Insulin regulates the expression of the GLUT5 transporter in L6 skeletal muscle cells

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Abstract Skeletal muscle, a primary insulin target tissue, expresses the GLUT5 fructose transporter. Although insulin has no acute effect on GLUT5 expression and function in muscle, we show here that long-term (24 h) insulin treatment of L6 muscle cells induces a dose-dependent increase in GLUT5 protein (by up to two-fold), leading to a concomitant increase in fructose uptake. The increase in GLUT5 expression and function was suppressed by inhibitors of gene transcription and protein synthesis, suggesting that insulin promotes de novo carrier synthesis. Transfection of the GLUT5 gene promoter fused to luciferase into L6 cells revealed that insulin induced a 1.8-fold increase in GLUT5 promoter activity. Our findings indicate that insulin is capable of increasing the abundance and functional activity of GLUT5 in skeletal muscle cells and that this is most likely mediated via activation of the GLUT5 promoter.

Key words: Fructose; Glucose; GLUT5 promoter; Luciferase

1. Introduction

Fructose is a major dietary sugar that, once absorbed across the intestinal barrier, passes into the portal blood stream, where it is delivered to the liver. The liver extracts much of the absorbed fructose and is the principal site of fructose metabolism. However, a significant proportion (~30%) remains available for utilisation by peripheral tissues, such as skeletal muscle and adipose tissue [1]. Indeed, whilst glucose is the major hexose substrate for skeletal muscle, several studies have demonstrated significant utilisation of fructose by this tissue. Early work showed that fructose was assimilated in peripheral tissues at least as quickly as glucose [2], and that in human muscle, glycogen synthesis rates were similar after infusion of either glucose or fructose [3]. Furthermore, in the absence of insulin, physiological concentrations of fructose have been shown to account for up to 30% of glycogen synthesis in human skeletal muscle [4]. Together, these studies indicate that fructose plays an important role in overall carbohydrate metabolism in skeletal muscle and that understanding how its transfer across the plasma membrane is regulated may shed further insight into its metabolic role in this tissue. We and others have shown that skeletal muscle and adipose tissue express the GLUT5 fructose transporter and that its expression is confined to the plasma membrane [5–8]. This cell surface localisation is in stark contrast to that of the GLUT4 glucose transporter, which resides in intracellular storage vesicles that are translocated to the plasma membrane in response to an acute insulin challenge [9,10]. Whilst the insulin-dependent redistribution of GLUT4 is central to the mechanism by which the hormone stimulates glucose uptake in skeletal muscle, acute insulin treatment has no effect on the plasma membrane abundance of GLUT5 or upon fructose transport [5,7]. However, there is no present knowledge as to whether insulin may regulate GLUT5 expression levels in muscle cells.

In this study, we show that, like adult rat and human skeletal muscle [6,7], GLUT5 is also expressed in the plasma membrane of cultured rat L6 skeletal muscle cells, where it mediates fructose uptake. We demonstrate for the first time that expression and function of this transporter can be enhanced by long-term exposure to insulin and that this is most likely mediated by an insulin-induced increase in GLUT5 promoter activity.

2. Materials and methods

2.1. Materials

All reagent-grade chemicals for buffers were obtained from BDH (Poole, Dorset, UK). Sterile trypsin/EDTA solution, fructose, cytochalasin B (CB) and insulin were obtained from Sigma (Poole, Dorset, UK). [^{14}C]fructose and [^{3}H]inulin were purchased from Amersham. α-Minimal Essential Media (α-MEM), foetal calf serum, antimycotic/antibiotic solution were from Life Technologies (Paisley, Renfrewshire, UK).

2.2. Cell culture

L6 muscle cells were grown as a monolayer as described previously to the stage of myotubes in α-MEM containing 2% foetal calf serum and 1% antimycotic/antibiotic solution at 37°C in a humidified atmosphere of 5% CO_2, 95% air [11].

