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Dexamethasone stimulates the expression of GLUT1 and GLUT4 proteins via different signalling pathways in L6 skeletal muscle cells

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Abstract It was recently demonstrated that dexamethasone treatment of L6 skeletal muscle cells resulted in an elevation of GLUT1 protein. However, the level of GLUT4 protein under these conditions was not examined. In addition, the signalling mechanism(s) leading to dexamethasone-induced expression of GLUT1 protein was not investigated. In the present study we investigated the effect of dexamethasone on the expression of GLUT1 and GLUT4 proteins in differentiated L6 muscle cells and the signalling mechanism(s) via which dexamethasone may act. Dexamethasone (300 nM) treatment for 24 h elevated GLUT1 and GLUT4 proteins by 68% and 94%, respectively, above control levels. These increases were due to de novo synthesis as shown by metabolic labelling with [^{35}S]methionine. Incubation of cells with 100 nM wortmannin or 30 ng/ml rapamycin prevented the dexamethasone-stimulated elevation of GLUT1 protein. In contrast, neither of these inhibitors affected the elevation of GLUT4 protein by dexamethasone. Furthermore, dexamethasone down-regulated insulin receptor substrate-1 protein content by 42% and insulin-induced tyrosine phosphorylation of insulin receptor substrate-1 by 28%. The p70 ribosomal S6 kinase was not activated by dexamethasone and instead, dexamethasone attenuated the stimulation of this enzyme activity by insulin. These results suggest that dexamethasone induces the expression of GLUT1 and GLUT4 protein by independent signalling mechanisms with a concomitant depression of intracellular signalling by insulin.

Key words: Glucose transporter; Phosphatidylinositol 3-kinase; p70 ribosomal S6 kinase; Mammalian target of rapamycin; PHAS-I

1. Introduction

Dexamethasone, insulin and cellular stressors such as 2,4-dinitrophenol (an uncoupler of oxidative phosphorylation) and hyperosmolarity have the common cellular effect of stimulating the expression of the glucose transporter GLUT1 in L6 skeletal muscle cells [1–3]. Upregulation of GLUT1 protein synthesis by insulin is completely abrogated by rapamycin [4] suggesting that in mediating this effect, insulin utilizes a signalling pathway involving mammalian target of rapamycin (mTOR). The serine/threonine kinase p70 ribosomal S6 kinase (p70 S6 kinase) lies downstream of mTOR and its activation by insulin is also inhibited by rapamycin [5]. Hence, these results have been interpreted as indicating that insulin induces GLUT1 protein synthesis by an mTOR/p70 S6 kinase signalling pathway [4]. The molecular mechanisms underlying the increase in GLUT1 protein expression stimulated by stressors have been described in a recent study [3]. Hyperosmolarity induces the expression of GLUT1 via the mTOR/p70 S6 kinase signalling pathway [3] as well as by the mitogen-activated protein kinase (MAPK)-dependent pathway [3]. In contrast, there are no studies describing the molecular mechanism(s) involved in the stimulation of GLUT1 expression by dexamethasone.

Understanding the action of dexamethasone on glucose transporters is attractive since an elevation of GLUT4 protein level has been demonstrated in skeletal muscle of dexamethasone-treated rats [6,7]. An increase in GLUT4 protein has been suggested to improve insulin sensitivity [8], yet dexamethasone also causes insulin resistance in vivo. Interestingly, the level of GLUT4 protein is not affected by either insulin or stressors in muscle cells in culture [2,3] while the effect of dexamethasone on the level of GLUT4 protein in these cells has not been described.

In the present study we investigated the intracellular signalling mechanisms by which dexamethasone induces the expression of GLUT1. Secondly, we examined the effect of dexamethasone on the expression of GLUT4. We show that 24-h treatment of L6 skeletal muscle cells with dexamethasone elevated GLUT1 and GLUT4 protein expression. Dexamethasone-induced expression of GLUT1 was inhibited by wortmannin, an inhibitor of phosphatidylinositol 3-kinase, and by rapamycin. In contrast, the expression of GLUT4 was not affected by either wortmannin or rapamycin. Finally, we show that dexamethasone down-regulated insulin signals.

