

Palmitoylation of the glucose transporter in blood-brain barrier capillaries

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Abstract

Palmitoylation of GLUT1 was investigated in brain capillaries. The glucose transporter was shown to be palmitoylated using [^3H]palmitate labeling and immunoprecipitation. The labeling was sensitive to methanolic KOH or hydroxylamine hydrolysis, indicating the presence of an ester or thioester bond. The released fatty acid was analyzed by reverse-phase HPLC and was identified as [^3H]palmitate. Specificity of the immunoprecipitation was assessed by competitive inhibition of anti-GLUT1 binding with a synthetic C-terminal peptide against which the antibody was raised. In vivo studies were performed using capillaries isolated from control rats, streptozotocin-induced diabetic rats and diet-induced hyperglycemic rats. Glycemia was increased 2- and 5-fold in the hyperglycemic and diabetic groups, respectively. GLUT1 expression was evaluated in the three groups by Western blot analysis. A 36% decrease in GLUT1 expression was observed in the diabetic group, while there was no significant variation in GLUT1 expression in the hyperglycemic group. Palmitoylation of GLUT1 was increased in both diet-induced hyperglycemic and diabetic groups. These results suggest that palmitoylation may be involved in the regulation of glucose transport activity in hyperglycemia.

Keywords: Palmitoylation; GLUT1; Brain capillary; Hyperglycemia; Blood-brain barrier

1. Introduction

Under normal physiological conditions, glucose is the only source of energy utilized by the brain [1]. Entry of glucose from the blood to the brain requires its transport across the capillary endothelial cells that form the blood-brain barrier [2–4]. In this tissue, glucose uptake is mediated by the GLUT1 isoform of the sodium-independent glucose transporter [5,6]. Although the upregulation of GLUT1 expression during hypoglycemia has been well characterized [6,7], little is known about the different events occurring during hyperglycemia. The results obtained on GLUT1 expression are controversial since different studies have reported a decrease [8–10], no change [11,12] or an increase in GLUT1 during hyperglycemia

[13,14]. However, in most studies GLUT1 transport activity was decreased. This suggests that GLUT1 activity may be directly regulated by a mechanism which could be independent of its level of expression.

Palmitoylation is a post-translational modification which is involved in the regulation of the activity of several membrane proteins such as p21^{ras} [15], β_2 -adrenergic receptor [16], transferrin receptor [17] and rhodopsin [18]. GLUT1 has six cysteine residues, several of which are located near the cytoplasmic surface of the plasma membrane [19,20]. Such cysteines are known to be among the most common palmitoylation sites [21,22]. In a previous study, GLUT1 was found to be palmitoylated in erythrocytes [23]. However, in a recent study, palmitate incorporation in this protein could not be demonstrated [24]. A study on the palmitoylation of blood-brain barrier capillary proteins has shown that the major substrate is a 55 kDa protein [25]. Since GLUT1, a 55 kDa protein, is strongly expressed in brain capillaries [26], the possibility that this glucose transporter is palmitoylated was investigated.

In this study, palmitoylation of the glucose transporter (GLUT1) from brain capillaries was characterized. In-

Abbreviations: BSA, bovine serum albumin; BBBC, blood-brain barrier capillaries; ECL, enhanced chemiluminescence; HPLC, high pressure liquid chromatography; HRP, horseradish peroxidase-conjugated; PB, physiological buffer; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBST, TBS-Tween.

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creased GLUT1 palmitoylation was observed in both streptozotocin-induced diabetic and diet-induced hyperglycemic rats. These findings suggest that palmitoylation may be involved in the regulation of the activity of the brain capillary glucose transporter.

2. Materials and methods

2.1. Isolation of blood-brain barrier capillaries

Brains were isolated from rats and placed in ice-cold physiological buffer (PB) containing 147 mM NaCl, 4 mM KCl, 3 mM CaCl_2 , 1.2 mM MgCl_2 , 5 mM glucose and 15 mM Hepes (pH 7.4). Capillaries were isolated as described by Dallaire et al. [27]. The final pellet containing purified brain capillaries was resuspended in PB containing 1.4% sorbitol and 14% glycerol as cryopreservative agents [27]. The capillaries were either used immediately or stored in liquid nitrogen and used within 1 month.

