then immediately transported to the laboratory.

Table 1
Clinical characteristics and fasting blood chemistry of the study participants

	Mean ± SEM
Sex (male/female)	11/16
Age (y)	43 ± 3
BMI (kg/m^2)	27.0 ± 0.7
WHR	0.89 ± 0.0
Fat mass (%)	30.7 ± 1.7
Fat-free mass (%)	69.3 ± 1.7
Blood HbA_{1c} (%)[a]	4.2 ± 0.1
Serum insulin (mU/L)	9.4 ± 2.1
Serum glucose (mmol/L)	4.9 ± 0.1
Serum triglycerides (mmol/L)	1.3 ± 0.1
Serum cholesterol (mmol/L)	5.3 ± 0.2
Serum LDL cholesterol (mmol/L)	3.1 ± 0.2
Serum HDL cholesterol (mmol/L)	1.6 ± 0.1

Data are number of subjects or means ± SEM. BMI indicates body mass index; WHR, waist to hip ratio; HbA_{1c}, hemoglobin A_{1c}; LDL, low-density lipoprotein; HDL, high-density lipoprotein.
[a] Reference range, 3.3% to 5.0%.

2.3. Reagents

Medium 199, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and penicillin/streptomycin were from Gibco BRL, Life Technologies (Paisley, UK). Bovine serum albumin (fraction V) and N^6-(*R*-phenylisopropyl)adenosine (PIA) were purchased from Sigma Chemical (St Louis, MO). Collagenase A and adenosine deaminase (ADA) were purchased from Boehringer Mannheim (Mannheim, Germany). Human insulin (Actrapid) was purchased from Novo Nordisk (Copenhagen, Denmark). [^{14}C]-U-D-glucose (specific activity, ~200-300 mCi/mmol) was from Amersham Pharmacia Biotech (Freiburg, Germany). The anti–phospho-Akt1 (Ser^{473}) antibody was from Cell Signaling Technology (Beverly, MA) and the anti–IRS-1 and anti–IRS-2 polyclonal antibodies were from Upstate Biotechnology (Lake Placid, NY). Anti–GLUT4 polyclonal antibody was from Alpha Diagnostic Int (San Antonio, TX). Anti-Akt1/2 (PKB) polyclonal antibody and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.4. Adipocyte preparation and incubation

Needle biopsies of abdominal subcutaneous fat tissue were obtained at 2:00 PM after a 6-hour fast. Fat biopsies

were transported to the laboratory in warm (37°C) medium 199 supplemented with 5.6 mmol/L glucose and 40 mg/mL BSA. The fat tissue was washed with prewarmed medium (as above) and blood clots were removed. Isolated fat cells were prepared by shaking the tissue in polypropylene containers at 37°C for ~50 minutes in medium 199 supplemented with 5.6 mmol/L glucose, 40 mg/mL BSA, and 0.6 mg/mL collagenase. Cells were filtered through a nylon mesh (ϕ 0.3 mm) and washed 4 times with fresh medium 199 supplemented with 5.6 mmol/L glucose and 40 mg/mL BSA. The cell suspension was split and cultured in polystyrene flasks containing DMEM with 6 mmol/L (initial concentration) D-glucose, 10% fetal calf serum (containing ~8 μU/mL insulin), penicillin (100 U/mL), and streptomycin (100 μg/mL). In different flasks, medium glucose and/or insulin concentrations were raised (to 20 mmol/L and 10^4 μU/mL, respectively) the last 2, 6, or 24 hours of incubation. Cells were incubated at 37°C with gentle stirring (25 rpm) for a total of 24 hours under a gas phase of 95% O_2 and 5% CO_2. Cell size was determined in isolated adipocytes as previously described [29]. Cell viability was verified by trypan blue exclusion tests.

