RESEARCH ARTICLE

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The Effects of Insulin and Metformin on Glucose Uptake in L8 Myotubes

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ABSTRACT: The establishment of *in vitro* cell culture systems is a useful approach in biomedical research studies to assist understanding of *in vivo* events. In this study, we have explored the effects of insulin and the hypoglycemic drug metformin on glucose uptake activity, and the possibility of using L8 cells for the studies of glucose transport was investigated. Serum deprivation caused a 35% reduction in basal glucose uptake by L8 myotubes. In the absence of serum, insulin showed a dose-related increase glucose stimulation. Insulin (100 nM) stimulated glucose uptake approximately 2.27±0.40-fold by increasing membrane translocation of GLUT4 from the intracellular compartment. Metformin (1-2 mM) also mediated a 2-fold increase in glucose uptake in the absence of insulin. Exposure of L8 myotubes pretreated with metformin to 100 nM insulin caused further activation of glucose uptake. Our study demonstrated that L8 myotubes exhibit basal glucose transport activity and respond to glucose uptake stimuli of both insulin and metformin. The results suggest that L8 cells could be used as an alternative cell line for glucose transport studies.

KEYWORDS: myotube, GLUT4, glucose uptake activity, metformin, insulin.

INTRODUCTION

Glucose enters cells via a family of facilitative membrane proteins. These include many isoforms, GLUT1 to GLUT9, which are expressed either ubiquitously or predominantly in some tissues.[1-3] Muscle is the major site of glucose disposal, in which insulin increases glucose uptake by promoting the translocation of the glucose transporter isoform 4 (GLUT4) from an intracellular compartment to the plasma membrane[4] via a phosphatidylinositol 3-kinase (PI 3-kinase) pathway.[5] However, the enhancement of GLUT4 translocation by other stimuli, such as exercise, muscle contraction and K^+ depolarization, is mediated via distinctive (Ca^{2+}-dependent) signaling mechanisms.[6-8] Calcium can also activate glucose transportation. Although contraction-mediated GLUT4 translocation is Ca^{2+}-dependent, the increased intracellular calcium *per se* does not share all the characteristics effect of exercise.[9]

Several muscle cell lines, such as C2C12, L6, and H9c2 and the adipocyte cell line 3T3-L1, have been used extensively for *in vitro* cell culture studies of glucose transport and signaling mechanisms. Each individual cell line exhibits unique properties in response to different stimuli. The commonly-used L6 cells respond to insulin-induced glucose transport via PI 3-kinase, but do not exhibit Ca^{2+}-stimulated glucose uptake.[10] Conversely, both insulin and K^+ depolariza-tion enhance the membrane translocation of GLUT4 in cultured H9c2 myotubes.[6]

L8 muscle cells are widely used for studies of growth and differentiation,[11-12] but very few studies on glucose regulation have been reported. In L8 myocytes, a mononucleated terminally differentiated stage of L8 cells, glucose exerts its autoregulatory effect on hexose transport by modifying the incorporation of active glucose transporters into plasma membrane.[13-14] This basal autoregulatory transport in L8 myocytes has been shown to be GLUT1-mediated.[15] However, no studies on activation of GLUT4 have been reported. To provide an additional cell line for the study of glucose transport, we demonstrated here the effects of insulin and metformin, an anti-diabetic drug, on glucose uptake and GLUT4 translocation in L8 multinucleated cells (myotubes).

MATERIALS AND METHODS

Materials

All cell culture media and supplements were

purchased from Life Technologies, Inc. (Gaithersburg, MD, U.S.A.). Rat skeletal L8 myoblasts were purchased from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). Bovine insulin (Ins), protease inhibitors cocktail and phloretin were from Sigma Chemicals (St. Louis, MO, U.S.A.). 2-deoxy-D-[^{3}H] glucose was from ICN Biomedicals (Irvine, CA, U.S.A.). Goat polyclonal antiserum against GLUT4 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Electrophoresis chemicals were from Bio-Rad (Hercules, CA, U.S.A.).