2.3. Fructose and glucose uptake in L6 muscle cells

L6 myotubes were exposed to insulin, insulin-like growth factor-1 (IGF-1), CB, cycloheximide and actinomycin D (Act D) at concentrations and periods indicated in the figure legends. Cells were washed three times in HEPES-buffered saline (HBS, 20 mM HEPES, pH 7.4;

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Abbreviations: CB, cytochalasin B; Act D, actinomycin D; HBS, HEPES-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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140 mM NaCl; 5 mM KCl; 2.5 mM $MgSO_4$ and 1 mM $CaCl_2$) and incubated for 30 min in warm HBS containing either 50 μM fructose, 0.12 μCi/plate [^{14}C]fructose and 0.8 μCi/plate [^{3}H]inulin (used to assess non-specific cell-associated radioactivity) or 10 μM 2-deoxy-[^{3}H]-D-glucose transport (1 μCi/ml, 26.2 Ci/mmol) as described previously. Uptake was terminated by washing cells three times with ice-cold saline prior to lysis in 50 mM NaOH. Cell-associated radioactivity was determined by scintillation counting as described previously [5].

2.4. Subcellular fractionation of L6 muscle cells

Total L6 cell membranes, plasma and intracellular membranes were prepared from L6 cells as described previously [11]. Protein content in each membrane fraction was determined using the Bradford assay with bovine serum albumin as standard [12].

2.5. SDS–PAGE and immunoblotting

Isolated membrane fractions from L6 cells were subjected to SDS–PAGE on 10% resolving gels and immunoblotted as previously reported [11]. PVDF membranes (Millipore, Bedford, MA, USA) were probed with antisera against a peptide corresponding to the 13 carboxy-terminal amino acids of the rat GLUT5 protein (generously provided by Dr. Y. Oka, University of Tokyo, Japan; [13]), the α1 subunit of the Na,K-ATPase (kindly provided by Dr. K. Sweadner, Harvard University; [14]), and PKB (New England Biolabs, Herts, UK). Primary antibody detection was performed using either horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG (Diagnostics Scotland, Lanarkshire, Scotland, UK), or protein A (ICN) and visualised using chemiluminescence Supersignal (Pierce).

2.6. RNA extraction and RT-PCR

Total RNA was extracted from L6 myoblasts and myotubes using an RNA isolator kit (Qiagen). Reverse transcription and PCR were performed using the Promega Access RT-PCR kit and GLUT5 specific primers as described previously [6].

2.7. Transfection of L6 cells and luciferase assay

One day after seeding in six-well multi-dishes, L6 myoblasts were transfected with 1μg human GLUT5 promoter constructs [15], using FuGENE 6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany). Cells were then incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air for 24 h. After this period, cells were incubated with 1 μM insulin for 3 h and lysed in a buffer containing 10% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM dithiothreitol, 2 mM diaminocyclohexanetetraacetic acid and 25 mM Tris–phosphate pH 7.8 and centrifuged at $10\,000 \times g$ for 10 min. Supernatant was used for the determination of luciferase activity, using a Luciferase Assay System kit (Promega) and a TD-20/20 luminometer (Turner Designs), as described previously [16]. Luciferase activities were expressed as relative light units/mg of protein.

2.8. Statistical analyses

For multiple comparisons statistical analysis was performed using one-way analysis of variance (ANOVA). Data analysis was performed using GraphPad Prism software and considered statistically significant at P values < 0.05.

3. Results

3.1. Expression and subcellular localisation of GLUT5 in L6 muscle cells

Since no information was available regarding the expression of the GLUT5 transporter in L6 muscle cells, we initially performed RT-PCR using rat-specific GLUT5 primers [6] with total RNA isolated from both L6 myoblasts and myotubes. Fig. 1A shows that both produced a 496-bp specific PCR product that was not observed in samples incubated without reverse transcriptase, indicating the presence of GLUT5 mRNA. To assess whether GLUT5 protein was expressed, we immunoblotted total membranes prepared from L6 myoblasts and myotubes, as well as crude rat jejunal membranes (a positive immunoreactive control). Fig. 1B shows that all the membrane fractions reacted with the GLUT5 antibody, demonstrating that GLUT5 protein was expressed in L6 muscle cells. The variation in electrophoretic mobility observed in crude jejunal membranes has previously been observed [6,17] and is most likely due to differences in *N*-linked glycosylation of the protein. Since no significant differences were observed in GLUT5 abundance between L6 myoblasts and L6 myotubes, it is unlikely that expression of this carrier is modulated during cellular differentiation (Fig. 1B). Fig. 1B also shows that GLUT5 expression was restricted to the plasma membrane in L6 myotubes; the weak signal observed in the intracellular microsomal fraction most likely representing cross-contamination between the two membrane fractions.