2.5. Glucose uptake assay

After 24 hours of culture, adipocytes were washed 4 times and glucose uptake was assessed as previously described [30,31]. In brief, adipocytes (lipocrit, 3%-5%) were incubated in medium 199 without glucose with BSA (4%), ADA (1 U/mL), and PIA (1 μmol/L) for 15 minutes at 37°C in a shaking water bath, in the presence or absence of 1000 μU/mL insulin. After that, D-[U-^{14}C]glucose (7.8 MBq/L, 0.7-1.0 μM) was added. Cells were separated from the incubation medium after 45 minutes by centrifugation through silicone oil and the radioactivity associated with the cells were measured by scintillation counting. Under these experimental conditions, glucose uptake is mainly determined by the rate of transmembrane glucose transport [12]. The cellular clearance of glucose from the medium was calculated according to the following formula and taken as an index of the rate of glucose uptake. Cellular clearance of medium glucose = (Cpm of cells × volume)/(Cpm of medium × cell number × time).

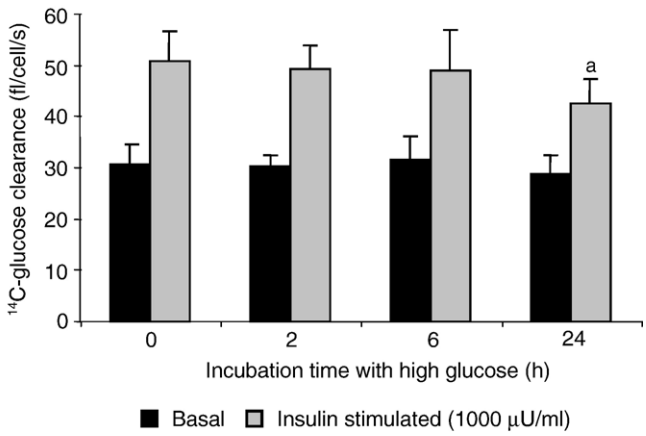

Fig. 1. Glucose uptake rate in isolated fat cells after exposure to high glucose levels. Cells were incubated at a high glucose (20 mmol/L) concentration during the final 2, 6, or 24 hours of a total period of 24 hours. Insulin (1000 μU/mL) was added for 15 minutes as indicated and then ^{14}C-glucose was added, and glucose uptake was assessed during 45 minutes as described in Materials and methods. Data are mean ± SEM (n = 11-14) and are expressed as cellular glucose clearance (fL per cell per second). $^{a}P < .05$ vs insulin-stimulated cells cultured under control conditions (0 hour), that is, 6 mmol/L glucose and no insulin, respectively.

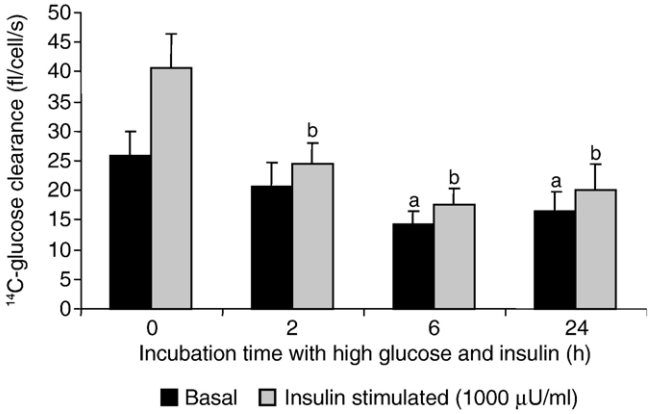

Fig. 2. Glucose uptake rate in isolated fat cells after exposure to high glucose and high insulin levels. Cells were incubated at a high glucose (20 mmol/L) and high insulin (10^4 μU/mL) concentration during the final 2, 6, or 24 hours of a total period of 24 hours. Insulin (1000 μU/mL) was added for 15 minutes as indicated and then ^{14}C-glucose was added, and glucose uptake was assessed during 45 minutes as described in Materials and methods. Data are mean ± SEM (n = 5-9) and are expressed as cellular glucose clearance (fL per cell per second). $^{a}P < .05$ vs non–insulin-stimulated and $^{b}P < .05$ vs insulin-stimulated cells cultured under control conditions (0 hour), that is, 6 mmol/L glucose and no insulin, respectively.