2. Materials and methods

2.1. Materials

Rabbit antisera containing polyclonal antibodies raised against carboxy-terminal sequences of GLUT1 and GLUT4 were obtained from East Acres Biological Laboratories (Southbridge, MA). Monoclonal anti-phosphotyrosine antibody and polyclonal antibodies to p70 S6 kinase and insulin receptor substrate-1 (IRS-1) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-p70 S6 kinase antibody (used to immunoprecipitate for activity assays), PKA and PKC inhibitory peptides and S6 peptide (RRRLLSSLRA) were from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody McK1 to the α1-subunit of the Na^+/K^+-ATPase was a kind gift from Dr. K. Sweadner (Massachusetts General Hospital). Antibodies used for immunoprecipitating GLUT1 and GLUT4 were previously described [9]. Protein A-Sepharose was from Pharmacia (Uppsala, Sweden). [^{35}S]Methionine and [γ-^{32}P]ATP were from Amersham (Oakville, Ont., Canada).

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2.2. Cell culture

A subclone of rat skeletal muscle L6 cells, selected for high fusion potential was used [10]. To obtain differentiated myotubes for experiments, cells were grown in α-MEM (2% FBS) [10]. For preparation of lysates the cells were seeded in 12-well plates (1×10^4 cells/ml) and prepared as in [11]. Cells were grown in 10-cm dishes (4×10^4 cells/ml) for preparation of total cellular membranes and p70 S6 kinase activity. Total membranes were prepared as previously described [12].

2.3. [^{35}S]Methionine labelling and immunoprecipitation of glucose transporters

L6 myotubes were treated with or without 300 nM dexamethasone in α-MEM supplemented with 0.2% (v/v) FBS for 24 h. During the last 2 h of incubation cells were incubated in methionine-free α-MEM supplemented with 0.2% (v/v) dialysed FBS in the continued presence or absence of dexamethasone. During the final 2 h the cells were pulsed for 2 h with 200 μCi of [^{35}S]methionine per dish. The labelling medium was removed, cells were washed twice with ice-cold phosphate buffered saline (PBS), solubilized with $C_{12}E_8$ and GLUT1 and GLUT4 glucose transporters were immunoprecipitated essentially as described [9].

2.4. p70 S6 kinase activity

L6 myotubes grown in 10-cm dishes were washed twice with ice-cold PBS and lysed in 1 ml Buffer A (50 mM HEPES, pH 7.5, 150 mM NaCl, 20 mM β-glycerophosphate, 10 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM Na_3VO_4 and 1% NP-40 (v/v)) containing a mixture of proteinase inhibitors (1 μM leupeptin, 1 μM pepstatin A, 10 μM E64, and 200 μM phenylmethylsulfonyl fluoride). After 15 min of slow agitation and centrifugation ($15\,000\times g$ for 15 min), the supernatant was subjected to immunoprecipitation. p70 S6 kinase was immunoprecipitated using 1 ml of cell extract and 1 μg of a rabbit polyclonal p70 S6 kinase antibody. The protein A-Sepharose complex was washed three times with Buffer B (50 mM Tris-acetate, pH 8, 50 mM NaF, 5 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 10 nM okadaic acid, 0.1% (v/v) β-mercaptoethanol) including all the proteinase inhibitors used above and twice with Buffer C (20 mM 4-morpholinepropanesulfonic acid, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 2 mM EDTA, 20 mM $MgCl_2$, 2 mM Na_3VO_4 and 1 mM dithiothreitol. p70 S6 kinase activity was assayed essentially as described [13] in a final volume of 50 μl of buffer C containing 1 μM protein kinase A and 1 μM protein kinase C inhibitor peptides, 0.2 mM S6 peptide, and 0.25 mM Mg-[γ-^{32}P]ATP at 30°C for 10 min. Aliquots (30 μl) were transferred onto Whatman p81 papers and washed 3 times for 15 min with 175 mM phosphoric acid [14]. ^{32}P incorporated into the S6 peptide was measured by liquid scintillation counting. One unit of protein kinase activity corresponds to 1 μM of ^{32}P incorporated into the substrate peptide under the assay conditions.

