Increased flux through the hexosamine biosynthesis pathway inhibits glucose transport acutely by activation of protein kinase C

Anthony FILIPPIS*, Stella CLARK† and Joseph PROIETTO*‡

*The University of Melbourne, Department of Medicine, Royal Melbourne Hospital, Parkville 3050, Victoria, Australia, and †Baker Medical Research Institute, Prahran 3181, Victoria, Australia

The hexosamine biosynthesis pathway and protein kinase C (PKC) activation mediate hyperglycaemia-induced impaired glucose transport, but the relative role of each pathway is unknown. Following a 2 h preincubation of rat adipocytes in the presence of either high glucose (30 mM) plus insulin (0.7 nM) or glucosamine (3 mM), both high glucose and glucosamine inhibited subsequent basal and insulin-stimulated glucose transport, measured at 5.0 mM glucose. Azaserine, an inhibitor of the enzyme glutamine:fructose-6-phosphate aminotransferase, abolished the effect of high glucose, but not that of glucosamine. Ro-31-8220, an inhibitor of PKC, reversed the effects of both high glucose and glucosamine, suggesting that flux through the hexosamine biosynthesis pathway impaired glucose transport

acutely by activating PKC. Both high glucose and glucosamine caused a 3-fold increase in PKC activity; this effect of high glucose, but not that of glucosamine, was partially decreased by azaserine. Neither high glucose nor glucosamine altered basal or insulin-stimulated plasma membrane GLUT1 levels, whereas both treatments decreased basal, but not insulin-stimulated, GLUT4 levels. Azaserine abolished the effect of high glucose, but not that of glucosamine, on basal plasma membrane GLUT4 levels. Ro-31-8220, which returned glucose transport to control values, caused a further decrease in plasma membrane GLUT4 levels. It is concluded that, in rat adipocytes, an acute increase in flux through the hexosamine biosynthesis pathway inhibits glucose transport by activation of PKC.

INTRODUCTION

High glucose concentrations have been shown to cause impaired glucose transport in both adipocytes and muscle cells *in vitro* [1] and *in vivo* [2]; however, the biochemical basis for this phenomenon is not well understood. Previous work has implicated increased glucose flux through the hexosamine biosynthesis pathway (HBP) [3,4]. Glucose enters the glycolytic pathway, where the majority is metabolized to pyruvate; however, a small quantity of fructose 6-phosphate combines with the amino acid glutamine to form glucosamine 6-phosphate. The rate-limiting enzyme controlling this step, and hence flux through the whole pathway, is glutamine:fructose-6-phosphate aminotransferase (GFAT; EC 2.6.1.16). Glucosamine 6-phosphate is further metabolized to UDP-*N*-acetylglucosamine, which serves as an essential precursor for the synthesis of glycoproteins, glycolipids and proteoglycans [5]. In the presence of hyperglycaemia, flux through the HBP increases. Adipocytes preincubated for 24 h with high glucose in the presence of insulin develop a defect in both basal and insulin-stimulated glucose transport [1]. This effect has been shown to be glutamine-dependent [6], and can be blocked by inhibitors of GFAT such as *O*-diazoacetyl-L-serine (azaserine) [3].

Glucosamine, like excess glucose, is metabolized via the HBP [3]; however, it enters the pathway distal to GFAT and is phosphorylated rapidly to give glucosamine 6-phosphate. It has been shown that glucosamine is 40 times more potent than glucose in mediating desensitization of the glucose transport system when used at a concentration 10 times lower than that of glucose [3]. While the evidence supporting a role for the HBP in

the glucose-induced desensitization of glucose transport is convincing, it is not clear how it causes these effects.