2.6. Western blot analysis

Cells were cultured for 24 hours as previously described. Thereafter, cells were washed 4 times with fresh medium and incubated 10 minutes at 37°C in a shaking water bath, in vials (lipocrit, ~15%) containing 5.6 mmol/L glucose, medium 199, BSA (4%), ADA (1 U/mL), and PIA (1 μmol/L) with or without a maximal insulin concentration (1000 μU/mL). Cells were collected, washed, and lysed with ~0.2 mL lysis buffer (25 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L EGTA, 25 mmol/L NaCl, 1% Nonidet P-40, 1 mmol/L Na_3VO_4, 10 mmol/L NaF, 0.2 mmol/L leupeptin, 1 mmol/L benzamidine, and 0.1 mmol/L 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochlorine [AEBSF]) and rocked for a minimum of 30 minutes at 4°C. The insoluble cell fractions were sedimented by centrifugation at 12 000g for 15 minutes at 4°C, and supernatants were collected and frozen in aliquots at −80°C. Protein content was measured with the BCA protein assay kit (Pierce Chemical, Rockford, IL) with BSA as standard.

Separation of proteins was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The total amount of protein added per lane was the same within each set of experiments. Proteins were transferred to an

Immobilon-P membrane (Millipore, Bedford, MA), which was blocked with 5% dry milk dissolved in phosphate-buffered saline (PBS) (pH 7.4) at 4°C overnight. Detection of the various signaling proteins and GLUT4 were performed using polyclonal antibodies designed for each protein. ECL Western blotting kit (ECL Plus, Amersham Biosciences, Buckinghamshire, UK) was used to visualize immunoreactive bands. Protein quantification was adjusted for the corresponding β-actin level.

2.7. Statistical analyses

Statistical analyses were performed using the SPSS package (SPSS, Chicago, IL). Results are given as means ± SEM. Analyses were based on comparisons made within each subject. Within-subject differences between incubation conditions were analyzed with the Wilcoxon signed rank tests. $P < .05$ was considered statistically significant. (The largest n always corresponds to the control culture situation.)

3. Results

3.1. Glucose uptake after culture with high glucose

Although individuals differ in glucose uptake capacity, the relative insulin response after a 24-hour culture was ~170% of basal, and this is very similar to what was previously reported in cultured human subcutaneous adipocytes [32,33]. Freshly isolated human adipocytes, on the other hand, typically have an insulin response of ~300% of basal [32,33]. Basal glucose uptake capacity did not differ after a 24-hour culture with high glucose (20 mmol/L) (Fig. 1). However, the maximal insulin-stimulated glucose uptake was decreased by ~20% compared with the control cells cultured in low glucose (6 mmol/L) ($P < .05$, Fig. 1).

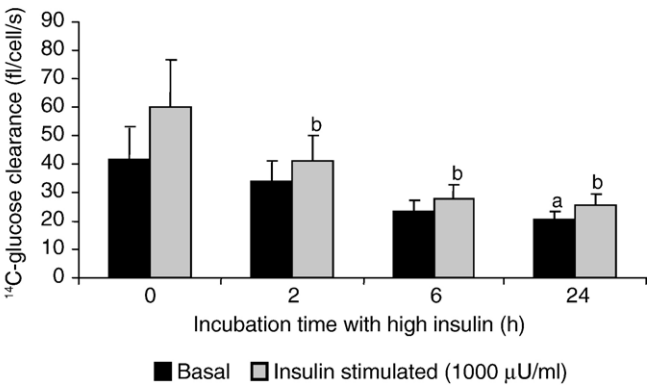

Fig. 3. Glucose uptake rate in isolated fat cells after exposure to high insulin levels. Cells were incubated at 6 mmol/L glucose and a high insulin (10^4 μU/mL) concentration during the final 2, 6, or 24 hours of a total period of 24 hours. Insulin (1000 μU/mL) was added for 15 minutes as indicated and then ^{14}C-glucose was added, and glucose uptake was assessed during 45 minutes as described in Materials and methods. Data are mean ± SEM (n = 5-6) and are expressed as cellular glucose clearance (fL per cell per second). $^{a}P < .05$ vs non–insulin-stimulated and $^{b}P < .05$ vs insulin-stimulated cells cultured under control conditions (0 hour), that is, 6 mmol/L glucose and no insulin, respectively.

