Regulation of Insulin-Stimulated Glucose Transport by Chronic Glucose Exposure in 3T3-L1 Adipocytes

TOSHIRO HOSAKA, KEN YAGA* AND YOSHIKOMO OKA

Third Department of Internal Medicine, Yamaguchi University School of Medicine, Ube, Yamaguchi 755, Japan
* Yamaguchi Rousai Hospital, Onoda, Yamaguchi, Japan

Abstract. Chronic hyperglycemia causes insulin resistance, termed glucose toxicity. Herein we studied chronic glucose-dependent regulation of the glucose transport system in adipocytes. 3T3-L1 adipocytes were incubated for up to 24 h with low (1 mM) or high (25 mM) glucose, and glucose transport was subsequently analyzed. 100 nM insulin was present throughout the experiments. 24 h incubation with 1 mM glucose caused a 2.3±0.4 fold increase in glucose transport activity, compared to the values obtained with 25 mM glucose. This difference was not observed when 24 h incubation was carried out without insulin. Glucose transport activity was not increased at 3 or 6 h incubation with 1 mM glucose, but was increased at 12 h, which closely paralleled increased expression of GLUT1. In addition to increased GLUT1 expression, more efficient translocation of GLUT1 to the plasma membrane was observed when incubated with 1 mM glucose compared to 25 mM glucose. The addition of azaserin or deprivation of glutamine at 25 mM glucose did not increase the glucose transport activity to the level obtained with 1 mM glucose. PD98059 did not affect glucose transport activity when incubated with 1 mM or 25 mM glucose. In conclusion, the present study is the first to show that, in 3T3-L1 adipocytes, chronic exposure to low (1 mM) and high (25 mM) glucose leads to different insulin-stimulated glucose transport activities. These differences result from the difference in the expression and plasma membrane distribution of GLUT1, but not of GLUT4, and the hexosamine biosynthesis pathway or extracellular signal-regulated protein kinase is not involved.

Key words: insulin resistance, glucose transporter, 3T3-L1 adipocytes

Insulin resistance exists in the pathological states including type 2 diabetes mellitus and obesity, which is largely due to decreased insulin-stimulated glucose transport activity in insulin-sensitive tissues including the adipose tissue and skeletal muscle [1, 2], but the molecular mechanism of insulin resistance is still not fully understood.

It was first demonstrated by Marshall and colleagues that prolonged exposure of primary cultured adipocytes to insulin decreased subsequent insulin-stimulated glucose uptake due to reduced insulin sensitivity [3]. This phenomenon is designated insulin-induced desensitization. In addition to insulin, glucose was also shown to affect insulin sensitivity. Correction of hyperglycemia by increasing glucosuria with phlorizin normalized insulin sensitivity in streptozotocine-induced diabetic rats without altering insulin secretion [4]. This phenomenon is termed glucose-induced desensitization or glucose toxicity. Marshall and colleagues, by using primary cultured rat adipocytes, have proposed that an increase in glucose efflux through the hexosamine biosynthesis pathway contributes to hyperglycemia-induced insulin desensitization [5]. They also found that the presence of insulin was required for hyperglycemia-induced desensitization of the glucose transport system [6].

Insulin-induced desensitization was further characterized by many investigators, with 3T3-L1 adipocytes [7-13]. Recently, Frost and colleagues...
reported that chronic insulin exposure of 3T3-L1 adipocytes caused insulin desensitization of the glucose transport system in the presence of high glucose concentrations, due to a decrease in GLUT4 expression, possibly at the post-translational level [14].

We examined whether chronic glucose exposure in the presence of high concentrations of insulin affected the subsequent insulin-stimulated glucose uptake in 3T3-L1 adipocytes. We report herein that insulin-stimulated glucose transport activity in chronic incubation with 1 mM and with 25 mM glucose significantly differed, which resulted from differences in the expression level and subcellular distribution of GLUT1, but not of GLUT4. We also examined whether the hexosamine biosynthesis pathway or mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) cascade was involved in these differences.

**Materials and Methods**

*Antibodies* – Anti-GLUT1 antiserum and anti-GLUT4 antiserum were raised against synthetic peptides corresponding to residues 478-492 of rabbit GLUT1 [15] and residues 495-509 of rat GLUT4 [16], respectively, as described previously.