2.5. Immunoblotting

Total cell lysates (15 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis on 7% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were probed with anti-phosphotyrosine antibody (1 μg/ml) followed by sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase (1:5000 dilution) or probed with anti-IRS-1 (1 μg/ml) or anti-p70 S6 kinase (1 μg/ml) antibodies followed by goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (1:5000 dilution). Proteins were visualized by the enhanced chemiluminescence method. GLUT1 and GLUT4 levels were determined in total membranes (50 μg protein) separated on 10% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were probed with anti-GLUT1 (1:2000 dilution) or anti-GLUT4 (1:2000 dilution) antibodies followed by ^{125}I-labeled protein-A (1 μCi/10 ml). Blots were visualized by autoradiography using XAR-5 film (Eastman Kodak, Rochester, NY). Laser scanning densitometry was done using a PDI model DNA 35 scanner with version 1 of the discovery series one-dimensional gel analysis software.

2.6. Statistical analysis

Data were compared using either the analysis of variance test (Fischer, multiple comparisons) or Student's paired *t*-test as indicated.

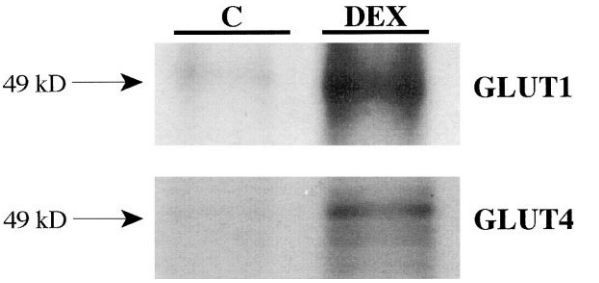

Fig. 1. Effect of dexamethasone treatment on GLUT1 protein. L6 muscle cells were incubated with or without 300 nM dexamethasone (DEX) for 24 h in α-MEM containing 0.2% FBS in the absence (1 μl/ml DMSO vehicle) or presence of 100 nM wortmannin (WORT) or 30 ng/ml rapamycin (RAPA). Total membranes were prepared and subjected to immunoblot analysis as described in Section 2. a: Representative blot showing the effect of the various conditions on GLUT1 protein. The content of Na^+/K^+-ATPase α1 subunit was determined to assess equality of protein loading. b: Mean ± S.E. of four independent experiments showing the content of GLUT1 protein. Total GLUT1 protein content in control cells in the absence of DEX was assigned a value of 1.0, and all other values are expressed relative to it. *Compared to untreated control. Compared to DEX-treated control (ANOVA).

3. Results

3.1. Effect of wortmannin and rapamycin on the expression of GLUT1 stimulated by dexamethasone

Differentiated L6 muscle cells were treated for 24 h with dexamethasone (DEX) in the absence or presence of 100 nM wortmannin, an inhibitor of phosphatidylinositol 3-kinase [15] and total cell membranes were prepared to examine the effect of this treatment on glucose transporter levels. As shown in Fig. 1A and quantified in Fig. 1B, GLUT1 level was increased by 68% above control in cells treated with DEX. We found that this increase in GLUT1 protein was blocked by incubation with wortmannin. To further characterize the molecular mechanism(s) that may be mediating the effect of dexamethasone on GLUT1, we utilized rapamycin. Rapamycin (30 ng/ml) inhibited the DEX-stimulated increase in GLUT1 protein. Hence, dexamethasone stimulated the expression of GLUT1 in L6 skeletal muscle cells through a wortmannin- and rapamycin-sensitive pathway.

3.2. Effect of wortmannin and rapamycin on the expression of GLUT4 stimulated by dexamethasone

The effect of DEX on GLUT4 protein level has not been reported in muscle cells in culture although an elevation of GLUT4 protein has been demonstrated in skeletal muscle of glucocorticoid-treated rats [6,16]. It is unknown if this effect is due to a direct action of DEX on skeletal muscle tissue. Therefore, we investigated the effect of dexamethasone on the level of expression of GLUT4 protein in L6 skeletal muscle cells in culture. Total membranes prepared from DEX-treated cells had an elevated GLUT4 protein level of 94% above vehicle treated control cells (Fig. 2). However, in contrast to the results obtained for GLUT1 this was not blocked by incubation with either 100 nM wortmannin or 30 ng/ml rapamycin.