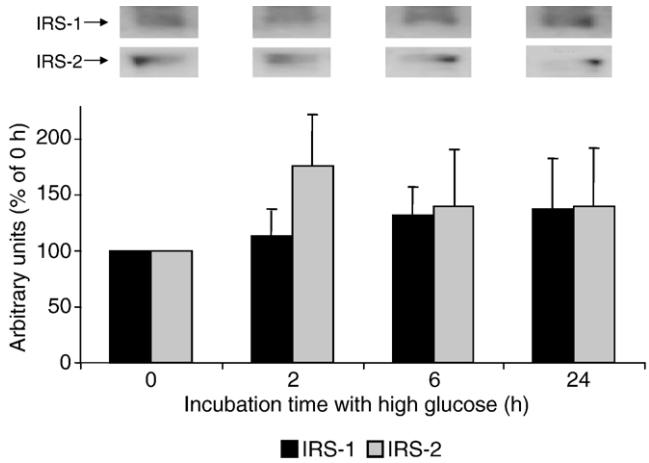

Fig. 4. Insulin receptor substrate 1 and 2 protein content in isolated fat cells after exposure to high glucose levels. Cells were incubated at a high glucose (20 mmol/L) concentration during the final 2, 6, or 24 hours of a total period of 24 hours. The results of densitometry analyses are shown, and data are expressed as arbitrary units in relation to the amount of target protein in the control situation (0 hour), that is, 24 hours preincubation with low glucose. Protein quantification was adjusted for the corresponding β-actin level. Data are expressed as means of 5 to 8 separate experiments. One representative blot is shown.

This is not likely caused by osmotic effects because, in control experiments, 14 mmol/L L-glucose added together with 6 mmol/L D-glucose did not alter glucose uptake capacity (data not shown and reference [7]). Shorter incubation periods (2 or 6 hours) with high glucose had no effect on the insulin-stimulated glucose transport (Fig. 1).

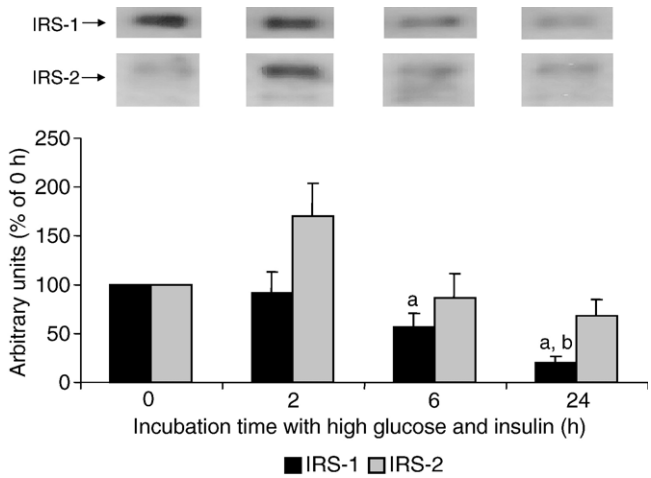

Fig. 5. Insulin receptor substrate 1 and 2 protein content in isolated fat cells after exposure to high glucose and high insulin levels. Cells were incubated at a high glucose (20 mmol/L) and high insulin (10^4 μU/mL) concentration during the final 2, 6, or 24 hours of a total period of 24 hours. The results of densitometry analyses are shown, and data are expressed as arbitrary units in relation to the amount of target protein in the control situation (0 hour), that is, 24 hours preincubation with low glucose and no insulin. Protein quantification was adjusted for the corresponding β-actin level. Data are expressed as means of 5 to 7 separate experiments. $^{a}P < .05$ vs cells cultured under control conditions (0 hour), that is, 6 mmol/L glucose and no insulin. $^{b}P < .05$ vs cells incubated at a high glucose/high insulin concentration during the final 6 hours. One representative blot is shown.