Cell Culture

L8 myoblasts were maintained in Dulbecco's-modified Eagle's medium with addition of 100 U/ml of penicillin, 100 µg/ml streptomycin, 3.7 g/l $NaHCO_3$ (DMEM complete medium, low glucose) and 10% fetal bovine serum (FBS) in a 5% CO_2 incubator at 37 °C. Cells were seeded at 4,000 cells/cm^2 either in 12-well plates (for 2-deoxyglucose uptake studies), or in 60x15 mm culture dishes (for isolation of intracellular fractions). The medium was changed every other day. When cells reached confluence, the medium was changed to 2% horse serum (HS) in DMEM to promote cell differentiation. Microscopic examination was performed to monitor cell growth and differentiation up to the stage of 90% fused myotubes (approximately 8-10 days).

Insulin Treatment and 2-Deoxyglucose Uptake Assay

Prior to insulin treatment and transport studies, cells were deprived of serum for 5 h in DMEM supplemented with 25 mM glucose (final concentration). After rinsing twice in HEPES buffered saline, pH 7.4 (HBS contains 140 mM NaCl, 20 mM HEPES, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, and 5 mM KCl), cells were incubated in HBS containing 10 µM 2-deoxy-[^{3}H] glucose (2-dGlc; 1 µCi/ml) in the absence and presence of 100 nM (standard protocol) or 50-800 nM insulin (in dose-response studies) at 37°C for 10 min. Insulin-stimulated glucose uptake was terminated by washing the cells 3 times with ice-cold saline solution. For time-course studies, glucose uptake was terminated after incubation with 2-dGlc in the absence and presence 100 nM insulin for 5-20 min. Phloretin (10 mM) was included for the determination of non-specific uptake and then subtracted from the total uptake of each assay. Cells were lysed in 0.5 ml of 0.05 N NaOH for 20 min and aliquot of cell lysate was taken to determine the total cellular protein by Lowry's method.[16] Total radioactivity of the cell lysates was measured in a scintillation counter.

Metformin-Stimulated Glucose Transport

Assessment of 2-dGlc uptake induced by metformin was performed according to Hundal *et al*[17] with slight modifications. Briefly, the differentiated L8 myotubes were incubated in fresh medium containing 15 mM glucose in the presence of 2% HS. After an initial priming for 3 h, the medium was removed, and cells were incubated in the same medium containing either 1 or 2 mM metformin for a period of 16 h. Following rinsing twice with HBS, glucose uptake was measured by a 10 min incubation of cells in HBS containing 10 µM 2-dGlc as described. In testing for the combined effects of insulin and metformin, culture medium was replaced with a serum-free HBS containing 15 mM glucose at the last hour of the 16-h incubation period with metformin. The 2-dGlc uptake was then measured in the absence and presence of 100 nM insulin for 10 min.

Separation of Intracellular Vesicles and Plasma Membrane

The plasma membrane-rich and intracellular-rich fractions from L8 myotubes were prepared according to Yu *et al* [6] with modification. In brief, after treatment with 100 nM of insulin for 10-30 min at 37°C, cells were rinsed twice with ice-cold PBS and scraped from the culture dishes. In homogenizing buffer (10 mM HEPES, pH 7.4, 150 nM NaCl, 1 mM EGTA, 0.1 mM $MgCl_2$, protease inhibitors), cells were treated with 15 strokes in Wheaton glass homogenizer. To remove cell nuclei, the whole lysates were centrifuged at 200 *g* for 5 min. The collected supernatant was further centrifuged at 16,000 *g* for 15 min. The resulting supernatant or soluble (S) fraction containing intracellular pool of transporter was collected. The pellets containing crude plasma membrane were dissolved in homogenizing buffer, and designated plasma membrane (PM) fraction. Protein content of these fractions was measured and the GLUT4 distribution was determined by Western blotting.

Gel Electrophoresis and Western Blotting

Cellular fractions were dissolved in SDS sample buffer, subjected to 10% polyacrylamide gel containing 0.1% SDS electrophoresis,[18] and transferred onto nitrocellulose membranes for the Western blot analysis. Non-specific binding sites on the membrane were blocked with 5% non-fat dry milk. The membranes were then incubated with a 1:500 dilution of anti-GLUT4 antibody. Antigen-antibody complexes were detected by an alkaline phosphatase-conjugated antibody. The immunoblots were then quantified using scanning densitometry and expressed in arbitrary units.

Statistical Analyses

Three independent experiments were conducted in all studies and all assay conditions were performed in triplicate. Data were analyzed using Student's *t* test and significant differences were inferred at a *p* value of 0.05.