It has been postulated that protein kinase C (PKC) activation may be involved in mediating impaired glucose transport [7]. Elevated glucose levels have been shown to increase the activity of PKC in bovine retinal epithelial cells [8], in rat glomeruli [9] and in rat adipocytes [10]. Studies in intact cells have demonstrated that high glucose concentrations can increase the *de novo* synthesis of diacylglycerol (DAG) [11], the natural activator of some isoforms of PKC. Activation of PKC has been shown by Bollag et al. [7] and Takayama et al. [12] to phosphorylate the insulin receptor and decrease its tyrosine kinase activity, which has downstream effects on signal transduction [13]. This suggests that PKC activation may interfere with glucose transporter translocation, causing impaired glucose transport. The relative contributions of glucose-mediated PKC activation and the metabolism of glucose via the HBP on the desensitization of glucose transport have not been examined. This was the aim of the present study, which focused primarily on the effects of short-term incubation with high glucose on glucose transport and GLUT1 and GLUT4 translocation.

EXPERIMENTAL

Materials

PVDF membranes were purchased from Schleicher and Schuell (Dassel, Germany), human insulin from Novo-Nordisk A/S (North Rocks, NSW, Australia) and 2-deoxy-D-[U-¹⁴C]glucose from Amersham International (Amersham, Bucks., U.K.). 3-*O*-Methyl-D-[U-¹⁴C]glucose was purchased from Dupont NEN

Abbreviations used: DAG, diacylglycerol; GFAT, glutamine:fructose-6-phosphate aminotransferase; HBP, hexosamine biosynthesis pathway; LDM, low-density microsomal membrane(s); PKC, protein kinase C; PM, plasma membrane(s).

‡ To whom correspondence should be addressed.

(Boston, MA, U.S.A.), and collagenase (Type 5) was from Worthington Biochemicals (Frehol, NJ, U.S.A.). Adenosine deaminase, azaserine and all other reagent-grade chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Animals

Male Sprague–Dawley rats (~200 g) were purchased from Monash University Animal Facility (Clayton, Victoria, Australia) and were fed on standard laboratory chow (Barastoc Products, Clark King, Victoria, Australia).

Adipocyte preparation

Rats weighing ~200 g were killed by CO₂ gassing and cervical dislocation. Rat epididymal fat pads were collected and adipocytes were obtained by collagenase digestion (1.5 mg/ml), using the procedure of Rodbell [14] as modified by Kelada et al. [15].

Adipocyte preincubation

Following digestion, cells (1×10^6 cells/ml) were distributed into 25 ml polypropylene flasks (10 ml/flask), with the adipocytes floating on a thin layer of buffer in an atmosphere of carbogen (5% CO₂ and 95% O₂) with shaking (100 rev./min) at 37 °C. Preincubations were initiated by the addition of low glucose (5 mM) or high glucose (30 mM) in the presence of insulin (0.7 nM) for 2 h. Glucose alone has a low rate of entry into adipocytes, and insulin is necessary to promote glucose entry into the cells. In some experiments, adipocytes were preincubated with glucosamine (3 mM) for 2 h. To keep the basal adenosine levels low, adenosine deaminase (2 units/ml) was added to each of the preincubation flasks. Following the 2 h preincubation, adipocytes were washed three times in equal volumes of glucose- and insulin-free Krebs–Ringer Hepes buffer. The washing procedure effectively removed all extracellular insulin, glucose or glucosamine and allowed the glucose transport system to be deactivated to basal levels. After this time, 2.5 ml aliquots were removed from each flask for determination of glucose uptake rates. The remainder of the cells from each quadruplicate incubation were pooled, washed and fractionated to yield plasma membrane (PM) fractions as described below.

