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UDP-N-acetylglucosamine transferase and glutamine: fructose 6-phosphate amidotransferase activities in insulin-sensitive tissues

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Summary Glutamine:fructose 6-phosphate amidotransferase (GFA) is rate-limiting for hexosamine biosynthesis, while a UDP-GlcNAc β-N-acetylglucosaminyltransferase (O-GlcNAc transferase) catalyses final O-linked attachment of GlcNAc to serine and threonine residues on intracellular proteins. Increased activity of the hexosamine pathway is a putative mediator of glucose-induced insulin resistance but the mechanisms are unclear. We determined whether O-GlcNAc transferase is found in insulin-sensitive tissues and compared its activity to that of GFA in rat tissues. We also determined whether non-insulin-dependent diabetes mellitus (NIDDM) or acute hyperinsulinaemia alters O-GlcNAc transferase activity in human skeletal muscle. O-GlcNAc transferase was measured using ^{3}H-UDP-GlcNAc and a synthetic cationic peptide substrate containing serine and threonine residues, and GFA was determined by measuring a fluorescent derivative of GlcN6P by HPLC. O-GlcNAc transferase activities were 2–4 fold higher in skeletal muscles and the heart than in the liver, which had the lowest activity, while GFA activity was 14–36-fold higher in submandibular gland and 5–18 fold higher in the liver than in skeletal muscles or the heart. In patients with NIDDM (n = 11), basal O-GlcNAc transferase in skeletal muscle averaged 3.8 ± 0.3 nmol/mg · min, which was not different from that in normal subjects (3.3 ± 0.4 nmol/mg · min). A 180-min intravenous insulin infusion (40 mU/m^2 · min) did not change muscle O-GlcNAc transferase activity in either group. We conclude that O-GlcNAc transferase is widely distributed in insulin-sensitive tissues in the rat and is also found in human skeletal muscle. These findings suggest the possibility that O-linked glycosylation of intracellular proteins is involved in mediating glucose toxicity. O-GlcNAc transferase does not, however, appear to be regulated by either NIDDM or acute hyperinsulinaemia, suggesting that mass action effects determine the extent of O-linked glycosylation under hyperglycaemic conditions. [Diabetologia (1997) 40: 76–81]

Keywords Hexosamines, insulin, glucose, diabetes mellitus.

Chronic hyperglycaemia or "glucose toxicity" is a major cause of insulin resistance in patients with insulin-dependent (IDDM) and non-insulin-dependent (NIDDM) diabetes mellitus [1–3]. Studies performed in rat adipocytes have suggested that overactivity of the hexosamine pathway may contribute to glucose induced insulin resistance [4]. Glutamine: fructose 6-phosphate amidotransferase (GFA) catalyses the formation of glucosamine 6-phosphate (GlcN6P) from fructose 6-phosphate and glutamine [5]. GlcN6P is then converted via a series of enzymatic steps to UDP-N-acetyl-glucosamine (UDP-GlcNAc) [6]. Transgenic mice overexpressing GFA in skeletal muscle are insulin resistant [7]. Hyperglycaemia increases GFA activity in cultured human muscle cells [8] and is also associated with increased GFA activity in human skeletal muscle in patients with NIDDM

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Abbreviations: IDDM, Insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; GFA, glutamine:fructose 6-phosphate amidotransferase; GlcN6P, glucosamine 6-phosphate; UDP-GlcNAc, UDP-N-acetyl-glucosamine; O-GlcNAc transferease, UDP-GlcNAc β-N-acetylglucosaminyltransferase; OPA, O-phthaldialdehyde; GalNAc, N-acetyl-galactosamine

[9]. Moreover, streptozotocin-induced diabetes and acute hyperglycaemia are accompanied by increased levels of hexosamine metabolites including UDP-GlcNAc, in skeletal muscle [10]. In the rat liver, UDP-GlcNAc is subsequently O-linked to serine and threonine residues on intracellular proteins via a UDP-N-GlcNAc β-N-acetylglucosaminyltransferase (O-GlcNAc transferase) [11]. This enzyme was purified from rat liver and was shown to have an extremely high affinity for UDP-GlcNAc (K_m 545 nmol/l). In rat liver, the enzyme exists as a heterotrimer complex with two α-subunits of 110 kDa and one β-subunit of 78 kDa [11]. It has been suggested, based on the multimeric state and large size of the enzyme, that its activity may be regulated within the cell [11]. Whether the enzyme exists in insulin-sensitive tissues such as in skeletal muscle, heart or adipose tissue is currently unknown. The presence of this enzyme is a prerequisite for the hypothesis that cytosolic O-linked glycosylation of one or several proteins is coupled to induction of a defect in the translocation of the insulin-sensitive glucose transporter GLUT4 [12]. The present studies were undertaken to determine if O-GlcNAc transferase activity is found in insulin-sensitive rat or human tissues, and if so how its activity is correlated with that of GFA. We also determined whether O-GlcNAc transferase is altered by NIDDM or insulin in vivo.