Results

Effect of Serum on 2-Deoxyglucose Uptake

The effect of serum withdrawal prior to the measurement of 2-deoxyglucose (2-dGlc) uptake in L8 myotubes is demonstrated in Table 1. Uptake of 2-dGlc was enhanced by serum. The 2-dGlc transport measured at 5, 10 and 20 min in the presence of serum was significantly higher than that in the absence of serum. The basal 10-min uptake of 2-dGlc in L8 myotubes declined by 35% after the 5-h period of serum deprivation suggesting a down-regulation of glucose transport activity.

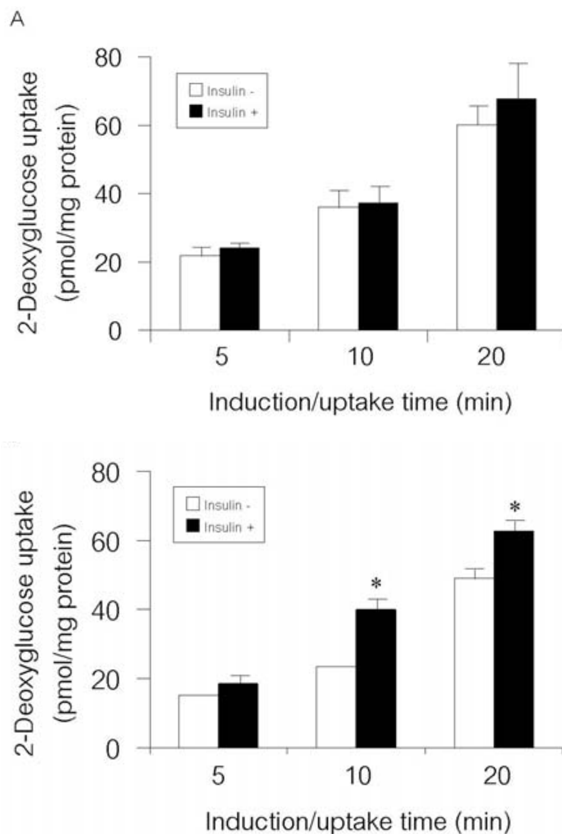

Fig 1. The effect of serum on insulin-stimulated 2-deoxyglucose uptake in L8 cells. L8 myotubes were incubated for 5 h in the presence (panel A) or absence (panel B) of 2% horse serum. Induction of 2-deoxyglucose uptake by insulin (100 nM) was measured at 5, 10 and 20 min. Data were expressed as means ± SD of the triplicates derived from one experiment. * $p < 0.05$ vs. uptake in the untreated cells.

Table 1. Effect of serum deprivation on basal glucose uptake of L8 myotubes.

Duration of uptake (minutes)	2-Deoxyglucose uptake (*pmol/mg protein*)		
	Serum +	Serum -	%reduction
5	19.59±1.18*	13.34±0.42	31.90
10	32.37±3.93*	20.79±2.06	35.77
20	53.42±3.06*	44.15±2.56	17.35

Data were expressed as means ± SD of the triplicates derived from one experiment.
* $p < 0.05$ vs. serum-depleted cells.

Effect of insulin on 2-deoxyglucose uptake and GLUT4 translocation

The effect of insulin on 2-dGlc uptake is shown in Fig 1. Insulin had no effect on serum-fed L8 myotubes (Fig 1A). A significant increase in 2-dGlc uptake was observed in the serum-deprived medium supplemented with 25 mM glucose (Fig 1B). A significant response of 2-dGlc transport was observed following either a 10 or 20 min induction/uptake period. The maximum 2-dGlc uptake was observed at 10 min after addition of insulin. The increment of glucose uptake ranged between 1.7- to 2.6-fold (2.27±0.40) as compared to that of basal uptake (data not shown). The dose-response relationship between insulin and 2-dGlc uptake is shown in Fig 2. Significant insulin activation of 2-dGlc uptake was observed at all concentrations ranging from 50-800 nM with the maximum stimulation detected at 100 nM insulin.