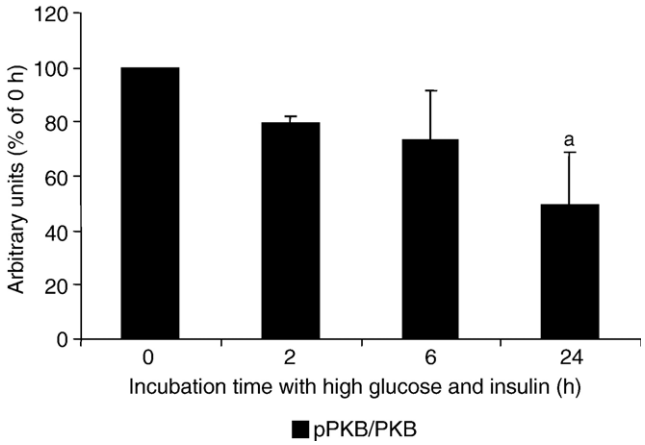

Fig. 6. Acute insulin-stimulated pPKB/PKB ratio after long-term exposure to high glucose and high insulin levels. Cells were incubated at a high glucose (20 mmol/L) and high insulin (10^4 μU/mL) concentration during the final 2, 6, or 24 hours of a total period of 24 hours. After washing, cells were treated for 10 minutes with or without insulin (1000 μU/mL) followed by total cellular lysate preparation as described in Materials and methods. Proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting using appropriate antibodies. The results of densitometry analysis are shown, and all proteins quantified were correlated against the corresponding β-actin level. Data are expressed as arbitrary units in relation to the pPKB/PKB ratio in the control culture condition (ie, pPKB/PKB ratio at 0 hours = 100). Data are expressed as means of 3 to 5 separate experiments. [a]$P < .05$ vs cells cultured under control conditions (0 hour), that is, 6 mmol/L glucose and no insulin.

3.2. Glucose uptake after culture with high glucose and high insulin

Exposure to a combination of high glucose and high insulin (10^4 μU/mL) for 6 hours or more was followed by a reduction of basal glucose uptake capacity by ~40% ($P < .05$, Fig. 2). Interestingly, insulin-stimulated glucose uptake capacity was reduced by ~40% already after 2 hours ($P < .05$) and reached a maximal decline (by ~50%, $P < .05$) after a 6-hour culture in high glucose and high insulin (Fig. 2).

3.3. Glucose uptake after culture with high insulin

Both basal and insulin-stimulated glucose uptake capacity was decreased after 24 hours of incubation with high insulin alone compared with the control situation (by ~50% and ~55% respectively, $P < .05$, Fig. 3), that is, in the same magnitude as when high insulin was combined with a high glucose concentration. Shorter incubations (2 and 6 hours) only decreased insulin-stimulated glucose uptake capacity (by ~30% and ~55%, respectively, $P < .05$; Fig. 3).

3.4. Analyses of insulin signaling proteins and GLUT4

High glucose alone for 2, 6, or 24 hours did not induce any significant alterations in IRS protein content (Fig. 4). Neither were there any significant alterations in insulin's ability to stimulate PKB (data not shown). Glucose transporter 4 protein content was decreased (by ~30%, $P < .05$) after 2-hour incubation in high glucose, whereas longer incubations (6 or 24 hours) did not alter GLUT4 protein content compared with control situation (Fig. 7).

Treatment with high glucose and insulin in combination for at least 6 hours reduced cellular IRS-1 protein content by ~45% or more ($P < .05$, Fig. 5). Insulin receptor substrate 1 protein was significantly lower after a 24-hour culture compared with a 6-hour culture with high glucose and high insulin (by ~65%, $P < .05$). There was a tendency for an