3.3. Dexamethasone stimulates the synthesis of GLUT1 and GLUT4

To determine if the elevation of GLUT1 and GLUT4 pro-

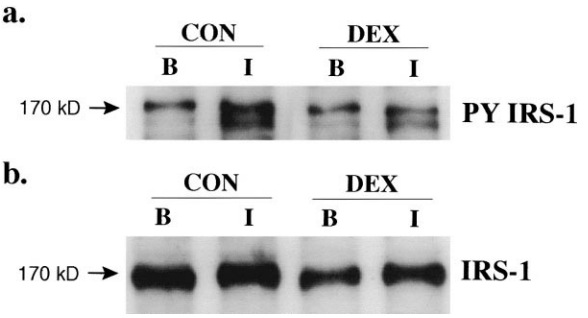

Fig. 2. Effect of dexamethasone treatment on GLUT4 protein. L6 muscle cells were incubated with or without 300 nM dexamethasone (DEX) for 24 h in α-MEM containing 0.2% FBS in the absence (1 μl/ml DMSO vehicle) or presence of 100 nM wortmannin (WORT) or 30 ng/ml rapamycin (RAPA). Total membranes were prepared and subjected to immunoblot analysis as described in Section 2. a: Representative blot showing the effect of the various conditions on GLUT4 protein. The content of Na^+/K^+-ATPase α1 subunit was determined to assess equality of protein loading. b: Mean ± S.E. of four independent experiments showing the content of GLUT4 protein. Total GLUT4 protein in control cells in the absence of DEX was assigned a value of 1.0, and other values are expressed relative to it. *Compared to untreated control (ANOVA).

tein content induced by DEX were due to de novo synthesis of glucose transporters, we investigated the ability of DEX to stimulate the metabolic incorporation of [^{35}S]methionine into the transporter proteins. As shown in Fig. 3, cells treated with DEX for 24 h showed increased metabolic labelling of both GLUT1 and GLUT4. These results indicate that the elevation of GLUT1 and GLUT4 protein levels observed with DEX treatment is a direct consequence of increased protein synthesis.

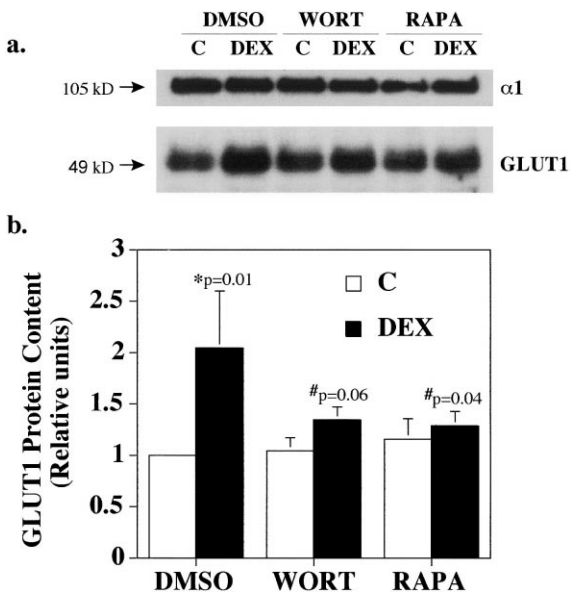

Fig. 3. Effect of dexamethasone treatment on the synthesis of GLUT1 and GLUT4 proteins. Cells treated for 24 h without (C) or with 300 nM dexamethasone in α-MEM containing 0.2% FBS were radiolabelled with [^{35}S]methionine as described in Section 2. GLUT1 and GLUT4 were immunoprecipitated and resolved on 10% SDS-PAGE. Gels were dried and the amount of ^{35}S-labelled glucose transporters determined by fluorography.

3.4. Effect of dexamethasone on IRS-1 protein level and phosphorylation

The increased expression of GLUT1 induced by DEX is reminiscent of the effect of prolonged exposure of cells to insulin [2,17]. However, it has been demonstrated that DEX treatment is associated with a down-regulation of insulin receptor substrate-1 (IRS-1) protein, an immediate downstream substrate of the insulin receptor [18], in several cell types [19–21]. To determine if DEX affects insulin signalling events in differentiated L6 muscle cells we examined whether DEX treatment altered IRS-1 protein content or its insulin-stimulated tyrosine phosphorylation. DEX treatment for 24 h reduced IRS-1 protein content to 57.2 ± 0.07% of the control levels (Fig. 4) ($P < 0.05$: Student's paired t-test). When these cells were stimulated with insulin for 5 min tyrosine phosphorylation of IRS-1 was reduced to 72.4 ± 0.04% of the control level by DEX treatment ($P < 0.05$: Student's paired t-test). These results suggest that dexamethasone treatment does indeed alter the insulin signalling cascade at an early step.