*Cell Culture* – 3T3-L1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose and 10% donor calf serum (Nalgene) in an atmosphere of 10% CO₂ at 37°C. Two days after the fibroblasts had reached confluence, differentiation was induced by treating the cells with DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine, 4 mg/ml dexamethasone, 25 mM glucose, and 10% fetal bovine serum for 48 h. The cells were refed with DMEM containing 5 mM glucose supplemented with 10% fetal bovine serum every other day for the next 6-10 days. More than 90% of the cells expressed the adipocyte phenotypes [17].

*Glucose Transport Assay* – 24 h prior to the start of the experiment, 3T3-L1 adipocytes in 24 well culture dishes were refed with DMEM containing 1 mM or 25 mM glucose, 10⁻⁷ M insulin and 10% fetal bovine serum. The cells were then incubated with Krebs-Ringer phosphate buffer containing 10⁻⁷ M insulin for 15 min at 37°C, and 2-deoxy-D-[¹H]glucose uptake was measured as described previously [17].

*Immunoblotting* – 3T3-L1 adipocytes were lysed and boiled in Laemmli buffer containing 10 mM dithiothreitol and subjected to 10% SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters. The filters were incubated with antisera, subsequently with anti-IgG antibody linked to horseradish peroxidase (Amersham), and exposed to ECL-hyper film (Amersham).

*Subfractionation of 3T3-L1 Adipocytes* – Fractionation of subcellular membranes from 3T3L-1 adipocytes was carried out essentially as described previously [18]. Aliquots of subcellular membrane fractions containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted as described above.

*Mitogen-activated protein (MAP) kinase assay* – The cells were pretreated for 24 h as described for glucose transport assay and then stimulated with 10⁻⁷ M insulin for 5 min at 37°C. The MAP kinase assay was then performed as previously described [18]. In short, the cells were washed, frozen at −70°C, and lysed in lysis buffer [20 mM tris(hydroxymethyl)aminomethane(Tris)-HCl, pH 7.5, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin, 2 mM dithiothreitol, and 100 mM NaCl], followed by partial purification with Q-Sepharose (Pharmacia). The MAP kinase activity was determined by means of a BIOTRAK MAP kinase enzyme assay (Amersham).

*Statistical analysis* – Data are presented as the means±S.D. The statistical significance of differences between mean values was assessed by Student’s t-test.

**Results**

**Chronic regulation of glucose transport activity by glucose**

In this study, 3T3-L1 cells were cultured at a glucose concentration of 5 mM for 6-10 days during the process of differentiation. The concentration of glucose was then changed from 5 mM to a low (1 mM) or a high (25 mM) concentration, and the adipocytes were incubated for 24 h in the presence of 10⁻⁷ M insulin. The glucose transport activity was
subsequently measured in the presence of $10^{-7}$ M insulin.

As shown in Fig. 1, cells incubated at a low (1 mM) glucose concentration for 24 h exhibited a significantly greater glucose transport activity ($2.3 \pm 0.4$ fold, mean±S.D., $n=4$, $p<0.01$) than those incubated at high (25 mM) glucose. The different effects of low and high glucose concentrations on subsequent glucose transport activity were insulin-dependent; no difference in glucose transport activity was observed when 24 h incubation was performed without insulin. A significant difference in glucose transport activity was also observed between the cells incubated with 1 mM and those with 5 mM glucose. The glucose transport values in cells incubated with 5 mM glucose were rather similar to those with 25 mM glucose (data not shown).

We then examined the time-course of the effects. Cells were incubated for 3, 6, 12 and 24 h with 1 mM or 25 mM glucose in the presence of insulin, and glucose transport activity was subsequently measured. The glucose transport activity was significantly increased at 12 h incubation, compared with 3 or 6 h incubation, at 1 mM glucose ($P<0.05$) (Fig. 2). The transport activity tended to be further increased at 24 h incubation with 1 mM glucose, but without statistical significance, compared to the values at 12 h. In contrast to the results obtained by incubation with 1 mM glucose, glucose transport activity of the cells incubated with 25 mM glucose stayed at similar levels during the 24 h incubation period.