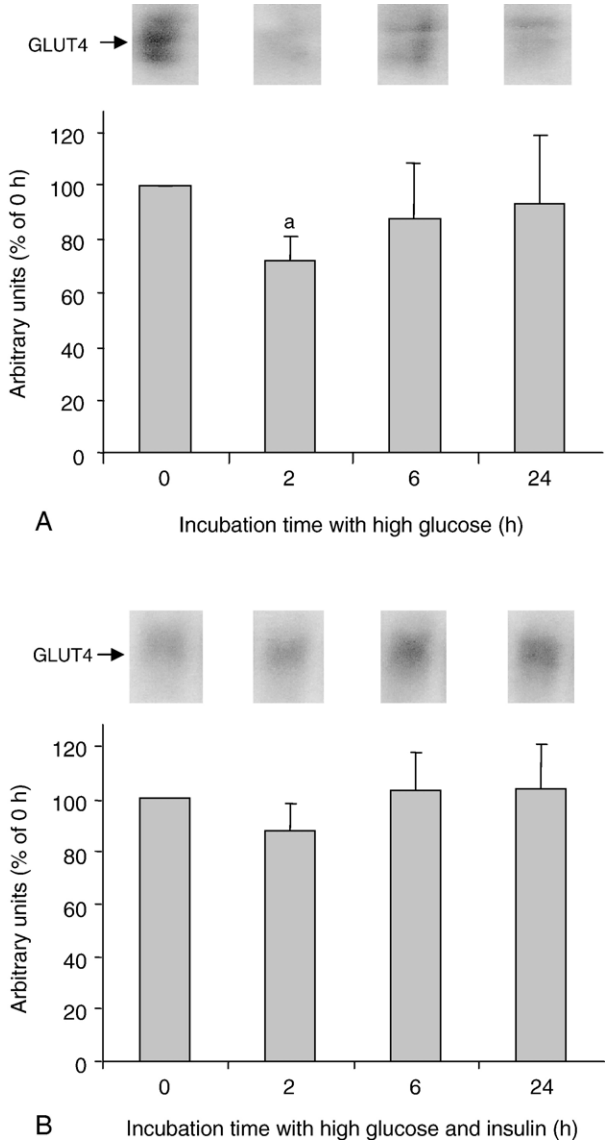

Fig. 7. Glucose transporter 4 protein content in isolated fat cells after exposure to high glucose (A) or high glucose and high insulin (B) levels. Cells were incubated at a high glucose (20 mmol/L) concentration with or without a high insulin (10^4 μU/mL) concentration during the final 2, 6, or 24 hours of a total period of 24 hours. The results of densitometry analyses are shown, and data are expressed as arbitrary units in relation to the amount of target protein in the control situation (0 hour), that is, 24 hours preincubation with low glucose and no insulin. Protein quantification was adjusted for the corresponding β-actin level. Data are expressed as means of 9 (A) and 4 to 7 (B) separate experiments. [a]$P < .01$ vs cells cultured under control conditions (0 hour), that is, 6 mmol/L glucose and no insulin. One representative blot is shown.

increase in IRS-2 ($P = .08$) after a 2-hour culture with high glucose and high insulin, whereas prolonged culture did not significantly alter IRS-2 content compared with the control situation, although there was a tendency for a decrease after 24 hours (by ~30%, P = NS). The high-glucose/high-insulin setting did not affect PKB content (data not shown), but the ability of insulin to phosphorylate PKB (ie, Ser473 phosphorylated protein kinase B [pPKB]/PKB ratio) was reduced by ~50% after 24-hour culture ($P \leq .05$, Fig. 6). In addition, the non–insulin-stimulated pPKB/PKB ratio was enhanced by ~3 to 7 times (data not shown) after high-glucose/high-insulin incubation, but there was no effect on the GLUT4 protein content compared with control situation (Fig. 7).

Cells cultured with high insulin in a physiologic glucose concentration (6 mmol/L) for up to 24 hours showed no significant alterations in IRS-1 protein content (118%, 101%, and 97% at 2, 6 and 24 hours of insulin, respectively, compared with the control situation, n = 4-5). Insulin receptor substrate 2 protein content, however, tended to decrease (by maximum 40% after 24 hours, n = 3, P = NS) much to a similar extent as when cells were preincubated with high glucose and high insulin. Glucose transporter 4 and PKB protein content, as well as insulin's ability to phosphorylate PKB, was not analyzed in the high-insulin setting because of the limited amount of available adipose tissue.

4. Discussion

The present in vitro study demonstrates for the first time that in adipocytes from healthy humans, high glucose and high insulin in combination for 2 hours or more induce pronounced insulin resistance with respect to glucose uptake, with a decrease in IRS-1 protein lagging behind. Pretreatment with high insulin alone for 2 hours or more induced a decrease in glucose uptake capacity comparable with the high-glucose/high-insulin setting; however, no diminishment of IRS-1 protein was evident. Treatment with high glucose alone for 24 hours induced a minute insulin resistance after a 24-hour culture.