Subjects, materials and methods

Experimental design

Animals. Overnight fasted male Sprague-Dawley rats weighing 200–300 g, were anaesthetized by sodium pentobarbital (50 mg/kg body weight i.p.) and the tissues (submandibular gland, epididymal fat, soleus, gastrocnemius, heart and liver) were rapidly removed by freeze-clamping in situ with aluminum tongs precooled in liquid nitrogen, and stored in liquid nitrogen until assay of O-GlcNAc transferase or GFA activity. These studies were approved by the Institutional Animal Care and Use Committee of the UTHSCSA.

Human studies. Eleven patients with NIDDM [body mass index (BMI) 31 ± 1 kg/m^2, seven women, four men, fasting plasma glucose 9.1 ± 0.6 mmol/l, fasting C-peptide 2.7 ± 0.3 nmol/l, glycosylated haemoglobin (HbA_{1c}) 8.0 ± 0.7 %] and eight normal subjects [BMI 29 ± 1 kg/m^2, four women, four men, fasting plasma glucose 5.3 ± 0.1 mmol/l, fasting C-peptide 2.2 ± 0.4 nmol/l, HbA_{1c} 4.7 ± 0.1 %] volunteered for the studies. After written informed consent was obtained, all subjects were physically examined and a 12-lead electrocardiogram was recorded. Blood was drawn for haematocrit, liver function tests, electrolytes, total protein and albumin, serum C-reactive protein and creatinine. A urine pregnancy test was performed in the females to exclude pregnancy. All subjects had a normal physical examination, electrocardiogram and blood tests. After 3 days on a weight-maintaining diet containing at least 200 g of carbohydrate per day, a 2-h oral glucose tolerance test [13] was performed to exclude patients with impaired glucose tolerance. The patients with NIDDM were treated with sulphonylureas. The medications were discontinued for 2 days before the study. Informed consent was obtained after the purpose, nature and potential risks were explained to the subjects. The experimental protocol was approved by the institutional review board of the University of Texas Health Science Center at San Antonio.

In each subject, insulin sensitivity was measured using the euglycaemic insulin clamp technique (insulin infusion rate 40 mU/m^2 · min for 180 min) [13]. Muscle biopsies for measurement of O-GlcNAc transferase activity were taken from vastus lateralis muscle immediately before and at the end of the insulin infusion.

Whole body insulin sensitivity, muscle biopsies. All studies were performed at 07.30 hours after a 10–12 h overnight fast. Prior to study, two 18-gauge catheters were inserted, one into an antecubital vein for infusion of 20 % glucose and insulin, and the second to a heated (65 °C) hand vein to obtain arterialized venous blood. A percutaneous muscle biopsy was obtained under local anaesthesia from vastus lateralis muscle using a Bergström needle, as previously described [9]. The muscle biopsy specimens were immediately frozen in liquid nitrogen and stored in liquid nitrogen until analysis. Immediately after the muscle biopsy, insulin (Humulin, Eli Lilly, Ind., USA) was infused in a primed-continuous manner for 180 min. Plasma glucose was measured at 5-min intervals with a Beckman glucose analyzer (Beckman Instruments, Fullerton, Calif., USA), and 20 % glucose was infused at a variable rate to maintain normoglycaemia. The muscle biopsy procedure was repeated at 180 min. The two biopsies were taken from opposite legs. Enough muscle tissue was obtained from 11 of 11 NIDDM and 6 of 7 normal subjects for analysis of basal O-GlcNAc transferase activity and in 7 of 11 NIDDM and 5 of 7 normal subjects for measurement of O-GlcNAc transferase activity at the end of the insulin infusion. In the patients with NIDDM, normoglycaemia was reached during the second hour of the insulin infusion. Whole body glucose uptake was calculated from the glucose infusion rate required to maintain normoglycaemia during the last hour of the insulin infusion after correcting for changes in the glucose pool size [14].