Glucose uptake assays

2-Deoxyglucose was used as the index of glucose transport and was measured as previously described [15,16]. After washing, adipocytes were resuspended in Krebs–Ringer Hepes buffer (2.5 ml) at pH 7.4 and incubated in the absence or the presence of various insulin concentrations with 5.0 mM glucose in a shaking water-bath for 30 min at 37 °C. Adipocytes were incubated with 2-deoxy-D-[U-¹⁴C]glucose (0.1 mM at 0.25 μ Ci/tube) for 10 min. To confirm that changes in glucose uptake were due to changes in transport and not phosphorylation, 3-*O*-methylglucose transport was measured in some experiments, as described previously [17,18]. Briefly, aliquots (200 μ l) of adipocytes (1×10^6 cells/ml) were mixed with 500 μ l of 3-*O*-methylglucose (final concentration 0.5 mM) and 0.1 μ Ci of 3-*O*-methyl-D-[U-¹⁴C]glucose. The assay was commenced by the addition of cells and terminated by the addition of 1 mM ice-cold phloretin. Cells were incubated for 10 s to measure basal and for 2 s to measure insulin-stimulated uptake rates. Cells were separated from buffer by centrifugation through oil (14250 *g*; 1 min), and cell-associated radioactivity was determined using a liquid scintillation counter.

PKC assays

Resting adipocytes were treated with high glucose or with glucosamine as described above. Cell suspensions were washed with calcium- and magnesium-free homogenization buffer consisting of 20 mM Tris, 1 mM EDTA, 0.25 M sucrose, pH 7.4, 400 μ M PMSF and 10 μ g/ml leupeptin. The cell suspension was homogenized in a Janke and Kunkel (Staufen, Germany) homogenizer and the homogenate was centrifuged at 6000 *g* for 2 min. The soluble fraction was retained and centrifuged at 31000 *g* for 20 min. The membrane pellet was solubilized with 0.1% Triton X-100 at 4 °C for 45 min and was centrifuged (31000 *g* for 20 min) to obtain the detergent-solubilized membrane fraction. Cytosolic and membrane fractions were subjected to Mono-Q chromatography in order to remove interfering materials and excess detergent [19]. PKC activity was measured using the Biotrak® PKC assay system from Amersham.

DAG assays

The accumulation of DAG in adipocytes incubated in the presence of low or high glucose and insulin was determined by measuring the conversion of [U-¹⁴C]glucose into [¹⁴C]DAG, as described by Dunlop et al. [20].

Subcellular fractionation of adipocytes

PM and low-density microsomal membranes (LDM) were prepared from the incubated adipocytes by the standard technique of differential ultracentrifugation as employed by Kelada et al. [15]. Following incubation, cells were washed three times in Krebs–Ringer Hepes buffer and homogenized (14 strokes at setting 7; equivalent to 1400 rev./min) in a Janke and Kunkel homogenizer, in a buffer containing 20 mM Tris, 1 mM EDTA and 0.25 M sucrose, pH 7.4. All subsequent steps were carried out at 4 °C using this buffer. The homogenate was then centrifuged as previously described [15]. The membrane pellets were resuspended in buffer containing two protease inhibitors (400 μ M PMSF and 10 mg/ml leupeptin) and stored at –70 °C.

Determination of glucose transporter levels

Proteins from the PM and LDM fractions were separated by SDS/PAGE on 10% gels using the system of Laemmli [21] and electrophoretically transferred to PVDF membranes before immunoblotting using a modification of the methods described by Towbin et al. [22] and Burnette [23]. The PVDF membranes were blocked with 5% skim milk and incubated with either an anti-GLUT4 polyclonal antibody (R1159) generated to a peptide consisting of the last 12 amino acids of the C-terminal region of GLUT4 [15] or an anti-GLUT1 polyclonal antibody (AP 4/93), generously supplied by Professor David James (University of Queensland, Brisbane, Queensland, Australia). Enhanced chemiluminescence was used for detection, and autoradiograms were quantified by laser densitometry.

Protein determination

Protein concentrations were determined by the Coomassie Brilliant Blue method described by Bradford [24], using crystalline BSA as the standard (Bio-Rad assay).

Statistical analysis

Significance was determined using Student's *t* test for paired data.