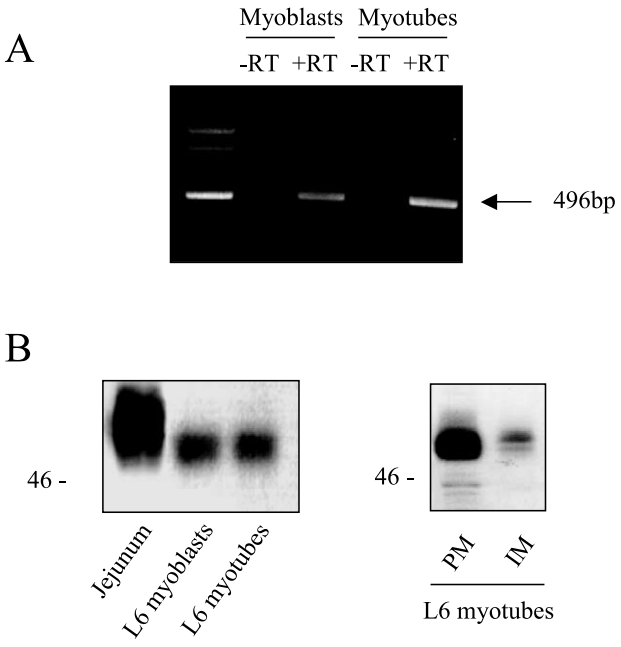

Fig. 1. Expression of GLUT5 mRNA and protein in L6 muscle cells. A: Total RNA was prepared from L6 myoblasts and L6 myotubes and used in RT-PCR with specific GLUT5 primers, as described in Section 2. The amplified product of 496 bp was detected on a 1.4% agarose gel stained with ethidium bromide. Control reactions were loaded lacking reverse transcriptase (−RT). B: Immunoblots showing GLUT5 abundance in rat jejunal crude membranes, L6 myoblast and myotube total membranes, L6 myotubes plasma membranes (PM) and L6 myotube internal membranes (IM). The blots are representative of three separate experiments.

3.2. Effects of insulin on cellular GLUT5 abundance

Next we investigated whether GLUT5 expression and function could be modulated by long-term exposure of cells to insulin. L6 myoblasts divide and grow until confluence, at which point they undergo spontaneous fusion to form multinucleated myotubes [11,18]. This cellular differentiation programme takes approximately 7 days. As fully differentiated myotubes, cell division and DNA synthesis ceases [19]. We thus elected to investigate the effects of insulin on GLUT5 expression/function at day 6 of the cellular differentiation programme, at which time cells are ~80–90% differentiated. Incubating L6 myotubes with insulin for periods up to 24 h did not cause any detectable increase in nuclei number (based on nuclei count of several randomly picked visual fields after Giemsa staining, data not shown). Incubating L6 myotubes with a supramaximal dose of insulin (1 μM) for 30 min had no detectable effect on GLUT5 abundance in plasma mem-

branes. However, when insulin was present in the culture medium for 24 h, we observed a dose-dependent increase in GLUT5 protein abundance (Fig. 2A). The increase in GLUT5 content after 24 h of 1 μM insulin treatment (~three-fold, Fig. 2B) does not represent a generalised increase in cellular protein expression, as under these circumstances insulin had no discernible effect on the expression of the α1 Na, K-ATPase subunit, another plasma membrane transport protein [14]. Likewise, the cellular abundance of protein kinase B (PKB), a predominantly cytosolic localised protein kinase [20], was unaffected by long-term insulin treatment (Fig. 2A). Moreover, it is noteworthy that a sustained (24 h) exposure to insulin has been shown previously not to increase cellular GLUT4 expression in L6 cells [21].