High glucose alone for 24 hours induce insulin resistance, but the down-regulating effect of high glucose was much slower and smaller than in the well-established model using rat adipocytes exposed to medium containing high glucose and high insulin [6,7,10,11]. It took 24 hours of incubation with high glucose before a small deterioration in glucose uptake was detected. An in vitro culture for about 24 hours, thus, is probably needed for this effect, and this is supported by data showing that a 16-hour culture with 25 mmol/L glucose did not induce a consistent impairment in glucose uptake in human adipocytes [34]. The present in vivo results can clearly be not directly transferred into the in vivo situation, where fat cell metabolism is influenced by interactions with other cells and tissues. Nonetheless, these data provide information on the direct cellular effects of an altered metabolic milieu. Furthermore, as high blood glucose levels are known to markedly impair glucose utilization in peripheral tissues in vivo, our results indicate that the underlying primary mechanism for this does not involve the adipocytes per se.

The down-regulating effect of high glucose and high insulin in combination is quicker and more pronounced than for glucose alone. Already after a 2-hour culture a significant reduction in insulin-stimulated glucose uptake was evident, reaching a maximum reduction of ~50% at 6 hours of culture in high glucose and high insulin. Interestingly, we found that high insulin alone for 24 hours could negatively affect insulin responsiveness in human adipocytes to the same extent as in the combined high-glucose/high-insulin setting. This is very different to what we have previously found regarding primary rat adipocytes, which are unaffected by long-term insulin treatment [7]. This emphasizes the species difference between rat and human adipocytes, further supported by our present results regarding IRS-2 levels, which in human cells are quite unaffected by the high-glucose/high-insulin setting but markedly down-regulated in primary rat adipocytes [7].

The supraphysiologic concentration of insulin (10^4 μU/mL) in our cultures was used to ensure a maximal insulin stimulation throughout the incubation period despite some degradation of the hormone. We have also performed 24-hour experiments with 100 μU/mL insulin (data not shown, n = 6) and they demonstrate a similar decrease in both basal and insulin-stimulated glucose uptake as with 10^4 μU/mL. This suggests that impaired glucose uptake capacity in adipocytes is seen also after prolonged exposure to high physiologic levels of insulin. The present results are obtained under somewhat nonphysiologic conditions with long-term in vitro cultures that per se may affect the adipocyte by, for example, decreasing glucose uptake capacity (see Glucose uptake after culture with high glucose) and GLUT4 messenger RNA expression [35,36]. However, despite these alterations, cells are still phenotypically adipocytes with respect to both morphology and hormonal responses [32,37-39].

Several studies have shown that seemingly healthy first-degree relatives of type 2 diabetic patients display insulin resistance. The presence of such individuals in the present study could generate misleading results. However, after dividing the subjects into 2 groups, with and without a family history of type 2 diabetes mellitus, respectively, no obvious difference between the 2 groups could be detected regarding glucose uptake capacity or levels of IRSs upon treatment.

Several ways for glucose and insulin to induce insulin resistance are plausible. Because insulin activation of the insulin signaling pathway leading to redistribution of insulin-responsive GLUT4 transporters is necessary for glucose transport in insulin-sensitive tissues like fat and muscle, we studied key proteins in this process. Previously, it has been shown that people with type 2 diabetes mellitus have reduced adipocyte IRS-1 protein levels, whereas IRS-2 was unchanged [19]. To our surprise, we found no effects of