3.5. Effect of dexamethasone on p70 S6 kinase activity

The stimulation of GLUT1 protein synthesis by insulin [2] has been suggested to be mediated by the mTOR/p70 S6 kinase signalling pathway [4]. Rapamycin inhibited the DEX-stimulated increase in GLUT1 protein (Fig. 1, Fig. 2). This suggested that DEX may be utilizing the mTOR/p70 S6 kinase pathway. To test this possibility we investigated the ability of DEX to activate p70 S6 kinase. Cells treated for 30 min or 3 h with 300 nM DEX showed no activation of p70 S6 kinase (Fig. 5). Intriguingly, in cells treated with DEX for 3 h and then stimulated with 100 nM insulin for 5 min, DEX actually

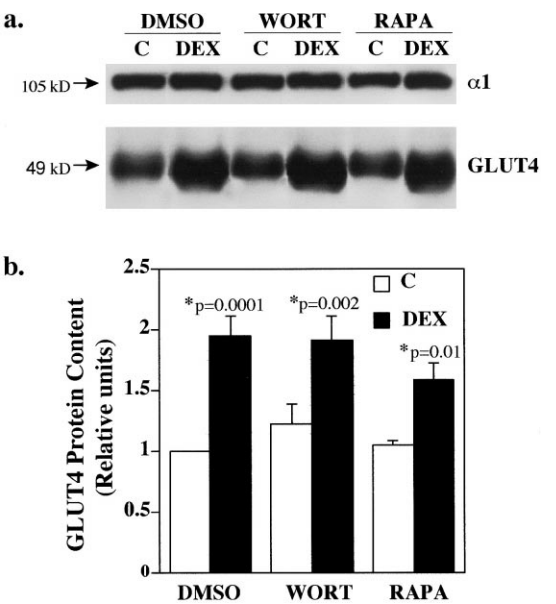

Fig. 4. Effect of dexamethasone treatment on IRS-1 protein content and tyrosine phosphorylation. L6 muscle cells were incubated without (CON) or with 300 nM dexamethasone (DEX) for 24 h in α-MEM containing 0.2% FBS. Cells were then treated without (Basal, B) or with 100 nM insulin (I) for 5 min, and IRS-1 protein and tyrosine phosphorylation were detected in whole cell lysates as described in Section 2. Representative blots for (a) phosphotyrosine and (b) IRS-1 protein levels are shown. Changes in IRS-1 protein content and tyrosine phosphorylation of four independent experiments were determined by densitometric scanning and are discussed in the text.

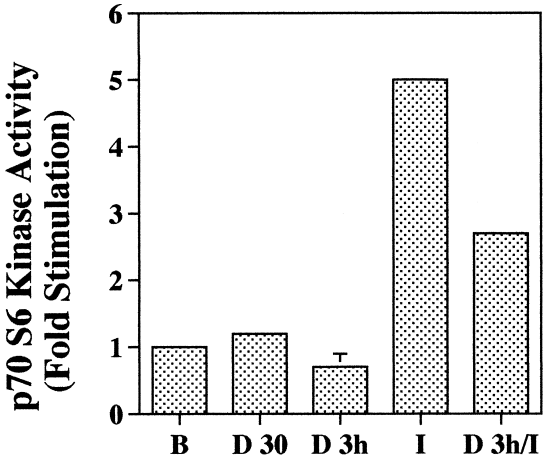

Fig. 5. Effect of dexamethasone on p70 S6 kinase activity. L6 muscle cells were deprived of serum for 12 h and then treated without (Basal, B) or with 300 nM DEX for 30 min (D 30) or 3 h (D 3h). Treated or untreated cells were stimulated with 100 nM insulin for 5 min (I). p70 S6 kinase activity was subsequently determined using whole cell lysates as described in Section 2. Results are the mean of two or three experiments presented as a percent change from control cells.

reduced insulin-stimulated p70 S6 kinase activity. There was no alteration in the total protein content of p70 S6 kinase in cells treated with 300 nM DEX for up to 24 h (104.2 ± 13% of control). This is the first time that the effect of DEX on insulin-stimulated p70 S6 kinase activity has been investigated.