3.3. Inhibitors of gene transcription and protein synthesis block the insulin-dependent increase in fructose uptake

The dose-dependent increase in GLUT5 expression elicited by chronic insulin treatment (Fig. 2A) was associated with an attendant increase in fructose uptake (Fig. 3A). It is plausible that the observed increases in GLUT5 expression and fructose uptake that we observe in response to the higher hormone concentrations used are as a result of insulin signalling through the IGF-1, rather than the insulin receptor. It has been previously reported that insulin (<100 nM) and IGF-1 (<10 nM) signal largely (>80%) through their respective receptors in L6 myotubes [22–24]. Given that insulin enhanced GLUT5 expression/function at concentrations as low as 10 and 100 nM (Figs. 2 and 3A), the observed effects are likely to be mediated via the insulin receptor. Nevertheless, to address this issue further we assayed glucose and fructose uptake in L6 myotubes after a 24-h incubation with IGF-1 (at concentrations between 0.3 and 10 nM). Incubation of L6 myotubes with 10 nM IGF-1 has previously been shown to up-regulate glucose transporter expression via the IGF-1 receptor [23]. Consistent with this we found that chronic incubation of muscle cells with IGF-1 induced an increase in glucose uptake by over 2.5-fold, whereas there was no enhancing effect on fructose uptake (Fig. 3B). This finding provides further support for the idea that signalling via the insulin, and not the IGF-1, receptor is likely to direct changes in GLUT5 expression and function in L6 myotubes.

The characteristics with which glucose and fructose are taken up in muscle cells can be further discriminated based on the sensitivity of the transport process to an acute bout of insulin treatment. Whilst long-term insulin exposure stimulates fructose uptake, the hormone fails to elicit any increase in the uptake of this sugar when presented to cells acutely (Fig. 3C). In contrast, glucose uptake was stimulated by over two-fold and was sensitive to inhibition by CB (Fig. 3C). We subsequently investigated whether the insulin-mediated up-regulation in GLUT5 function was dependent upon gene transcription and ongoing protein synthesis. To assess this we pre-treated L6 myotubes with Act D, an inhibitor of DNA transcription or cycloheximide, a protein synthesis inhibitor, 15 min prior to incubation with 1 μM insulin for 24 h. Each inhibitor was present during the 24-h insulin treatment period. Fig. 3D shows that the increase in cellular fructose uptake induced by 24 h of insulin treatment was both transcription- and translation-dependent. The inclusion of CB (10 μM) in the uptake assay failed to suppress the basal or the insulin-induced increase in fructose uptake, consistent with the suggestion that fructose uptake and the increase elicited by insulin is unlikely to arise as a result of changes in the expression of other transporters, such as GLUT1 and GLUT4.