Materials. Chemicals and other supplies were provided by: UDP-D-6-[^{3}H]-N-GlcNAc (specific activity 925 GBq/mmol) (Amersham, Arlington Heights, Ill., USA), Ready Protein Scintillation fluid (Beckman), O-GlcNAc transferase peptide substrate (Indiana University School of Medicine, Department of Biochemistry Biotechnology Facility, Ind., USA), Bicinchoninic acid protein reagent (Pierce, Rockford, Ill., USA), P81 ion exchange paper (Whatman, Clifton, N.J., USA), other reagents, reagent grade or better (Sigma, St. Louis, Mo., USA).

Assay of O-GlcNAc transferase activity. Tissue samples (25–50 mg) were homogenized in 4 × volume (μl/mg) of homogenizing buffer containing 20 mmol/l Hepes, 10 mmol/l Mg_2Cl_2, 0.4 mmol/l Na_3VO_4, 1 mmol/l EDTA, 4 μmol/l benzamidine, 4 μmol/l phenylmethyl sulphonyl fluoride, 1 μg/ml pepstatin, 1 ng/ml leupeptin and 0.5 ng/ml pepstatin and 250 mmol/l sucrose (HBS), pH 7.0 in a glass homogenizer for 30 s on ice. The homogenate was centrifuged at 100 000 × *g* for 25 min at + 4 °C. The supernatant was mixed with an equal volume of 30 % polyethylene glycol-8000 and incubated on ice for 30 min to precipitate proteins. The precipitated material was pelleted by brief centrifugation in an Eppendorf table-top centrifuge. The supernatant was discarded and the pellet was washed once with HBS and resuspended in half the original volume of HBS. O-GlcNAc transferase was measured as described by Haltiwanger et al. [11] with slight modifications.

Both methods derive their specificity by utilizing peptide substrates that are modelled after O-glucosaminylation motifs of known O-GlcNAcylated proteins [11]. The method employed here utilizes an Arg-tagged peptide which binds firmly to phosphocellulose paper. Reactions were conducted in 45-μl mixtures containing 1–5 μg extract protein in 20 mmol/l Hepes (pH 8.0)/250 mmol/l sucrose/5 mmol/l peptide substrate (TITSETPSSTTTQITKR)/0.5 mmol/l EDTA/0.2 μCi UDP-[6-^{3}H]-N-acetylglucosamine (25 Ci/mmol). The reactions were incubated for 60 min at 25 °C. At the end of the incubation 40 μl portions were spotted onto 1-cm^2 Whatman P81 ion exchange paper. The paper squares were immediately immersed in 50 mmol/l formic acid, washed extensively with 4 × 4 ml 50 mmol/l formic acid per square and then transferred while still moist into scintillation vials. The samples were counted in Beckman Ready Protein scintillation cocktail (5 ml/square). All assays were conducted in the linear range for time, peptide concentration and extract protein (data not shown). Protein concentrations in muscle homogenates were measured using the bicinchoninic acid method with a kit from Pierce using bovine serum albumin as the standard.