**Glucose transporter expression and subcellular distribution**

Because glucose transport activity is mediated by facilitative glucose transporters, GLUT1 and GLUT4, in 3T3 L1 adipocytes, expression of these transporter isoforms was examined by immunoblotting. As shown in Fig. 3A, expression of GLUT1 was increased at 12 h incubation with 1 mM glucose, and further increased at 24 h incubation when compared with the expression level at 3 or 6 h. No change in the GLUT1 expression level was observed during 24 h incubation when cells were incubated with 25 mM glucose. GLUT4 expression levels of cells incubated with 1 and 25 mM glucose for 24 h were similar (Fig. 3B). Interestingly, the increase in GLUT1 expression was insulin-dependent; no increase was observed when 24 h incubation was performed without insulin (data not shown).

Incubation with 1 mM glucose in the presence of $10^{-7}$ insulin for 24 h not only increased GLUT1 expression but also changed its subcellular distribution. The cells were homogenized and fractionated by differential centrifugation, yielding the plasma mem-

![Fig. 1. Effects of 24 h incubation with 1 mM and 25 mM glucose on subsequent glucose transport activity.](image-url)

**Fig. 1.** Effects of 24 h incubation with 1 mM and 25 mM glucose on subsequent glucose transport activity. Differentiation of 3T3-L1 fibroblasts to adipocytes was induced as described in Materials and Methods. The cells were cultured at a glucose concentration of 5 mM during the differentiation process (6–10 days) and then further incubated with 1 mM glucose or 25 mM glucose for 24 h in the presence or absence of $10^{-7}$ M insulin. The cells were then incubated with Krebs-Ringer phosphate buffer containing $10^{-7}$ M insulin for 15 min at 37°C, and 0.1 mM 2-deoxy-D-[3H]glucose uptake for 4 min at 37°C was measured. Data are expressed as the mean±S.D. of four independent experiments each performed in triplicate.
brane fraction and the low density microsome fraction which represents the intracellular membrane. As shown in Fig. 4, immunoblotting of these membrane fractions revealed that subcellular distribution of GLUT1 in the plasma membrane and the low density microsomes occurred more in the plasma membrane in cells incubated with 1 mM glucose than in those incubated with 25 mM glucose. Taking into account that the amount of protein recovered in low density microsomes was approximately one third that in plasma membrane, more than 90% of cellular GLUT1 was estimated to be present in the plasma membrane when incubated with 1 mM glucose, whereas only 60-70% of GLUT1 was in the plasma membrane when incubated with 25 mM glucose.

**Hexosamine biosynthesis pathway and MEK/ERK pathway**

It has been proposed that glucose-induced reduc-
tion in glucose transport is induced by stimulation of the hexosamine biosynthesis pathway in primary rat adipocytes, in which fructose-6-phosphate (F6P) is converted to glucosamine-6-phosphate (Gln6P), with glutamine acting as a donor of its amido group [5, 19, 20]. We therefore examined the possible involvement of the hexosamine biosynthesis pathway in 3T3-L1 adipocytes under our experimental conditions.

When cells were incubated for 24 h with 1 mM glucose plus 2 mM glucosamine, glucose transport activity was markedly decreased compared to that in cells incubated with 1 mM glucose alone, and rather lower, but with no statistical significance, than that obtained with 25 mM glucose (Fig. 5). These results suggested that the hexosamine biosynthesis pathway might contribute, at least in part, to the difference in glucose transport activity observed between incubation with 1 mM and 25 mM glucose. We therefore tried to inhibit the hexosamine biosynthesis pathway at 25 mM glucose by incubation with azaserin (20 \(\mu\)M) or by omitting glutamine from the incubation medium. Glucose transport activity was not, however, increased by these treatments. The glucose transport activities in cells incubated for 24 h with 25 mM glucose, 25 mM glucose plus 20 \(\mu\)M azaserin,
and 25 mM glucose without glutamine in the media, were similar and much lower than the value observed in cells incubated with 1 mM glucose (Fig. 6).