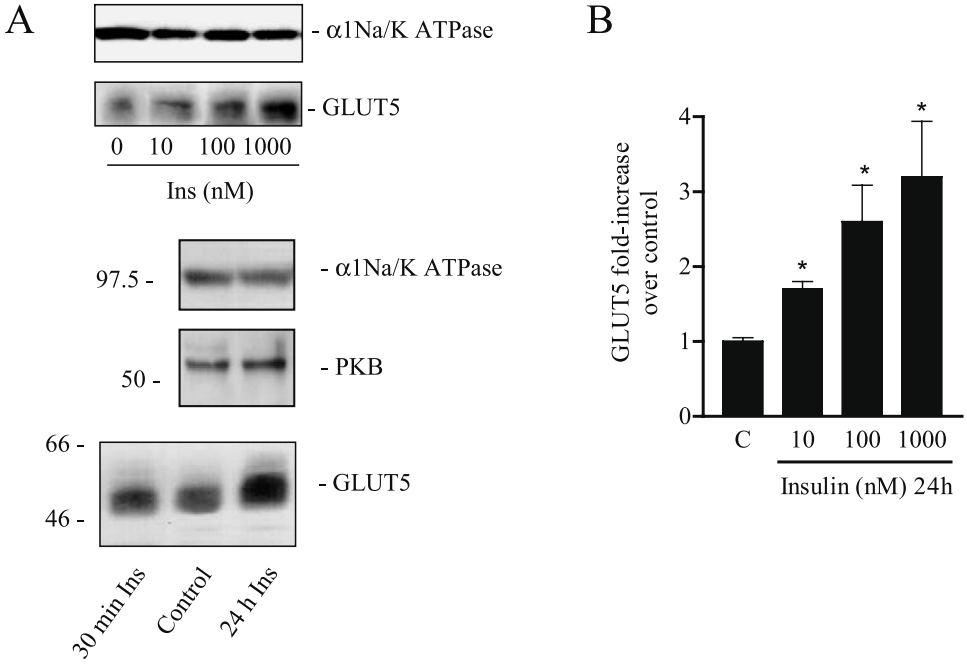

Fig. 2. Effect of acute and chronic insulin treatment on GLUT5 expression in L6 muscle cells. L6 myotubes were incubated with insulin for times and concentrations indicated. L6 plasma membranes were prepared and 15 μg membrane protein analysed by SDS–PAGE and immunoblotting as described in Section 2. A: Representative immunoblots from up to four separate experiments showing plasma membrane abundance of GLUT5, the α1 subunit of the Na^+,K^+-ATPase and cytosolic content of PKB in untreated (control) and insulin-incubated L6 myotubes. B: Densitometric quantification of GLUT5 abundance in L6 plasma membranes from 24-h insulin-treated cells. GLUT5 signal density from untreated cells was assigned a value of 1. Values represent means ± S.E.M. ($n = 4$). * Statistically significant change compared with control cells ($P < 0.05$).