The effect of insulin on GLUT4 translocation was demonstrated in the fractionation study. The isolated plasma membrane (PM) and soluble (S) fractions from insulin-treated L8 myotubes were subjected to

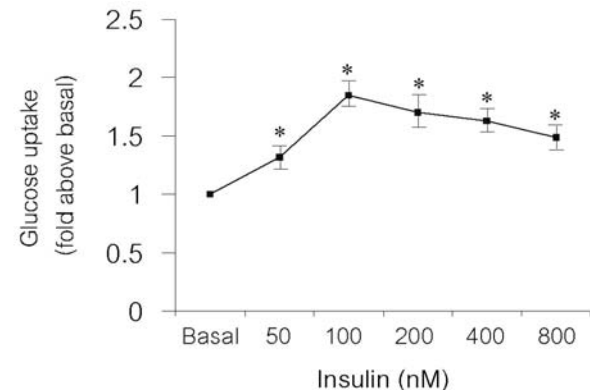

Fig 2. Stimulation of 2-deoxyglucose uptake by insulin. Serum-starved L8 myotubes were treated with 0-800 nM insulin. Uptake of 2-deoxyglucose was measured at 10 min after addition of insulin. Data were derived from the triplicates of three independent experiments, and expressed as means ± SD. * $p < 0.05$ vs. uptake in the untreated cells.

electrophoresis and immunoblotting. As shown in Fig 3, the increase in GLUT4 reactivity in the PM fractions is in agreement with the corresponding decrease in GLUT4 reactivity of the S fractions, suggesting a shift of transporters from the intracellular compartment to the plasma membrane in response to insulin.

Effect of metformin on 2-deoxyglucose uptake

The effect of metformin on 2-dGlc uptake by L8 myotubes in the absence and presence of 100 nM insulin is shown in Fig 4. In the absence of preincubation with metformin, insulin caused a 1.9-fold stimulation of 2-dGlc uptake. Pre-incubation with 1-2 mM metformin alone increased basal uptake by more than 2-fold. This suggests that metformin was capable of activating glucose transport independently of insulin. Those increases did not differ significantly from the effect caused by insulin. Uptake in response to insulin, for 10 min, after 16-h pre-incubation with 2 mM metformin was increased by 18% above that caused by metformin alone, and elicited a significant increase (by 80%) above that caused by insulin alone. These data suggest that the effects of insulin and metformin on 2-dGlc transport were additive at maximum doses of metformin (2 mM).

Discussion

The presence of serum in the medium promoted L8 cell growth and proliferation. In contrast, differentiation required a switch from a serum-rich (10% FBS) medium to a serum-poor (2% HS) medium. Under these conditions the cells aligned, fused into multi-nucleated fibers (myotubes), and expressed

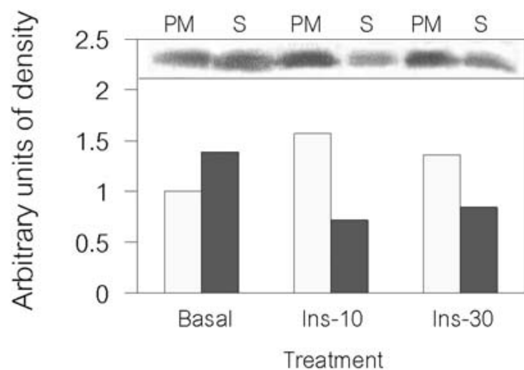

Fig 3. Effect of insulin on subcellular distribution of GLUT4 protein in L8 myotubes. L8 myotubes were serum-deprived for 5 h in the presence of 25 mM glucose and left untreated (Basal) or stimulated with 100 nM insulin for 10 and 30 min (Ins-10 and Ins-30). The plasma membrane (PM) and soluble (S) fraction were prepared. Sample (100 mg) were electrophoresed, Western blotted, and probed for GLUT4. The GLUT4 immunoblot was the picture from one of the three independent experiments.

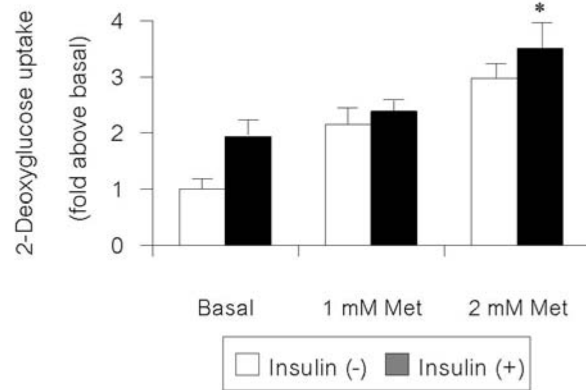

Fig 4. Effect of combined treatments of metformin and insulin on 2-deoxyglucose transport in L8 myotubes. Cells were preincubated with metformin (Met) at 1, 2 mM for 16 h in the medium containing 2% horse serum and 15 mM glucose. The 2-deoxyglucose was measured for 10 min in the presence and absence of 100 nM insulin. Data were expressed as means ± SD of triplicates derived from two independent experiments. * $p < 0.05$ vs. basal cells in the presence of insulin.