long-term (24-hour) treatment with high glucose on the protein levels of IRS-1 and IRS-2. This finding was in contrast to what we recently reported in primary rat adipocytes, as IRS-1 was down-regulated, whereas IRS-2 was up-regulated in that study [7]. Interestingly, after a high-glucose/high-insulin setting in our present study, IRS-1 content was down-regulated much in accordance with the previously mentioned study in rat adipocytes [7]. This is not due to insulin's ability to induce down-regulation of IRS-1 protein through the proteasome degradation pathway, as shown in rodent cell lines [40-42], because IRS-1 protein levels were unaltered by insulin alone in the present study. Worth mentioning is that a high-glucose/high-insulin setting in rat adipocytes also induces a significant decrease in IRS-2, which could not be seen in human adipocytes. An intriguing discovery was that insulin resistance preceded IRS-1 depletion in the present study, much in accordance with what we recently reported in rat adipocytes [10]. The major difference was that the time course for IRS-1 depletion was quicker in human adipocytes. These data strongly suggest that in adipocytes, IRS-1 depletion is not a primary factor leading to the development of impaired glucose uptake capacity in this model of insulin resistance. Further support for this conclusion comes from the fact that insulin alone induced a decrease in glucose uptake capacity without any alterations in IRS-1 protein content. This also seems true regarding IRS-2 because protein levels were mainly unaffected by the high-glucose and/or high-insulin setting. However, the involvement of the IRSs should not be dismissed because altered phosphorylation and/or activity status of the IRSs could be important for the development of impaired glucose transport and should be investigated further. For example, insulin is able to regulate serine/threonine vs tyrosine phosphorylation of IRS-1 that in the end may affect insulin sensitivity [40,42].

Protein kinase B has been proposed as a candidate for deficient insulin signaling mainly in skeletal muscle [21,22]. The role of PKB in defective insulin signaling in adipocytes is, however, less clear. In adipocytes from patients with type 2 diabetes mellitus, serine phosphorylation of PKB in response to insulin was considerably reduced, although PKB content was left intact [43]. In line with this, our present data indicate that a high-glucose/high-insulin setting impairs insulin's ability to phosphorylate and stimulate PKB in human adipocytes without affecting PKB protein content.

The effect of high glucose with or without high insulin on GLUT4 has not been studied in human adipocytes previously. Although our present report demonstrates that there is no alteration in GLUT4 content after long-term high-glucose/high-insulin treatment in vitro, studies in 3T3-L1 adipocytes cultured in high glucose (25-30 mmol/L) and insulin (~100 μU/mL) suggest alterations in basal cycling or intrinsic activity of GLUT4 [44,45]. This dearrangement is not evident in 3T3-L1 cells cultured in high glucose alone. Our results could accordingly reflect an apparent insulin resistance due to an altered localization of GLUT4, which might be overcome after a reincubation period under physiologic conditions. In our previous report [10], primary rat adipocytes were preincubated for 6 hours in high glucose and high insulin to induce insulin resistance. Subsequent incubation under physiologic conditions for up to 16 hours could only partially restore insulin responsiveness. Studies on GLUT4 recycling kinetics in response to insulin stimulation reveal a rather fast system. For instance, 30 to 60 minutes after subsequent removal of insulin, GLUT4 levels in the plasma membrane are back to basal levels [46,47]. Taken together, it seems unlikely that the observed insulin resistance in solely due to a transient alteration in GLUT4 localization or intrinsic activity. We cannot exclude that alterations in GLUT4 cycling or intrinsic activity occur, and indeed contribute to the observed insulin resistance. Future studies monitoring GLUT4 traffic in human adipocytes are necessary to throw new light on this issue. Because our data show that high concentrations of glucose with or without insulin do not affect the cellular level of GLUT4 in human adipocytes, hyperinsulinemia and/or hyperglycemia per se may not be the reason behind the observed decrease in GLUT4 in adipose tissue in obesity and type 2 diabetes mellitus [24,25].

In conclusion, after only 2 hours of exposure to high glucose and high insulin, adipocytes from healthy humans display insulin resistance with respect to glucose uptake and this precedes IRS-1 depletion, whereas IRS-2 protein content was left unchanged. Thus, IRS-1 depletion appears to be a secondary phenomenon in this model of insulin resistance. High insulin alone decreased glucose uptake to the same extent as when high insulin is combined with a high glucose concentration, but without any alteration in IRS-1 protein content. High glucose alone only induced a subtle down-regulation of glucose uptake capacity, and this was not seen until after 24 hours of exposure, suggesting that hyperglycemia per se could not directly produce major insulin resistance in adipocytes. Importantly, the data suggest that IRS-1 depletion in human adipocytes is a secondary phenomenon in the development of insulin resistance.

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