RESULTS AND DISCUSSION

Preincubation of adipocytes with high glucose (30 mM) (in the presence of 0.7 nM insulin) or glucosamine (3.0 mM) for only 2 h inhibited both basal and submaximal (0.7 nM) insulin-stimulated glucose transport (Table 1). The decrease in glucose transport in the insulin-stimulated state was due to a lower basal transport rate, as the fold stimulation by insulin remained the same. High glucose did not inhibit maximal (7.0 nM) insulin-stimulated glucose transport (results not shown). 2-Deoxyglucose is readily taken up by adipocytes and undergoes hexokinase-mediated phosphorylation on entering the cell interior. Although hexokinase activity is normally not rate-limiting in adipocytes, the argument can be made that, under certain experimental conditions, phosphorylation could become rate-limiting and thus confound accurate measurements of glucose transport activity [18]. To address this potential problem, additional studies were performed using 3-*O*-methylglucose, which cannot undergo phosphorylation. As shown in Table 2, the results obtained using 3-*O*-methylglucose mirrored those using 2-deoxyglucose, confirming that the changes observed were due to changes in glucose transport and not phosphorylation. Preincubation of adipocytes with glucose alone, insulin alone or 30 mM mannitol for 2 h had no effect on basal or insulin-stimulated glucose transport (results not shown). The GFAT inhibitor azaserine (20 μ M) prevented the glucose-mediated inhibition of glucose transport, but not that caused by glucosamine, which enters the pathway beyond GFAT (Table 1). In fact, in cells preincubated with both glucose and azaserine there was a significant increase above basal glucose transport rates (Table 1), an effect that was blocked by Ro-31-8220 (control, 1.01 ± 0.0 ; high glucose + azaserine + Ro-31-8220, 1.0 ± 0.02). Surprisingly, the PKC inhibitor Ro-31-8220 prevented the inhibition of glucose transport by both glucose and glucosamine (Table 1), suggesting that flux through the HBP might inhibit glucose transport via activation of PKC.

To test whether flux through the HBP activates PKC, total PKC activity in the particulate fraction was measured following treatment of adipocytes with 30 mM glucose or 3 mM glu-

Table 1 Effects of glucose and glucosamine on 2-deoxyglucose transport in rat adipocytes

Adipocytes were preincubated in the absence or in the presence of high glucose (30 mM) plus insulin (0.7 nM), or with glucosamine (3 mM), for a total of 2 h at 37 °C. In other experiments cells were preincubated in the presence of the GFAT inhibitor azaserine (20 μ M) for 30 min or the PKC inhibitor Ro-31-8220 (10 μ M) for 10 min. Cells were then washed to remove extracellular insulin, glucose or glucosamine prior to further stimulation with 0.7 nM insulin for 30 min. 2-Deoxyglucose transport rates were then measured in quadruplicate in 5–15 separate experiments as described in the Experimental section. Results are presented as fold change over basal activity in the absence of any additions. * $P < 0.05$ compared with respective control. Basal transport rates were 338 ± 49 , 346 ± 51 and 352 ± 35 pmol/min per 10^6 cells in control, azaserine and Ro-31-8220 conditions respectively.

	2-Deoxyglucose transport (fold increase)		
	Control	High glucose	Glucosamine
Basal	1.0 ± 0	$0.74 \pm 0.04^*$	$0.50 \pm 0.20^*$
+ Insulin	2.79 ± 0.31	$2.11 \pm 0.32^*$	$1.86 \pm 0.30^*$
+ Azaserine (20 μ M)			
Basal	1.0 ± 0	$1.33 \pm 0.08^*$	$0.55 \pm 0.16^*$
+ Insulin	3.02 ± 0.26	2.91 ± 0.18	$1.93 \pm 0.16^*$
+ Ro-31-8220 (10 μ M)			
Basal	1.0 ± 0	1.03 ± 0.03	1.05 ± 0.03
+ Insulin	2.77 ± 0.14	2.76 ± 0.18	2.75 ± 0.17

Table 2 Effects of glucose and glucosamine on 3-*O*-methylglucose transport in rat adipocytes

Adipocytes were preincubated as described in the legend to Table 1. 3-*O*-Methylglucose transport was measured in duplicate in four to five separate experiments as described in the Experimental section. Results are presented as fold change over basal activity in the absence of any additions. * $P < 0.05$; ** $P < 0.005$ compared with respective control. The basal transport rate was 539 ± 41 pmol/min per 10^6 cells.