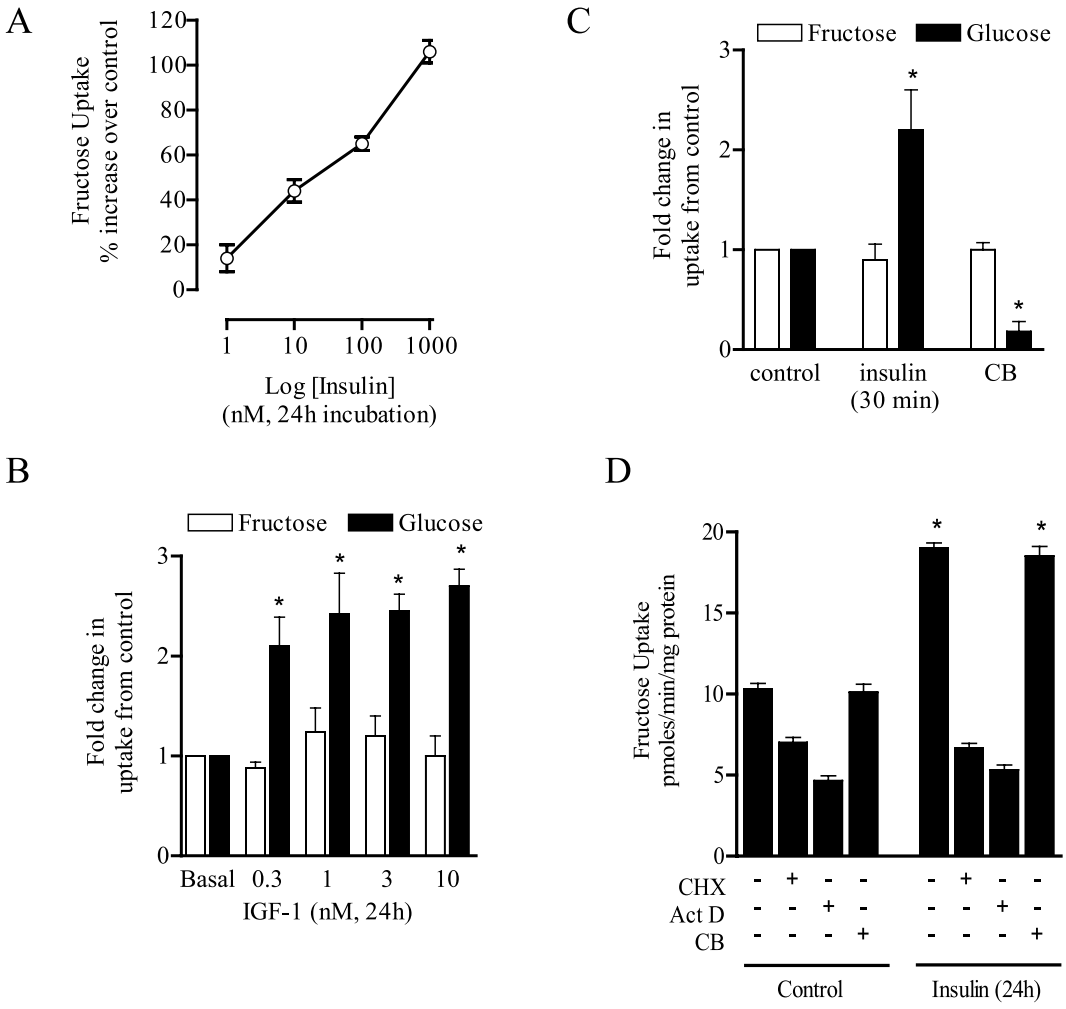

Fig. 3. Effects of insulin and inhibitors of gene transcription and protein synthesis on fructose uptake in L6 myotubes. A: L6 myotubes were incubated with different insulin concentrations (between 1 nM and 1 μM) for 24 h prior to assaying fructose uptake as described in Section 2. Uptake was expressed as a percentage increase over that measured in cells not treated with insulin. B: L6 cells were incubated with IGF-1 at concentrations shown for 24 h prior to assaying fructose and glucose uptake. C: Fructose and glucose uptake were assessed in muscle cells in the absence and presence of 100 nM insulin. D: Muscle cells were incubated with 3.5 μM cycloheximide (CHX) or 4 μM Act D 15 min before treatment with 1 μM insulin for 24 h and fructose uptake assay. In some experiments 10 μM CB was included during the uptake assay. Values represent means ± S.E.M. ($n = 3$–4). * Statistically significant change compared with fructose uptake measured in untreated (control) cells ($P < 0.05$).

The finding that Act D suppresses insulin's ability to up-regulate fructose uptake suggests that the hormone may enhance GLUT5 gene expression largely through a transcriptional mechanism, perhaps by stimulating the activity of the GLUT5 promoter. To test this possibility, we transfected L6 cells with a human GLUT5 promoter fused to luciferase. Since transfection efficiency is low in fully differentiated L6 myotubes, we used L6 myoblasts, which also express GLUT5 (see Fig. 1). Myoblasts were transfected with 1 μg plasmid containing GLUT5 promoter and then maintained at 37°C in a CO_2 incubator for 24 h prior to study, so as to allow sufficient promoter expression. Luciferase activity was found to be ~five-fold higher when the GLUT5 promoter was fused to the reporter gene, compared to cells transfected with a promoter-less construct (Fig. 4A, inset). Fig. 4 shows that incubation of transfected cells with insulin for 3 h led to a two-fold activation in GLUT5-driven luciferase activity (Fig. 4A). An analysis of the GLUT5 promoter sequence revealed the presence of a number of putative insulin responsive elements (IREs at regions around −1705, −1600, −480 and −320). Three different 5′-deleted promoter–luciferase constructs (which lacked these putative IREs) were transfected into L6 cells. Fig. 4B shows that deletion to −385 bp produced a 50% decrease in insulin-induced luciferase activity. Almost complete inhibition of insulin-stimulated luciferase activity was observed when cells were transfected with the −272/+41-bp GLUT5 promoter construct (Fig. 4B).

4. Discussion

It is now evident that at least three of the classical facilitative sugar transporters, each having distinct biochemical properties and functional roles with regard to maintenance of sugar homeostasis, are expressed in insulin-sensitive tissues (i.e. skeletal muscle and fat). GLUT1 and GLUT4 function as the principal mediators of glucose uptake in these tissues [25], the latter being responsible for the acute enhancement in glucose uptake in response to insulin. In rat adipocytes, an acute