2.2. SDS-polyacrylamide gel electrophoresis

BBBC (blood-brain barrier capillaries) proteins (10 μg) [28] were resuspended in Laemmli's sample buffer containing 60 mM Tris-HCl (pH 6.8), 12.5% glycerol, 1% SDS, 5 mM β -mercaptoethanol and 0.05% Bromophenol blue. The samples were placed under agitation for 15 min in the sample buffer and were loaded on 10 or 12.5% acrylamide/bisacrylamide (29.2:0.8) gels [29]. Electrophoresis was carried out at a constant voltage of 120 V for 2 h. The gels were stained with Coomassie Brilliant Blue or transferred electrophoretically onto Immobilon-P membranes for Western blot analysis. Molecular weight determinations were based on a calibration curve prepared with the following standards: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa) and α -lactalbumin (14 kDa).

2.3. Western blot analysis

Proteins were separated by SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) and the proteins were transferred electrophoretically onto 0.45 μm pore size Immobilon-P membranes (Millipore). The blots were blocked overnight with 10% powdered milk in 20 mM Tris (pH 7.0), 150 mM NaCl and 0.2% Tween 20 (TBST). Polyclonal anti-GLUT1 antibody was kindly provided by Dr. W. Pardridge from UCLA or purchased from Eastacres (RaGLUTrans) (1:5000) was used as primary antibody and incubation was carried out for 2 h at room temperature. After four washes in TBST, the blots were incubated with anti-rabbit horseradish peroxidase-conjugated antibody (Pierce) (1:1000) for 1 h at room temperature, washed four times in TBST, and developed with an

enhanced chemiluminescence detection system (Amersham).

2.4. Immunoprecipitation

Brain capillaries (200 μg protein in 10 μl) were lysed by addition of 1 ml of 1% Triton X-100, 0.1% SDS, 1% BSA, 20 mM Tris (pH 7.5), 150 mM NaCl, and placed under agitation for 30 min at room temperature. Lysates were transferred to microcentrifuge tubes and centrifuged at $12\,500 \times g$ for 10 min at 4° C. Supernatants were transferred to new microcentrifuge tubes and incubated overnight at 4° C with 5 μl anti-GLUT1 antibody. Protein A-Sepharose 4 fast flow (Pharmacia) was added (40 μl) and the incubation continued at 4° C for another 5 h. Beads were washed once with 1% Triton X-100, 0.1% SDS, 1% BSA, 150 mM NaCl, 20 mM Tris (pH 7.5) and three times with TBS. The final pellets were prepared for SDS-PAGE as described previously, but the samples were placed under agitation for 15 min in the sample buffer.

2.5. Peptide synthesis

The 15-amino acid peptide CEELFHPLGADSQV was synthesized using Fmoc chemistry and an Applied Biosystems automated peptide synthesizer following the recommendations of the manufacturer. This peptide corresponds to the 14-amino acid C-terminal portion of the human erythrocyte glucose transporter (GLUT1) [30], an N-terminal Cys residue was added to allow coupling of the peptide. The peptide was purified on a semi-preparative Vydac C18 high performance liquid chromatography column and its identity was verified by fast atom bombardment mass spectrometry. This peptide corresponds to the sequence recognized by the anti-GLUT1.

2.6. Palmitoylation

Palmitoylation was performed by adding 100 μg of BBBC proteins to an incubation medium containing: 25 μM [^3H]palmitic acid (150 $\mu\text{Ci}/\text{assay}$) and 0.1% Triton X-100 or BSA in PB (pH 7.4). In some experiments, palmitoylation was carried out in PB containing 0.1% Triton X-100, 20 mM MgCl_2 , 20 mM MnCl_2 , 20 mM ATP and 0.2 mM CoA. The mixture was incubated for 3 h at 37° C and the reaction was stopped by the addition of lysis buffer for a final concentration of 1% Triton X-100, 1% BSA, 0.1% SDS, 20 mM Tris (pH 7.5) and 150 mM NaCl. The glucose transporter was then immunoprecipitated and proteins resolved by SDS-PAGE, as described previously. For fluorography, the gels were treated with En^3Hance (NEN) and dried. Before exposure, a Kodak radiographic film was pre-flashed to obtain 0.1 to 0.2 absorbance unit. The dried gels were exposed at -80°C for 3 to 40 days. The fluorograms were scanned with an LKB Ultrosan XL enhanced laser densitometer. In some

experiments, the gels were sliced in 2 mm strips placed in individual vials. The strips were then treated with NCS tissue solubilizer (Amersham) and the palmitate incorporation was measured by liquid scintillation counting.