Assay of GFA activity. GFA activity was assayed as previously described [8, 9]. The tissue specimen (50–150 mg) was homogenized for 10 s on ice and sonicated for 10 bursts in a Branson 250 Sonifier (Branson, Danbury, Conn., USA) in 1 ml of extract buffer (100 mmol/l KCl, 1 mmol/l EDTA, 50 mmol/l Na phosphate, pH 7.5). The sample was then centrifuged at 60000 *g* for 15 min at + 4 °C and the supernatant was used for assay of GFA activity. All samples were assayed immediately after homogenization. We incubated 50 μl of extract in a reaction mix (final volume 100 μl) containing 12 mmol/l fructose 6-phosphate, 12 mmol/l glutamine, 40 mmol/l Na phosphate (pH 7.4), 1 mmol/l EDTA and 1 mmol/l dithiotreitol at 37 °C for 45 min in the presence and absence of 500 mmol/l of UDP-GlcNAc. The reaction was terminated with 50 μl of 1 mol/l perchloric acid, vortexed, and centrifuged (16 000 *g*, 4 °C) for 15 min. The deproteinized supernatant (145 μl) was then treated with 258 μl of a 1:4 mixture of tri-N-octylamine:1,1,2-trifluoroethane, vortexed and centrifuged as above for 1 min. The aqueous phase was then filtered through a 0.2 μmol/l filter and 50 μl was derivatized with an equal volume of O-phthaldialdehyde (OPA) solution (4 mg OPA dissolved in 50 μl ethanol and added to 5 ml of 0.1 mol/l sodium borate and 10 μl mercaptoethanol). Immediately thereafter samples were separated over a reverse-phase C_{18} column (25 cm × 4.6 mm Spherisorb ODS Phase Sep, Norwalk, Conn., USA) equilibrated with 15 mmol/l sodium phosphate, pH 7.2, 5 % acetonitrile and 5 % isopropanol. Absorbance of the sample eluent was analysed using a fluorescent detector and the peak area was integrated. OPA-derivatized GlcN6P standards were run separately to determine the retention time and to generate a standard curve to correlate area to activity. The correlation coefficient between the concentration of GlcN6P standards and the area under the GlcN6P peak was 0.999 or higher. The recovery of samples spiked with GlcN6P prior to derivatization was 100 %. Activity is expressed as U/mg protein where 1 U represents the generation of 1 pmol of GlcN6P/min. The coefficient of variation of GFA measurements performed in two separate pieces of rat muscle was less than 2 %.

Other methods. Plasma glucose concentration was measured in duplicate using the glucose oxidase method [15]. Serum insulin was determined before and every 30 min during the 240-min insulin infusion using a radioimmunoassay (Coat-a-Count insulin kit, Diagnostic Products Corporation, Los Angeles, Calif., USA).

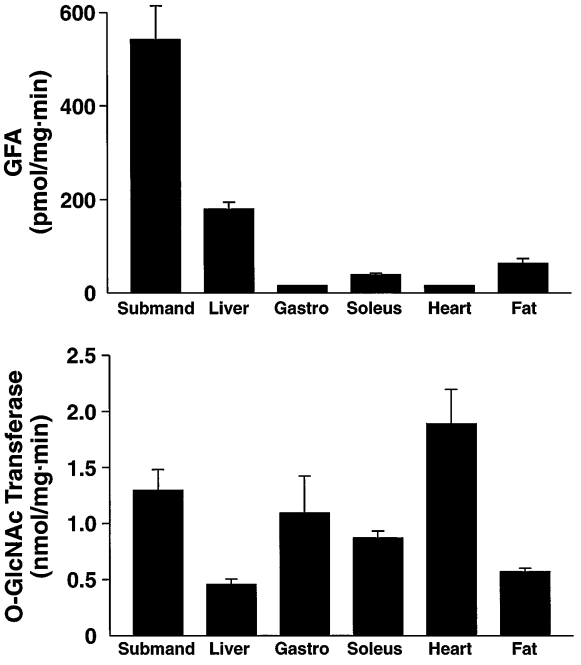

Fig. 1. Distribution of GFA (pmol/mg protein · min) and O-GlcNAc transferase (nmol/mg protein · min) activities in rat tissues (n = 4–7 per tissue). For significances between tissues, see text. Data represent mean ± SEM

Statistical analyses

Enzyme activities between the groups were compared using analysis of variance followed by Bonferroni's test for pairwise comparison. Data between the study groups in the human studies were analysed using the unpaired *t*-test. Simple correlations between selected study variables were calculated using Pearson's correlation coefficient for normally distributed variables. Multiple linear regression analysis was used to analyse the causes of variation in insulin sensitivity. All calculations were made using the SYSTAT statistical package (SYSTAT Inc., Evanston, Ill., USA). All data are expressed as means ± SEM.