Since insulin is required to induce an increase in glucose transport activity and GLUT1 expression at the 1 mM glucose concentration, possible insulin signaling toward GLUT1 expression was studied. Insulin was shown to activate Ras, and microinjection of constitutively active Ras into 3T3-L1 adipocytes and overexpression of Ha-Ras into adipocytes in transgenic mice were reported to increase GLUT1 expression [21, 22]. One of the downstream signaling cascades from Ras activation is Ras $\rightarrow$ Raf$-1$ $\rightarrow$ MAPK/ERK kinase (MEK) $\rightarrow$ ERK cascade. We therefore studied whether this cascade is involved in our observation. MAP kinase activity was increased approximately 1.5-fold by 1 mM glucose incubation, compared with 25 mM glucose incubation, and the increase was blocked by incubation of cells with 50 $\mu$M PD98059, MAPK/ERK kinase (MEK) specific inhibitor [23] (data not shown). Nevertheless, the presence of 50 $\mu$M PD98059 during the chronic incubation period did not affect glucose transport activity (Fig. 7) or GLUT1 expression (data not shown).

**Fig. 6.** Effects of incubation with azaserin or omitting glutamine from the incubation media on subsequent glucose transport activity. 3T3-L1 adipocytes were incubated for 24 h with 1 mM glucose, 25 mM glucose, 25 mM glucose plus 20 $\mu$M azaserin, or 25 mM glucose but without glutamine in the incubation media, in the presence of $10^{-7}$ insulin. 0.1 mM 2-deoxy-D-[3H]glucose uptake was then measured in the presence of $10^{-7}$ M insulin. Data are expressed as the mean±S.D. for three independent experiments each performed in triplicate.

**Fig. 7.** Effects of PD98059 on subsequent glucose transport activity. 3T3-L1 adipocytes were incubated for 24 h with 1 mM glucose or 25 mM glucose in the presence of $10^{-7}$ M insulin, with or without 50 $\mu$M PD98059, a MAP-kinase-kinase specific inhibitor. 0.1% dimethylsulfoxide (DMSO) was needed to dissolve PD98059, and thus incubation with 0.1% DMSO alone was also included as another control. Data are expressed as the mean±S.D. for three independent experiments each performed in triplicate.
Discussion

We found that insulin-stimulated glucose transport activity in 3T3-L1 adipocytes incubated for 24 h with low (1 mM) glucose was significantly greater than that in cells incubated with high (25 mM) glucose, but only when insulin was present during 24 h incubation. This finding was not previously reported, even though many studies have been performed on insulin resistance with 3T3-L1 adipocytes. This is mainly because most of the previous studies with 3T3-L1 adipocytes have focused on insulin-induced desensitization of the glucose transport system [3, 7-13]. Effects of glucose on subsequent glucose transport activity were previously characterized in 3T3-L1 adipocytes, but by a comparison of the absence and presence of glucose [24], and therefore the results most likely reflected glucose-deprivation effects. Glucose deprivation increased glucose transport activity in 3T3-L1 adipocytes and this increase was associated with the appearance of a lower molecular weight form of GLUT1, which probably represented a deglycosylated form of GLUT1 [24, 25]. In our experiments at 1 mM glucose, however, no appearance of a lower molecular weight form of GLUT1 was observed. In addition, the results were similar whether or not the incubation media were replaced every 8 hours (data not shown). These results indicate that our observation did not reflect glucose deprivation effects.

GLUT1 protein levels were increased when incubated with 1 mM glucose in the presence of insulin, compared to incubation with 25 mM glucose. The increase in GLUT1 protein during 24 h incubation closely paralleled the increase in glucose transport activity, strongly suggesting that increased cellular GLUT1 protein levels contribute to increased glucose transport activity. Although hyperosmolarity (300 mM) was shown to increase GLUT1 expression 1.7-fold through activation of signaling modules including p38 MAPK in L6 muscle cells [26], the present results were opposite; low glucose (1 mM) increased GLUT1 expression. In addition, the difference in glucose transport activity was observed only in the presence of insulin. The osmotic difference between 1 and 25 mM glucose is therefore highly unlikely to account for the difference in glucose transport activity and GLUT1 expression. Of interest is the finding that subcellular distribution of GLUT1 was also different in incubation with 1 mM glucose from that with 25 mM glucose. GLUT1 was more preferentially present in the plasma membrane when incubated with 1 mM glucose. The major mechanism whereby insulin acutely stimulates glucose transport activity is translocation of glucose transporter from the cell interior to the cell surface. Cells incubated with 1 mM glucose is therefore more sensitive to insulin in terms of translocation of glucose transporter than cells incubated with 25 mM glucose. These results indicate that, in addition to increased GLUT1 protein levels, efficient translocation of GLUT1 to the plasma membrane contributes to increased glucose transport activity when incubated with 1 mM glucose.