2.7. Metabolic labeling with [^{35}S]methionine

BBBC proteins (100 μg) were incubated in PB containing 0.3 μM [^{35}S]methionine (30 $\mu\text{Ci}/\text{assay}$). The mixture was incubated for 1 h at 37°C and the reaction was stopped by the addition of lysis buffer. The glucose transporter was then immunoprecipitated, the proteins separated by SDS-PAGE and the gel treated for fluorography as described previously.

2.8. Analysis of labeled fatty acids

After immunoprecipitation and SDS-PAGE, the radioactive fatty acids were liberated from GLUT1 by treating the gel slices with 1.5 M KOH in 25% methanol. Fatty acids were extracted with chloroform/methanol (2:1) and then analyzed on a C-18 reverse phase HPLC column (Vydac C18 #100) using a 75–100% acetonitrile gradient as described by Christgau et al. [31].

2.9. Animal treatments

Male Sprague-Dawley rats (300–400 g) were divided into the following three groups: Control (Co), Diabetic (Da) and Hyperglycemic (Hy). The first two groups (Co and Da) received Purina rat chow and water ad libitum, while the third group (Hy) received only a 50% glucose solution. Diabetes was induced with a single intraperitoneal injection of streptozotocin (65 mg/kg) dissolved in PB. All animals were killed by decapitation after 14 days and brain capillaries were purified as described previously.

2.10. Blood glucose analysis

Blood was collected immediately after decapitation and the samples placed into glass tubes at room temperature for 30 min. The tubes were centrifuged at $5000 \times g$ for 30 min and glucose concentration was measured in serum with a glucose oxidase-peroxidase assay [32].

3. Results

Immunoprecipitation of brain capillary proteins by anti-GLUT1 antibody, after pre-incubation of capillaries with [^3H]palmitate, resulted in the detection of two major bands corresponding to proteins with molecular masses similar to that of GLUT1, as detected by Western blot analysis (Fig. 1). The same immunoprecipitation procedure was used with [^{35}S]methionine-labeled capillary proteins and revealed labeled bands corresponding to polypeptides

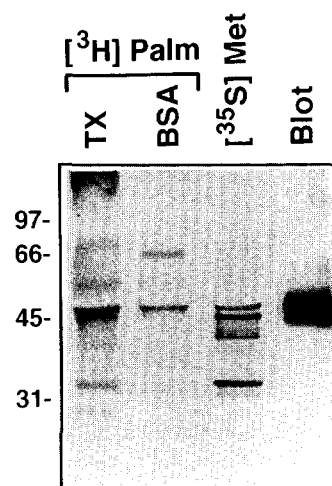


Fig. 1. Immunodetection and radiolabeling of GLUT1 using [^3H]palmitate and [^{35}S]methionine. Capillary proteins (100 μg) were labeled with [^3H]palmitate for 3 h at 37°C using Triton X-100 (TX) or bovine serum albumin (BSA) as palmitate solubilizing agents, or labeled with [^{35}S]methionine for 1 h at 37°C ([^{35}S]Met). At the end of the incubation period, the glucose transporter was immunoprecipitated using a polyclonal antibody directed against the C-terminal portion of the protein. After separation of immunoprecipitated proteins by SDS-PAGE, the gels were treated for fluorography and exposed to preflashed Kodak films for 82 ([^3H]palmitate) or 6 days ([^{35}S]methionine). GLUT1 was also detected by Western blot analysis (Blot) with the same antibody as described under Materials and methods.

with the same molecular masses as those detected with [^3H]palmitate labeling. Palmitoylation was performed using either BSA or Triton X-100 as palmitate solubilizing agents. In some experiments BSA alone was used because it has no disruptive effects on the plasma membrane, in order to verify if GLUT1 is palmitoylated in normal physiological conditions. The use of BSA alone prevents alterations in protein structure or cellular integrity that may be caused by detergents. However, since Triton X-100 resulted in an increased palmitoylation, this condition was preferred in several characterization experiments.