	3- <i>O</i> -Methylglucose transport (fold increase)		
	Control	High glucose	Glucosamine
Basal	1.0 ± 0	$0.74 \pm 0.06^*$	$0.66 \pm 0.05^{**}$
+ Insulin	5.63 ± 0.42	$4.32 \pm 0.30^*$	$4.25 \pm 0.33^*$

cosamine. Both high glucose and glucosamine stimulated PKC activity approx. 3-fold (Table 3). The GFAT inhibitor azaserine caused a significant decrease in PKC activity in adipocytes pretreated with high glucose, but had no effect on those treated with glucosamine (Table 3), confirming that flux through the HBP is involved in glucose-mediated PKC activation. The residual PKC activity in glucose-treated cells could be mediated via an increase in the *de novo* synthesis of DAG, the natural activator of some isoforms of PKC [25,26]. We measured the *de novo* synthesis of DAG from labelled glucose under our incubation conditions, and found an approx. 2-fold increase in DAG synthesis ($9.5 \pm 0.9\%$ compared with $22.6 \pm 4.1\%$ of total glucose carbon incorporated into lipids in low- and high-glucose conditions respectively; $P < 0.05$, $n = 4$). Taken together, these results suggest that glucose activates PKC both by an increase in DAG and also via a novel and possibly more potent mechanism dependent on flux through the HBP.

To investigate further the mechanism by which increased flux through the HBP inhibits glucose transport, GLUT1 and GLUT4 levels were measured in the PM and LDM fractions. Incubation of adipocytes with either high glucose (in the presence of insulin) or glucosamine did not alter GLUT1 levels on the PM under either basal (1.0 ± 0 -, 1.02 ± 0.04 - and 0.95 ± 0.19 -fold changes over control basal values in control, high glucose and glucosamine conditions respectively) or insulin-stimulated (3.99 ± 0.06 -, 4.20 ± 0.17 - and 3.74 ± 0.53 -fold increases respectively) conditions. In contrast, both glucose and glucosamine decreased basal levels of PM GLUT4, but did not prevent the insulin-

Table 3 Stimulation of PKC activity by high glucose and by glucosamine

Adipocytes were preincubated as described in the legend to Table 1. The cells were fractionated and total PKC activity was measured in the particulate fraction, as described in the Experimental section. PKC activity was measured in triplicate in four separate experiments. * $P < 0.05$ compared with control without azaserine; † $P < 0.05$ compared with high glucose without azaserine.

Treatment	PKC activity (pmol/min per μ g of protein)	
	— Azaserine	+ Azaserine
Control	0.19 ± 0.02	0.21 ± 0.03
High glucose (30 mM)	$0.61 \pm 0.15^*$	$0.31 \pm 0.04^{*\dagger}$
Glucosamine (3 mM)	$0.63 \pm 0.13^*$	$0.68 \pm 0.16^*$

Figure 1 Representative immunoblots showing the effects of high glucose and of glucosamine on PM GLUT4 levels

(A) Adipocytes were treated as described in the legend to Table 1. Following insulin stimulation, cells were homogenized and PM fractions obtained by differential ultracentrifugation. (B) As for (A), except that cells were preincubated in the presence of 20 μ M azaserine. (C) As for (A), except that cells were preincubated in the presence of 10 μ M Ro-31-8220. C, control; HG, high glucose; Gln, glucosamine.

stimulated translocation of GLUT4 (Figures 1A and 2A). The decrease in basal GLUT4 levels in the PM fraction was accompanied by a concomitant increase in LDM GLUT4 levels (results not shown), suggesting intracellular retention rather than increased degradation of the transporter. Azaserine blocked the glucose-induced, but not the glucosamine-induced, decrease in GLUT4 levels on the PM (Figures 1B and 2B). However, when cells were preincubated with the PKC inhibitor Ro-31-8220, which reversed the defect in glucose transport (Table 1), GLUT4 levels were further decreased on the PM (Figures 1 and 2).