Results

O-GlcNAc transferase and GFA rat tissues. O-GlcNAc transferase activities averaged 0.45 ± 0.05, 1.29 ± 0.19, 1.09 ± 0.33, 0.87 ± 0.06, 1.88 ± 0.31 and 0.57 ± 0.03 nmol/mg protein · min in the liver, submandibular gland, gastrocnemius, soleus, heart and adipose tissue, respectively. The activity in the liver was significantly lower than in any of the other tissues ($p < 0.05$ to $p < 0.001$, Fig. 1). The activities were significantly ($p < 0.05$ or less) higher in the heart, submandibular gland and soleus muscle than in adipose tissue, which had higher activity than the liver ($p < 0.05$). GFA activity was highest in the submandibular gland (542 ± 60 pmol/mg protein · min) followed by the liver (194 ± 14, $p < 0.001$), adipose tissue (62 ± 11, $p < 0.001$ vs submandibular gland and liver),

soleus (39 ± 4, $p < 0.001$ vs submandibular gland, liver and adipose tissue), heart (16 ± 1, $p < 0.001$ vs submandibular gland, liver, adipose tissue and soleus), and gastrocnemius (15 ± 1, $p < 0.001$ vs submandibular gland, liver, adipose tissue and soleus).

Studies in humans

O-GlcNAc transferase in human skeletal muscle. To assess the reproducibility of the O-GlcNAc transferase measurements in human skeletal muscle specimens, duplicate biopsies were processed and assayed for O-GlcNAc transferase on two separate days. The coefficient of variation for measurement of O-GlcNAc transferase activity from two separate biopsies was 8 ± 2 % ($n = 4$). Basal O-GlcNAc transferase averaged 3.3 ± 0.3 nmol/mg protein · min in the normal subjects ($n = 6$). In the five subjects in whom O-GlcNAc transferase was measured before and at the end of the insulin infusion, O-GlcNAc transferase averaged 3.0 ± 0.3 and 3.5 ± 0.3 nmol/mg protein · min (NS). The coefficient of variation of O-GlcNAc transferase measured before and at the end of the insulin infusion was 10 ± 4 %. In patients with NIDDM, basal O-GlcNAc transferase averaged 3.8 ± 0.3 nmol/mg protein · min ($n = 11$), which was not different from that in the normal subjects. O-GlcNAc transferase was unchanged by insulin (3.9 ± 0.3 vs 4.3 ± 0.3 nmol/mg protein · min, basal vs insulin, NS) in seven NIDDM patients. The coefficient of variation of O-GlcNAc transferase in the latter subjects was 12 ± 3 %.

Insulin sensitivity. During the last hour of the insulin infusion (120–180 min), plasma glucose (5.1 ± 0.1 vs 5.0 ± 0.1 mmol/l, NIDDM vs normal subjects) and insulin (516 ± 19 vs 456 ± 28 pmol/l, respectively) were similar in both groups. The rate of whole body glucose disposal was 56 % lower in the patients with NIDDM (10.4 ± 1.0 μmol/kg · min) than in the normal subjects (23.9 ± 6.2 μmol/kg · min, $p < 0.01$). Whole body insulin sensitivity was inversely correlated with BMI ($r = -0.44$, $p < 0.05$) and the fasting plasma glucose concentration ($r = -0.65$, $p < 0.005$), but not with O-GlcNAc transferase activity in either group.

Discussion

The present study was undertaken to characterize the tissue distribution and regulation of two enzymes, which are necessary for UDP-GlcNAc formation and O-linked attachment to proteins, GFA and O-GlcNAc transferase, respectively. O-GlcNAc transferase activity was found in all insulin-sensitive tissues examined. Tissue distribution of O-GlcNAc transferase activities significantly differed from that of GFA, in keeping with tissue-specific differences in N- and O-linked glycosylation. O-GlcNAc transferase was also present in human skeletal muscle but its activity was not altered by NIDDM or by 3 h of hyperinsulinaemia suggesting that O-linked glycosylation is a substrate-driven event.

Early studies utilizing the colorimetric spectrophotometric assay to assess GFA found no significant activity in either heart or skeletal muscle [16, 17] but did find as in the present study, that GFA activity was higher in the submandibular gland than in the adult rat liver [16]. The highest GFA activities have been reported in the gut [16]. We have confirmed the latter data by measuring GFA activity in the mucosa of the duodenum and terminal ileum, in which GFA activities averaged 688 and 1128 pmol/mg protein · min, i.e. up to 75-fold higher than in skeletal muscle. Both the submandibular gland and the gut are extensively engaged in production of mucins to which N-acetyl-galactosamine (GalNAc) is attached via galactosyltransferase [18]. UDP-GalNAc production is also dependent upon GFA [6]. These data are consistent with the idea that mucin production is the major reason for the high GFA activity in submandibular gland and that O-GlcNAc only accounts for a small fraction of total macromolecular incorporation of labelled glucosamine [18].