The nature of the linkage between [^3H]palmitate and the labeled proteins was determined by hydrolysis with hydroxylamine or methanolic KOH treatments (Fig. 2). Radioactive labeling was abolished by both treatments suggesting the presence of an ester or thioester bond. The nature of the cleavage products was studied by cutting out the portion of the gel corresponding to the immunoprecipitated radiolabeled proteins, treating it with methanolic KOH and extracting the resulting supernatant with chloroform/methanol (1:1). Free fatty acids were resolved by HPLC on a reverse-phase column. The cleaved radioactive fatty acids eluted from the column with the same retention time as unlabeled palmitate (Fig. 3).

Competitive inhibition of GLUT1 immunoprecipitation was performed after [^3H]palmitate labeling, using a synthetic peptide against which the antibody was raised. The immunoprecipitation was inhibited by 10^{-9} M peptide (Fig. 4A) at the same concentration needed to inhibit

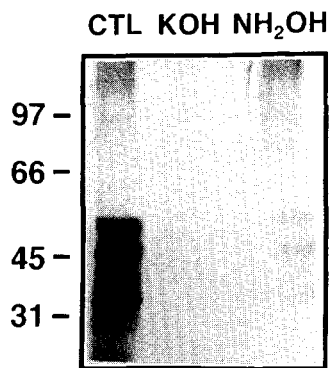


Fig. 2. Properties of GLUT1-bound palmitate. Capillary proteins were labeled for 3 h at 37°C in the presence of Triton X-100, as described in Fig. 1. At the end of the incubation period, the glucose transporter was immunoprecipitated using a polyclonal antibody directed against the C-terminal portion of the protein. The different lanes represent: assays done under standard conditions (CTL), samples incubated in 1 M methanolic KOH for 20 min at room temperature after immunoprecipitation (KOH) and gel that was placed in 1 M hydroxylamine (pH 7.0) for 12 h before fluorography (NH₂OH). For fluorography, the gel was exposed 78 days with a preflashed Kodak film.

GLUT1 immunodetection (Fig. 4B). Densitometric scanning showed that, in both cases, 90% of the signal is displaced by 10⁻⁹ M peptide.

The effect of hyperglycemia on the palmitoylation of the glucose transporter was investigated by comparing the levels of [³H]palmitate incorporation in control rats (Co), streptozotocin-induced diabetic rats (Da) and diet-induced (50% glucose) hyperglycemic rats (Hy). Both Da and Hy rats lost about 50 g in body weight, while the control rats gained 45 g (Table 1). The plasma glucose levels of Hy

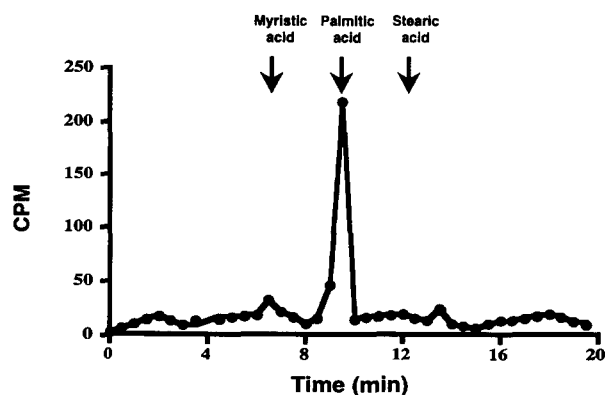


Fig. 3. Identification of palmitic acid as the GLUT-linked lipid. Brain capillary proteins (100 µg) were labeled with [³H]palmitate, GLUT1 was immunoprecipitated and subjected to alkaline methanolysis. The released fatty acids were analyzed by reverse-phase HPLC as described under Materials and methods. The palmitate peak was identified by co-elution with standards.

and Da rats were 2- and 5-fold higher than that of Co rats, respectively. Brain capillaries were isolated from each group and GLUT1 levels were measured by Western blot analysis. A significant reduction in GLUT1 was observed in capillaries isolated from diabetic rats (36%), while no significant change was noted in capillaries isolated from hyperglycemic rats (Fig. 5). Palmitoylation was evaluated by fluorography or gel slicing and scintillation counting. A significant increase in palmitoylation of the glucose transporter was observed in both Da and Hy groups. Palmitate incorporation was increased to 152 and 138% of Co in Hy and Da groups, respectively. A representative fluorogram demonstrating this results is shown in Fig. 6. Since immunodetection showed a decrease in GLUT1 expression in