muscle-specific proteins including GLUT4. Studies with L6 myotubes[10] have shown that the sensitivity to serum was pronounced after cell fusion, and cells responded to serum by increasing rate of sugar uptake. Conversely, the increased transport elicited by serum could be reversed by serum deprivation. Transport stimulation was also observed in 3T3-L1 preadipocytes as cells progressed through differentiation.[19] Inhibition of serum-stimulated glucose transport in L6 myotubes[10] and L8 myocytes[14] by cycloheximide suggested that active protein synthesis is required to maintain the steady-state of basal glucose transport.

The effect of insulin was not observed in L8 cells maintained in the presence of serum, and serum deprivation prior to insulin administration conferred insulin-stimulated glucose transport (Fig 1). This study supported the previous finding in L6 myotubes that depression of the basal glucose transport by serum withdrawal in high glucose medium was a prerequisite for obtaining a significant response in insulin-sensitive muscle cells.[10] One possible explanation is that the transport systems were operating at maximum capacity in serum-fed cells and could not be stimulated further by insulin. In addition, increase of the glucose uptake by insulin in the serum-deprived conditions to the same magnitude as that observed in the serum-fed medium suggested the similar effect of serum and insulin on glucose transport into L8 cells. However, it should be noted that serum and insulin could differentially increase glucose uptake by involving distinct transporter isoforms. Previous observations that cell surface content of GLUT1 was higher than that of GLUT4 at basal state of insulin-sensitive cells

could suggest the involvement of the GLUT1 isoform in serum-regulated transport.[20-23]

There is a controversy concerning the insulin response. Although studies on GLUT4 localization in response to insulin in L6 myotubes,[23] rat adipocyte[24] and our findings in L8 myotubes (Fig 3) have suggested that the GLUT4 isoform accounts for most of the effect of insulin on glucose transport, a modest translocation of GLUT1 upon insulin stimulation was also observed in other studies.[21, 25-26] In contrast to serum stimulation, the effect of acute insulin was not prevented by cycloheximide in L6 cells and rat adipocytes, suggesting that the regulation of glucose transport activity by a brief exposure to insulin is protein synthesis-independent and involving subcellular redistribution of the GLUT1 and GLUT4 proteins.[10, 27] Studies in L6 muscle cells have shown that the cell surface contents of GLUT1 and GLUT4 did not differ significantly in the insulin-stimulated stage,[21] and prolonged exposure to insulin elevated GLUT1 mRNA and diminished GLUT4 mRNA levels.[25] Furthermore, a large reduction in basal glucose transport and a complete loss of insulin-induced glucose uptake of GLUT4 has been demonstrated in GLUT4 knockout mice.[28] These may be suggested that GLUT4 plays a key role in insulin-regulated glucose transport systems. However, over-expression of GLUT4 in C2C12 skeletal muscle cells, the insulin-insensitive cell line that expresses low amount of GLUT4, does not convert its sensitivity to insulin.[29] This implied that insulin stimulation of glucose transport is not solely dependent on the presence of the insulin receptor and the GLUT4 protein.

A number of studies have reported the metformin's effect on glucose transporter translocation. The change in sub-cellular distribution of GLUT1 mediated by 800 mM of metformin with no effect on GLUT4 was demonstrated in L6 myotubes.[17] However, metformin at higher concentrations in millimolar range appeared to mediate a translocation of both GLUT1 and GLUT4 in cardiomyocytes and adipocytes.[30-31] Increased glucose transport in quadriceps muscle and rat adipocytes has been shown to occur without changes in either mRNA or total membrane protein of both GLUT1 or GLUT4 isoforms,[32-33] suggesting that this drug did not affect the expression of glucose transporters. Moreover, metformin enhanced the glucose transport activity of GLUT1 and GLUT4 transporters,[22, 33] and the quantitative effect induced by metformin was observed above the values accounted for only by recruited transporters.[33]

We have reported here that in L8 myotubes, prolonged exposure to metformin increases glucose transport significantly and further activation is observed upon treatment with insulin in the presence of metformin (Fig 4). Although the means value shows 18% increase in glucose uptake after treatment with insulin, this difference was not statistically significant. Moreover, the fact that metformin enhances glucose uptake by activation of insulin receptor[34] and by modulation of insulin action through insulin receptor substrate-1 (IRS-1) and PI 3-kinase activity[34-35] could also explain the lack of additional effect of insulin in the presence of metformin. Based on this study and on other observations,[17, 22, 30-36] it appears that the effects on glucose transport could be attributed to a net gain in transporters mobilized to the plasma membrane by both insulin and metformin and improved transporter intrinsic activity or capacity by metformin.