In the present study GFA was measured using HPLC-detection of fluorescent glucosamine, which is more sensitive and reproducible than the colorimetric method [8–10]. In keeping with recent reports on GFA in skeletal muscle [8–10], we found GFA in skeletal muscle as well as in the heart, and found GFA to be significantly higher in soleus than in gastrocnemius muscle. Although GFA activity is much lower in these tissues than in mucin-producing tissues, its activity was significantly different between several insulin-sensitive non-mucin-producing tissues. The functional significance of these differences remain to be established. In the heart, GFA activity was as high as in gastrocnemius muscle, although the heart does not exhibit glucose-induced insulin resistance in patients with IDDM [19]. In this tissue, however, glucose phosphorylation rather than glucose transport is rate-limiting for glucose uptake [20–22]. In rats, induction of insulin resistance by glucosamine is associated with a translocation defect in the insulin-sensitive glucose transporter, GLUT4 [12]. If this represents the key defect controlling glucose uptake under chronic hyperglycaemic conditions, it would not affect insulin-sensitive glucose uptake in the heart where glucose phosphorylation is rate-limiting. Whether the high GFA activity found in the liver regulates glucose uptake or merely serves to regulate UDP-GlcNAc availability for hepatic glycoprotein and glycolipid synthesis is currently unknown.

Except for the liver, no data are currently available regarding O-GlcNAc transferase activity. In the present study we found significantly higher O-GlcNAc transferase activity in heart, skeletal muscle and adipose tissue than in the liver. The tissue-distribution of an O-GlcNAc selective N-acetyl-β-D-glucosaminidase, which catalyses the selective removal of UDP-GlcNAc from proteins, was also determined recently [23]. Interestingly, except for the spinal cord, the activity of this enzyme was also lowest in the liver of all rat tissues examined [23]. The functional significance of the tissue-specific differences in these enzymes are presently unknown but the finding of significant O-GlcNAc transferase activity in many tissues is consistent with the view that attachment of GlcNAc to proteins has more in common with cellular regulation such as protein phosphorylation than with other classical types of protein glycosylation. The latter is typically extracellular and catalysed by galactosyltransferases [24]. In keeping with this, O-GlcNAc is attached to many proteins that are typically also phosphorylated and multimeric. O-GlcNAc attachment sites are similar in amino acid sequence to growth factor kinase phosphorylation sites [25], and the expression of growth factors such as transforming growth factor β expression can be increased via glucose-induced activation of the hexosamine biosynthetic pathway [26]. Thus, the consequences of hyperglycaemia-induced activation of the hexosamine pathway may not be restricted to glucose metabolism.

O-GlcNAc transferase activities could be reproducibly measured in human skeletal muscle. O-GlcNAc transferase activity was not altered by 3 h of normoglycaemic hyperinsulinaemia in normal subjects and was not different between NIDDM patients and normal subjects either basally or during normoglycaemic hyperinsulinaemic conditions. Although no time course of insulin action on O-GlcNAc transferase activity was performed, these data imply that neither chronic hyperglycaemia (NIDDM) nor acute hyperinsulinaemia was associated with changes in O-GlcNAc transferase activity, which indicates that glucose-induced insulin resistance is mediated via mechanisms other than altered O-GlcNAc activity. These mechanisms include an increase in GFA activity in skeletal muscle, as shown by the studies performed in transgenic mice [7], and patients with NIDDM [9]. Activation of the hexosamine pathway by glucose mass-action also seems to be involved as both acute and chronic hyperglycaemia increase levels of hexosamine metabolites in skeletal muscle [10]. Furthermore, infusion of glucosamine to rats induces insulin resistance in vivo. Glucosamine bypasses GFA and increases levels of UDP-GlcNAc in skeletal muscle [27]. Whether these alterations are accompanied by changes in O-linked glycosylation of specific proteins linked to down-regulation of glucose transport is currently unknown. The existence of O-GlcNAc in insulin-sensitive tissues is obviously a prerequisite for such intracellular glycosylation. The present data show that O-GlcNAc transferase activity is present in large quantities in tissues such as adipose tissue and skeletal muscle which are subject to glucose-induced downregulation of the glucose transport system. As O-GlcNAc transferase activity does not itself appear to be regulated by chronic hyperglycaemia, our data would support the view that increases in hexosamine metabolites are of critical importance for the development of glucose toxicity.

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