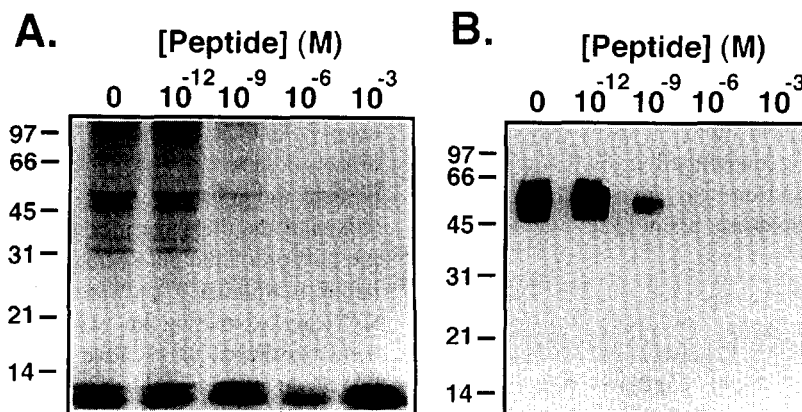


Fig. 4. Competitive inhibition of the immunoprecipitation of the palmitoylated GLUT1 and immunodetection by a synthetic peptide recognized by the antibody. (A) Capillary proteins (100 µg) were labeled with [³H]palmitate in the presence of ATP, CoA and Mg²⁺ before GLUT1 immunoprecipitation and separation by SDS-PAGE, as described in Fig. 1. For fluorography, the gel was exposed 62 days with a preflashed Kodak film. (B) Capillary proteins (1 µg) were separated by SDS-PAGE and transferred onto a PVDF membrane. Immunodetection was performed using a polyclonal antibody directed against the C-terminal portion of GLUT1. Anti-GLUT1 binding was detected using a horseradish peroxidase-conjugated goat anti-rabbit IgG and detection performed using enhanced chemiluminescence. Competitive inhibition was performed by adding a synthetic C-terminal peptide recognized by the antibody in the immunoprecipitation medium (A) or by mixing the peptide with the anti-GLUT1 antibody for immunodetection (B).

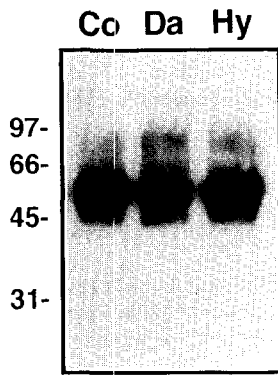


Fig. 5. Western blot analysis of GLUT1 from different rat populations. Capillaries were isolated from normal rats (Co), streptozotocin-induced diabetic rats (Da) and diet-induced hyperglycemic rats (Hy). Capillary proteins (1 μ g) were separated by SDS-PAGE and the immunodetection was performed using a polyclonal anti-GLUT1 antibody, as described in Fig. 4.

the Da group, the real increase in GLUT1 palmitoylation may be corrected to 216% of Co.

4. Discussion

The present results indicate that the glucose transporter of blood-brain barrier capillaries is covalently modified by palmitic acid. The lipid is attached to GLUT1 by an alkaline- and hydroxylamine-sensitive linkage, as are the majority of palmitoylated proteins [33]. The nature of the covalently bound palmitate was confirmed by alkaline hydrolysis followed by reverse phase HPLC. The exact location of the modification has not been determined. However, of the six cysteine residues present in the glucose transporter, three may represent a possible palmitoylation site. Hydropathy analysis has suggested that Cys-201, -207 and -347 are located close to the cytoplasmic surface of the plasma membrane [19]. Such localized cysteines are the most common palmitoylation sites [21,22] and are found on many well-characterized palmitoylated proteins [15,17,34,35].