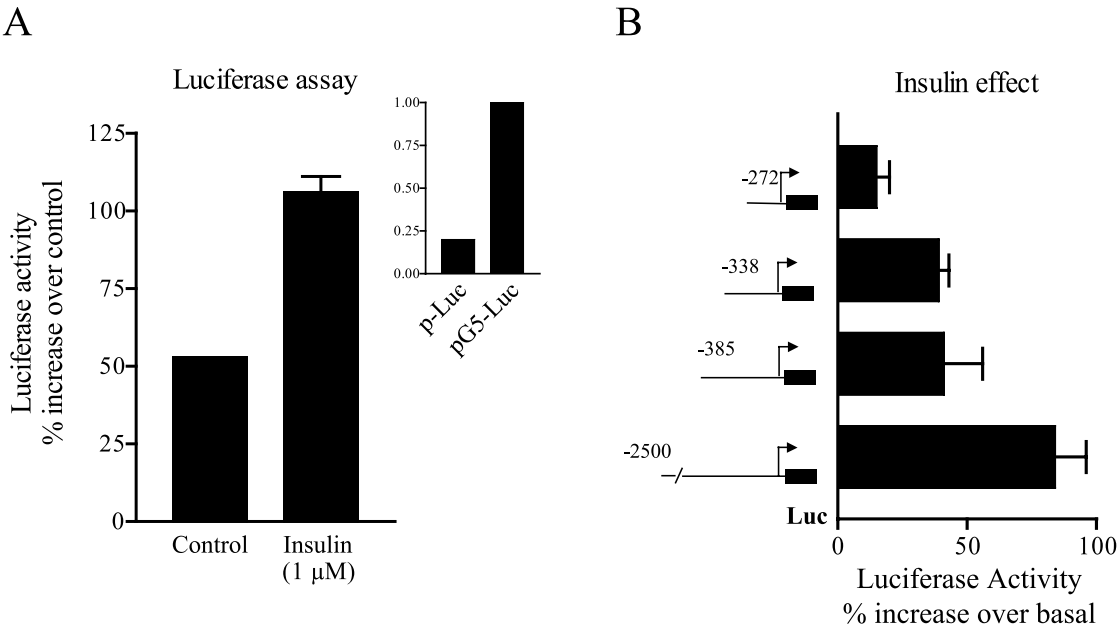

Fig. 4. Effect of insulin on GLUT5 promoter activity in L6 myoblasts. A: L6 myoblasts were transfected with 1 μg of different constructs of pGLUT5-Luc. Cells were allowed to recover for 24 h post transfection with the GLUT5 promoter containing the −2500/+41 region prior to incubation with 1 μM insulin for 3 h and assay of luciferase activity. The inset shows results obtained from L6 cells transfected with the GLUT5 promoter-less–luciferase (p-Luc) construct compared to cells transfected with the −2500/+41 promoter construct (pG5-Luc) in the absence of insulin. B: Luciferase activity obtained in cells transfected with GLUT5 promoters encoding the −2500/+41, −385/+41, −338/+41 and −272/+41 regions following incubation with 1 μM insulin for 3 h. Luciferase values represent means ± S.E.M. from at least three separate experiments. * Statistically significant change compared with untreated transfected cells ($P < 0.05$).

insulin challenge induces a marginal (15–20%) increase in fructose uptake, which can be suppressed by CB, an inhibitor of facilitative glucose transport, indicating that GLUT4 has a modest capacity to mediate fructose uptake [5]. However, the major fraction of total fructose uptake in both rat adipocytes [5] and skeletal muscle [6] is insensitive to CB, suggesting that most of the fructose is transported via another carrier. Heterologous expression studies in *Xenopus* oocytes indicate that GLUT5 functions primarily as a fructose transporter and that it is insensitive to inhibition by CB [26]. Given that the presence of fructokinase has previously been demonstrated in skeletal muscle [27,28], the uptake of fructose across the plasma membrane of muscle cells via GLUT5 represents the first committed step in fructose utilisation in this tissue. Whilst glucose metabolism becomes predominant over that of fructose in the presence of insulin, physiological concentrations of fructose contribute significantly to lactate production and can account for up to 30% of total glycogen synthesis when circulating insulin is low [4]. Such observations underscore the important contribution that GLUT5 is likely to make in supporting the use of fructose as a carbohydrate source in skeletal muscle.

Insulin not only exerts acute effects on numerous responses in skeletal muscle (e.g. stimulation of glucose transport), but also imparts anabolic effects in the long-term through regulation of gene expression [29]. Our data indicate that insulin has no acute stimulatory effect on GLUT5 abundance or upon fructose uptake in L6 muscle cells, but that both parameters are enhanced by long-term exposure of cells to insulin in a dose-dependent manner, and that this event relies upon gene transcription. The observed effects on transporter expression were most notable at supramaximal concentrations of insulin (1 μM). At these concentrations the hormone is also likely to signal through IGF-1 receptors, although our data suggest that signalling via the IGF-1 receptor per se is unlikely to induce the up-regulation in GLUT5 expression/function that we see in response to insulin. This proposition is further supported by the finding that significant increases in transporter function (~15–20%) were observed in response to insulin at more physiological concentrations (i.e. 1 nM; Fig. 3A) that are unlikely to invoke activation of the IGF-1 receptor. Our findings imply that a positive correlation exists between insulin availability and GLUT5 expression and raise the interesting possibility that circulating levels of insulin may be very important for maintaining the normal expression and function of GLUT5 in skeletal muscle. In this regard, it is worth stressing that we have previously observed a profound reduction in adipocyte GLUT5 expression in rats rendered diabetic with streptozotocin, which lack the ability to synthesise insulin and hence are hypo-insulineamic [5]. Furthermore, recent work in our laboratory has shown that fat cell expression of GLUT5 is dramatically down-regulated during insulin resistance, consistent with our suggestion that the hormone may provide a tonic stimulus to help maintain GLUT5 expression (Litherland, Hajduch and Hundal, unpublished data). Since both skeletal muscle and adipose tissue contribute significantly to fructose utilisation [1], it is plausible that changes in GLUT5 expression elicited in response to insulin availability or insulin resistance may have a significant impact on circulating fructose levels. Indeed, Kawasaki et al. [30] have reported very recently that serum levels of fructose are 50% higher in diabetic than in non-diabetic or healthy human subjects. Whilst the primary defect that gives rise to this increase in blood fructose remains unclear, sustained increases in serum concentrations of the ketose have been associated with adverse metabolic changes such as glucose intolerance, hyperlipidaemia