Glucose effects have been more extensively studied in primary rat adipocytes than in 3T3-L1 adipocytes. We therefore looked for experimental conditions similar to ours in previous studies with primary rat adipocytes. When the previous results were analyzed carefully, insulin-stimulated glucose transport after 24 h exposure to 1 mM glucose in the presence of insulin was rather slightly decreased compared to that without insulin (Fig. 1 in ref. 6), although the results were not cited in the text. These results apparently differ from our results obtained in 3T3-L1 adipocytes: increased glucose transport activity in the presence of insulin. It should be pointed out that glucose transporter expression in primary rat adipocytes and 3T3-L1 adipocytes differ, although both cells contain GLUT1 and GLUT4. GLUT4 represents 97% of the glucose transporter pool [15] in rat adipocytes, whereas GLUT4 represents only 33% of the glucose transporter pool in 3T3-L1 adipocytes [6] so that the contribution of GLUT1 to cellular glucose transport activity is much greater in 3T3-L1 adipocytes than in primary rat adipocytes. This difference in glucose transporter isoform expression may, at least in part, account for the different results obtained in 3T3-L1 adipocytes and rat adipocytes.

Chronic glucose effects on subsequent glucose transport activity were first demonstrated by Marshall and colleagues in rat adipocytes [6]. They later proposed that increased glucose efflux into the hexosamine biosynthesis pathway is responsible for hyperglycemia-induced insulin resistance [5].
presence of glucosamine in the incubation medium with 1 mM glucose abolished the difference between incubation with 1 mM and 25 mM glucose in glucose transport activity. This result is not inconsistent with the notion that the results obtained with 25 mM glucose incubation resulted from an increased hexosamine biosynthesis pathway, but neither adding azaserin nor omitting glutamine restored the low glucose transport activity with 25 mM glucose to the level obtained with 1 mM glucose. These results indicate that a difference in the hexosamine biosynthesis pathway, if it exists, is not involved in the difference between 1 mM and 25 mM in glucose transport activity. Similar to our observation in 3T3-L1 adipocytes, no involvement of the hexosamine biosynthetic pathway in glucose-induced reduction in glucose transport was reported in cultured L6 myotubes [19].

Since cells incubated with 1 mM glucose were more sensitive to insulin in terms of glucose transporter translocation than cells incubated with 25 mM glucose, we speculated that insulin signaling toward the nucleus was also increased in cells incubated with 1 mM glucose, thus leading to increased expression of GLUT1. Nevertheless, the results obtained with PD98059 indicate that one of the insulin signaling cascades to the nucleus, Ras-Raf-MEK-ERK, does not appear to be involved in an increase in GLUT1 expression. Similar results were reported in L6 myoblasts [27]. Chronic insulin treatment of these cells increased glucose transport activity due to increased biosynthesis of GLUT1 and GLUT3, but the introduction of dominant negative Ras failed to inhibit the insulin-induced increase in GLUT1 [27].

The molecular mechanisms whereby different concentrations of glucose (1 mM and 25 mM glucose) causes different insulin sensitivity of glucose transporter translocation as well as different expression levels of GLUT1, but not GLUT4, are not yet clear, and further investigation is needed. One of the possible mechanisms is protein kinase C (PKC) activation by high concentrations of glucose, which was shown to parallel inhibition of insulin receptor kinase [28, 29]. In any case, the cells presented in this study provide an intriguing model to investigate the molecular mechanism for the regulation of glucose transport, which is apparently determined by complex interplay between insulin and glucose.

In conclusion, the present study is the first to show that in 3T3-L1 adipocytes chronic exposure to different concentrations of glucose, low (1 mM) and high (25 mM) glucose, in the presence of insulin, leads to different insulin-stimulated glucose transport activities. The different transport activities are attributed to the difference in the expression and plasma membrane distribution of GLUT1, but not of GLUT4, and the hexosamine biosynthesis pathway or MEK-ERK cascade is not involved in these differences.

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References

GLUCOSE EFFECTS ON ADIPOCYTE GLUCOSE TRANSPORT