In conclusion, L8 myotubes displayed the characters of basal glucose transport, exhibited sensitivity to insulin-stimulated glucose uptake, and responded to metformin. Besides their well-known use for growth and differentiation studies, L8 muscle cells could also be employed for the study of glucose uptake and transport studies.

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References

1. Mueckler M (1994) Facilitative glucose transporters. *Eur J Biochem* **219**, 713-25.
2. Doege H, Bocianski A, Joost H-G and Schurmann A (2000) Activity and genomic organization of human glucose transporter (GLUT9), a novel member of sugar transport facilitators predominantly expressed in brain and leukocytes. *Biochem J* **350**, 771-6.
3. Doege H, Schurmann A, Bahrenberg G, Brauers A and Joost H-G (2000) GLUT8, a novel member of the sugar transport facilitator family with glucose transport activity. *J Biol Chem* **275**, 16275-80.
4. Stephens JM and Pilch PF (1995) The metabolic regulation and vesicular transport of GLUT4, the major insulin-responsive glucose transporter. *Endocrine Rev* **16**, 529-46.
5. Czech MP and Corvera S (1999) Signaling mechanisms that regulate glucose transport. *J Biol Chem* **274**, 1865-8.
6. Yu B, Poirier LA and Nagy LE (1999) Mobilization of GLUT-4 from intracellular vesicles by insulin and K^+ depolarization in cultured H9c2 myotubes. *Am J Physiol* **277**, E259-67.
7. Hayashi T, Hirshman MF, Kurth EJ, Winder WW and Goodyear LJ (1998) Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* **47**, 1369-73.
8. Coderre L, Konstantin V, Kandror KV, Vallega G and Pilch PF (1995) Identification and characterization of an exercise-sensitive pool of glucose transporters in skeletal muscle. *J Biol Chem* **270**, 27584-8.
9. Hayashi T, Wojtaszewski JF and Goodyear LJ (1997) Exercise regulation of glucose transport in skeletal muscle. *Am J Physiol*