4. Discussion

Glucocorticoids induce a state of insulin resistance when administered to humans or experimental animals [22]. Studies in whole animals preclude the assessment of the site of action of glucocorticoids. It is possible that these hormones act directly on peripheral tissues resulting in insulin resistance, or alternatively through changes in the blood levels of glucose, insulin or fatty acids that occur during glucocorticoid excess. The use of cells in culture provides a means to focus on the effect of hormones directly. In the present study we examined the effect of dexamethasone (DEX) on the expression of the glucose transporters GLUT1 and GLUT4, in L6 skeletal muscle cells. We show by metabolic labelling with [^{35}S]methionine that DEX increased the biosynthesis of GLUT1 and GLUT4 proteins. In addition, DEX down-regulated two insulin-dependent signals: insulin receptor substrate-1 (IRS-1) phosphorylation and activation of p70 ribosomal S6 kinase. We hypothesize that the elevation in GLUT1 and GLUT4 proteins by DEX might be an adaptive response to the down-regulation of insulin signals.

Rapamycin inhibited the induction of GLUT1 protein by DEX. This indicates that either mTOR or some downstream effector that is not necessarily p70 S6 kinase, participates in this response. DEX did not stimulate p70 S6 kinase activity (Fig. 5). Therefore the sensitivity to rapamycin could be due to a direct participation of mTOR itself and not p70 S6 kinase. Brunn et al. [23] have recently demonstrated that the translational repressor PHAS-I (protein that is heat and acid stable-insulin responsive) is phosphorylated by mTOR and the phosphorylation of the latter is inhibited by rapamycin. Phosphorylation of PHAS-I is an important step mediating insulin-stimulated protein synthesis [24,25]. In addition, von Manteuffel et al. (1997) have clearly demonstrated that PHAS-I and p70 S6 kinase are located on parallel signalling pathways, downstream of mTOR [26]. Hence, the ability of rapamycin to inhibit DEX-stimulated expression of GLUT1 protein may implicate the mTOR/PHAS-I pathway. Rapamycin has also been suggested to interfere with signal transduction from the glucocorticoid receptor by interacting with immunophilins that associate with the glucocorticoid receptor [27]. These immunophilins (several heat shock proteins) facilitate proper translocation of the glucocorticoid signalling complex to the nucleus [28]. This is unlikely in our study since if rapamycin was interfering with activation of the glucocorticoid receptor, then there should not be any preference for the inhibition of GLUT1 protein expression over GLUT4. However, the elevation of GLUT4 protein by DEX was not inhibited by rapamycin. This indicates that different pathways are involved in the regulation of GLUT1 and GLUT4 protein expression. This is not surprising since Sivitz and Paisley (1995) demonstrated that GLUT1 mRNA and protein are elevated by DEX. However, there was no change in the amount of GLUT4 mRNA. Here we show that GLUT4 protein increases in response to DEX.

Wortmannin treatment inhibited the stimulation of GLUT1 protein expression by DEX without affecting the DEX-induced expression of GLUT4. DEX treatment reduces IRS-1 associated phosphatidylinositol 3-kinase (PI 3-kinase) activity in rat skeletal muscle [29] and reduces IRS-1 associated PI 3-kinase activity stimulated by IGF-1 in L6 muscle cells by 50% [30]. Hence, DEX is not expected to act via a PI 3-kinase-dependent pathway to induce GLUT1 protein. The sensitivity of GLUT1 protein expression to wortmannin may be due to a non-specific action of wortmannin. Indeed, wortmannin has been shown to inhibit phospholipase A2 [31], mitogen activated protein kinases [32], phosphatidylinositol 4-kinase [33], mTOR autokinase activity [34] and PHAS-I phosphorylation [23]. We suggest that the observed wortmannin sensitivity could be due to a direct inhibitory effect on mTOR or the mTOR-mediated signalling pathway involving PHAS-I.

From the above discussion we conclude that direct exposure of muscle cells to DEX reduced total IRS-1 protein, impaired its insulin-dependent tyrosine phosphorylation and reduced insulin-dependent activation of p70 S6 kinase. In addition, DEX treatment increased biosynthesis of GLUT1 and GLUT4 proteins. This action did not utilize known elements of insulin signalling pathways known to regulate glucose transporters synthesis. The increased transporter levels may arise in an attempt to counteract a detrimental effect of DEX on insulin signal transduction.

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