The specificity of the immunoprecipitation was confirmed by competitive inhibition of anti-GLUT1 binding with a synthetic C-terminal peptide recognized by the antibody. This peptide inhibited both immunoprecipitation

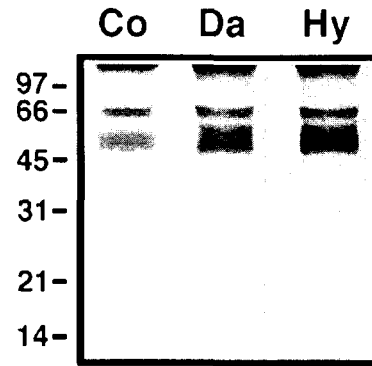


Fig. 6. Palmitoylation of GLUT1 in different rat populations. Freshly isolated capillaries from: normal rats (Co), streptozotocin-induced diabetic rats (Da) and diet-induced hyperglycemic rats (Hy) were incubated with [3 H]palmitate for 3 h at 37° C in the presence of BSA (absence of Triton X-100), before GLUT1 immunoprecipitation, as described in Fig. 1. For fluorography, the gel was exposed 102 days with a preflashed Kodak film.

and immunodetection at the same concentration (1 nM), showing that in both cases the immunoreactive protein was GLUT1.

An increase in GLUT1 palmitoylation was detected in both diabetics (Da) and diet-induced hyperglycemic (Hy) rats after immunoprecipitation of this transporter. The increase was specific to GLUT1 since, the palmitoylation profile of all other brain capillary proteins remained unchanged (results not shown). This increase in palmitoylation in both Da and Hy rats does not correlate with GLUT1 expression since glucose transporter levels were decreased in diabetic rats, as previously reported [6,9,36], and remained unchanged in the Hy groups. Like glycemia, palmitoylation was stronger in Da rats than in Hy rats.

Even if incubation was performed at 3 h which represents the time required to reach steady state of palmitoylation for most brain capillary substrates [25], the real turnover of GLUT1 palmitoylation remains unknown. No other studies have looked at the relative palmitoyl-transferase and -esterase activity present in the brain capillaries. It is possible that the increased palmitoylation is the result of an augmentation of free cysteine residues available for reaction with exogenous palmitate. Thus, the increased palmitoylation observed *in vitro* could result from a decreased *in vivo* palmitoylation. However, the

Table 1
General characteristics of the experimental groups

Group	n	Δ weight (g)	Serum glucose (mg/100 ml)	GLUT1 expression (% of control)	GLUT1 palmitoylation (% of control) ^a
Control	24	+45 \pm 4.7	108 \pm 4.7	100 \pm 0	100 \pm 0
Streptozotocin treated	15	-52 \pm 5.2 *	534 \pm 28 *	64 \pm 5.8 *	215 \pm 3.3 *
Glucose diet	24	-47 \pm 3.4 *	203 \pm 6.7 *	89 \pm 3.5	152 \pm 11 *

For GLUT1 expression and palmitoylation rats were divided in three groups and capillaries were purified from 5 to 7 rats. Results are means \pm S.E.

* Significantly different from control value $P < 0.05$ (paired *t*-test).

^a Values were corrected for variations in GLUT1 expression in the different groups.

palmitoylation of the glucose transporter varied in the same direction in both models of hyperglycemia. This variation could correspond to an increased or decreased *in vivo* palmitoylation.

The role of palmitoylation in the regulation of the activity of the glucose transporter remains unknown. Palmitoylation is a post-translational process which modifies cysteine residues [33]. Cysteine modifiers (Cys-maleimide) have been shown to inhibit the transport activity of GLUT1 without affecting either substrate binding or the ability of the carrier to change conformation [37]. Furthermore, palmitoylation is known to be a reversible process with a rapid turn over [38,39]. This suggests that this modification may provide a rapid mechanism by which glucose uptake could be decreased to prevent brain damage caused by hyperglycemia [40,41]. The implication of palmitoylation in GLUT1 regulation could also explain the observations of Kahn et al. [43] who reported an inhibition of GLUT1 transport activity in diabetic rats which could not be associated with transporter endocytosis or with a reduction in GLUT1 expression. However, it is difficult to establish a direct correlation between the level of palmitoylation and alteration of glucose transport, since the levels of alteration vary strongly (from 0 to 100%) between previous studies and depend upon the procedure which is used to measure glucose transport [8,10,12,42].

In conclusion, this study shows that the glucose transporter (GLUT1) of brain capillaries is covalently modified by palmitate. Palmitoylation was increased in both diabetic and diet-induced hyperglycemic rats. These results suggest that palmitoylation may be involved in the regulation of GLUT1 activity when the blood glucose concentration is rising.

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