In contrast with the effects of high glucose and of glucosamine on PM GLUT4 levels in the basal state, the effects of these agents of decreasing glucose transport in the presence of insulin (Table 1) was not associated with a decrease in PM GLUT1 or GLUT4. Furthermore, inhibition of the HBP or of PKC, which clearly modified the changes in GLUT4 levels caused by glucose in the basal state, had no effect in the insulin-stimulated state (Figures 1B and 1C). Thus, under these incubation conditions, the inhibition of glucose transport in the insulin-stimulated state does not appear to be related to a defect in the insulin-stimulated translocation of GLUT4 or GLUT1.

Our results demonstrate clearly that the impairment in basal glucose transport cannot be due to changes in GLUT4 distribution or function. However, interference with the function of GLUT1 may provide a possible mechanism. PKC is thought to phosphorylate GLUT1 [27], which could either interfere with its intrinsic activity or alter its PM localization, such that, while GLUT1 may be present in the PM, it may not be capable of

Figure 2 Effects of high glucose and of glucosamine on PM GLUT4 levels

Autoradiographs were scanned using laser densitometry. (B) and (C) show data in the presence of 20 μ M azaserine and 10 μ M Ro-31-8220 respectively. C, control; HG, high glucose; Gln, glucosamine. Results are means $\pm$ S.E.M. of five separate experiments; * P < 0.05; # P < 0.005 compared with control.

transporting glucose. This is supported by recent work with 3T3-L1 adipocytes showing that glucose deprivation results in an 8–10-fold increase in basal glucose transport by activation of GLUT1 [28]. Furthermore, in an *in vivo* study in rats in which hyperglycaemia was induced by streptozotocin, it was shown that the decrease in basal glucose transport was primarily associated with a decrease in activity of the transporters [29]. The fact that the internalization of GLUT4 did not contribute to impaired glucose transport suggests that, in rat adipocytes in the basal state, most glucose transport is mediated via GLUT1.

The effects reported in the present study were obtained with a preincubation of just 2 h in high glucose or glucosamine. It is likely that additional effects will be obtained with longer preincubation times. Indeed, in hyperglycaemic streptozotocin-diabetic rats, a decrease in the intrinsic activity of glucose transporters was an early event but, with time, hyperglycaemia induced a defect in transporter translocation in response to insulin [29]. Furthermore, a defect in the insulin-mediated translocation of GLUT4 has recently been reported in rat muscle following a 4 h infusion of glucosamine [30].

Two other results require comment: (a) the further decrease in plasma membrane GLUT4 when PKC activity was inhibited (Figures 1 and 2), and (b) the stimulation above basal of glucose transport when high glucose and azaserine were used together

(Table 1). Both of these changes can be explained by the fact that PKC activation stimulates the translocation of GLUT4 to the PM [31,32]. We propose that the internalization of GLUT4 caused by high glucose is mediated by an effect of the HBP not involving the activation of PKC. In the presence of azaserine, flux through the HBP is blocked but glucose still partially activates PKC (Table 3), presumably by an increase in DAG. This PKC activation results in a modest translocation of GLUT4 (Figures 1B and 2B) which proceeds without the opposing effect of the HBP to internalize GLUT4. Inhibition of PKC by Ro-31-8220 removes the stimulatory effect of DAG-activated PKC on GLUT4 translocation and allows full expression of the internalization of GLUT4, a process that occurs via a non-PKC-mediated mechanism. It is not clear how the HBP causes the intracellular retention of GLUT4.

In conclusion, we have shown that acute hyperglycaemia activates PKC in two ways: via the *de novo* synthesis of DAG and, more powerfully, via activation of the HBP. The HBP causes the internalization of GLUT4 by a process that is independent of its activation of PKC. This effect on GLUT4, however, is not responsible for the impairment of basal glucose transport and, in the short term, is reversed by insulin.

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