273, E1039-51.
10. Kilp A, Li G and Logan WJ (1984) Induction of sugar uptake response to insulin by serum deprivation in fusing L6 myoblasts. *Am J Physiol* **247**, E291-6.
11. Barnoy S, Glaser T and Kosower NS (1997) Calpain and calpastatin in myoblast differentiation and fusion: effects of inhibitors. *Biochim Biophys Acta* **1358**, 181-8.
12. Cambron LD and Leskawa KC (1994) Glycosphingolipids during skeletal muscle differentiation: comparison of normal and fusion-defective myoblasts. *Mol Cell Biochem* **130**, 173-85.
13. Sasson S and Cerasi E (1986) Substrate regulation of the glucose transport system in rat skeletal muscle. Characterization and kinetic analysis in isolated soleus muscle and skeletal muscle cells in culture. *J Biol Chem* **261**, 16827-33.
14. Wertheimer E, Sasson S and Cerasi E (1990) Regulation of hexose transport in L8 myocytes by glucose: possible sites of interaction. *J Cell Physiol* **143**, 330-6.
15. Greco-Perotto R, Wertheimer E, Jeanrenaud B, Cerasi E and Sasson S (1992) Glucose regulates its transport in L8 myocytes by modulating cellular trafficking of the transporter GLUT-1. *Biochem J* **286**, 157-63.
16. Lowry O, Rosebrough N, Farr A and Randall R (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **193**, 265-75.
17. Hundal HS, Ramlal T, Reyes R, Leiter LA and Klip A (1992) Cellular mechanism of metformin action involves glucose transporter translocation from an intracellular pool to the plasma membrane in L6 muscle cells. *Endocrinology* **131**, 1165-73.
18. Laemmli UK (1970) Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5.
19. Liu F, Kim J, Li Y, Liu X, Li J and Chen X (2001) An extract of Lagerstroemia speciosa L. has insulin-like glucose uptake-stimulatory and adipocyte differentiation-inhibitory activities in 3T3-L1 cells. *J Nutr* **131**, 2242-7.
20. Douen AG, Ramlal T, Rastogi S, Bilan PJ, Cartee GD, Vranic M, Holloszy JO and Klip A (1990) Exercise induces recruitment of the "insulin-responsive glucose transporter". Evidence for distinct intracellular insulin- and exercise-recruitable transporter pools in skeletal muscle. *J Biol Chem* **265**, 13427-30.
21. Wilson CM, Mitsumoto Y, Maher F and Klip A (1995) Regulation of cell surface GLUT1, GLUT3, and GLUT4 by insulin and IGF-I in L6 myotubes. *FEBS Lett* **368**, 19-22.
22. Merrall NW, Plevin R and Gould GW (1993) Growth factors, mitogens, oncogenes and the regulation of glucose transport. *Cell Signal* **5**, 667-75.
23. Czech MP and Buxton JM (1993) Insulin action on the internalization of the GLUT4 glucose transporter in isolated rat adipocytes. *J Biol Chem* **268**, 9187-90.
24. Yang J and Holman GD (1993) Comparison of GLUT4 and GLUT1 subcellular trafficking in basal and insulin-stimulated 3T3-L1 cells. *J Biol Chem* **268**, 4600-3.
25. Koivisto UM, Martinez-Valdez H, Bilan PJ, Burdett E, Ramlal T and Klip A (1991) Differential regulation of the GLUT-1 and GLUT-4 glucose transport systems by glucose and insulin in L6 muscle cells in culture. *J Biol Chem* **266**, 2615-21.
26. Zorzano A, Wilkinson W, Kotliar N, Thoidis G, Wadzinkski BE, Ruoho AE and Pilch PF (1989) Insulin-regulated glucose uptake in rat adipocytes is mediated by two transporter isoforms present in at least two vesicle populations. *J Biol Chem* **264**, 12358-63.
27. Kono T, Suzuki K, Dansey LE, Robinson FW and Blevins TL (1981) Energy-dependent and protein synthesis-independent recycling of the insulin-sensitive glucose transport mechanism in fat cells. *J Biol Chem* **256**, 6400-7.
28. Zisman A, Peroni OD, Abel ED, Michael MD, Mauvais-Jarvis F, Lowell BB, Wojtaszewski JF and Harshman MF, et al (2000) Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nat Med* **6**, 924-8.
29. Kotliar N and Pilch PF. (1992) Expression of the glucose transporter isoform GLUT4 is insufficient to confer insulin-regulatable hexose uptake to cultured muscle cells. *Mol Endocrinol* **6**, 337-45.
30. Fischer Y, Thomas J, Rosen P and Kammermeier H (1995) Action of metformin on glucose transport and glucose transporter GLUT1 and GLUT4 in heart muscle cells from healthy and diabetic rats. *Endocrinology* **136**, 412-20.
31. Matthaei S, Hamann A, Klein HH, Benecke H, Kreymann G, Flier JS and Greten H (1991) Association of metformin's effect to increase insulin-stimulated glucose transport with potentiation of insulin-induced translocation of glucose transporters from intracellular pool to plasma membrane in rat adipocytes. *Diabetes* **40**, 850-7.
32. Thomas CR, Turner SL, Jefferson WH and Bailey CJ. (1998) Prevention of dexamethasone-induced insulin resistance by metformin. *Biochem Pharmacol* **56**, 1145-50.
33. Matthaei S, Reibold JP, Hamann A, Benecke H, Haring HU, Greten H and Klein HH (1993) In vivo metformin treatment ameliorates insulin resistance: evidence for potentiation of insulin-induced translocation and increased functional activity of glucose transporters in obese (fa/fa) Zucker rat adipocytes. *Endocrinology* **133**, 304-11.
34. Kumar N and Dey CS (2002) Metformin enhances insulin signaling in insulin-dependent and-independent pathways in insulin resistant muscle cells. *Br J Pharmacol* **137**, 329-36.
35. Pryor PR, Liu SC, Clark AE, Yang J, Holman GD, Tosh D (2000) Chronic insulin effects on insulin signaling and GLUT4 endocytosis are reversed by metformin. *Biochem J* **348**, 83-91.
36. Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, Zhou G and Williamson JM, et al (2002) Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes* **51**, 2074-81.