[31] and non-enzymatic fructosylation of proteins [32], all factors implicated in the pathogenesis of diabetic complications.

Although previous studies have shown that chronic insulin treatment up-regulates GLUT1 and glucose transport in L6 cells [21], our data represent the first demonstration that insulin increases GLUT5 abundance and function via activation of the GLUT5 promoter. This finding raises the possibility that the promoter driving the expression of the GLUT5 gene may possess insulin response elements. Indeed, sequence analysis of the GLUT5 promoter reveals the presence of putative insulin-responsive motifs at −1705, −1620, −535 and −320 of the promoter. Removal of the promoter region between −2500 and −385 bp (which results in the loss of three of the four putative IREs) resulted in a 50% decrease in luciferase activity, suggesting the presence of critical response element(s) that are indispensable for maintaining full GLUT5 promoter activity in this cell type. Furthermore, the truncation of the promoter up to −272 bp resulted in 85% loss of the insulin-stimulated luciferase activity, indicating that the −338 to −272 bp region of the promoter contains an additional insulin-responsive region (possibly the putative IRE identified at −320). The presence of hormone response elements in the GLUT5 gene promoter is not unprecedented. Matosin-Matekalo et al. have shown in Caco-2 cells, a human colon cancer cell line, that the GLUT5 gene promoter could be activated by triiodothyronine (T_3) and that the −308/−290 region of the promoter contained a T_3 responsive element [15]. These workers also demonstrated that glucose could up-regulate GLUT5 promoter activity in Caco-2 cells and that a glucose-responsive element was likely to also be present in the promoter [15]. However, in our cell-based studies increased glucose or fructose availability over a 24-h period does not enhance functional fructose uptake (data not shown). This finding is in line with our previous work showing that feeding rats a fructose-enriched diet does not up-regulate GLUT5 expression in rat skeletal muscle, but does so in the intestine and kidney [6]. Such differences in substrate-induced regulation of GLUT5 may reflect the distinct roles that the carrier plays in tissues where the primary function is to absorb or reabsorb fructose from the intestinal lumen and kidney tubule respectively, as opposed to utilising the sugar for metabolism in tissues such as muscle and fat.

In summary, there is now growing evidence that fructose can be utilised as a source of fuel in tissues that are primary targets of insulin action (i.e. skeletal muscle and adipose tissue). The ability to utilise this sugar is inherently dependent upon the expression of GLUT5 in these tissues. Whilst insulin is unable to acutely up-regulate fructose utilisation in muscle, it is likely to provide an important physiological stimulus that is required for the constitutive expression of GLUT5 in this tissue, probably by regulating the activity of the GLUT5 promoter. The molecular mechanism involved in this process remains currently unknown, but may involve modulated association of transcription factors with specific promoter regions. Indeed, use of TESS, a web-based search programme for transcription element binding sites (http://www.cbil.upenn.edu/tess), reveals putative binding sites for C/EBP, CREB, PPAR, MyoD and myogenin within the GLUT5 promoter, which may be involved in regulating the activity of the promoter in response to changes in hormone and substrate availability. Exploring the molecular regulation of the GLUT5 promoter represents an interesting topic for future study and may give further insights into the mechanism by which insulin regulates the expression of GLUT5 and whether its dys-regulation contributes to pathophysiological changes in whole-body fructose metabolism.

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