Regulation of Glucose Transport in Cultured Muscle Cells by Novel Hypoglycemic Agents

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The antidiabetic agent troglitazone (CS-045) and a metabolite designated M3 have potent blood glucose–lowering actions. The mechanism of the hypoglycemic effects of troglitazone and M3 was investigated in cultured L6 muscle cells. Short-term (2-hour) exposure of fully differentiated myotubes to troglitazone had no effect on glucose transport activity; M3 exposure caused a modest (50% to 60%) increase in basal and insulin-stimulated transport. Long-term (72-hour) treatment of myotubes with troglitazone resulted in a doubling of glucose transport in the absence of insulin, whereas M3 treatment resulted in a fivefold increase in basal glucose transport. Transport activity in M3-treated myotubes was greater than that seen after short-term insulin treatment. Insulin did not stimulate transport further in long-term M3-treated cells. A similar effect of prolonged exposure to M3 was observed in nondifferentiated myocytes. The agent had no influence on cell growth or the extent of differentiation. Augmentation of basal glucose transport by M3 was slow in onset, requiring 18 to 24 hours before significant effects were observed and 72 hours for full stimulation. M3 action on glucose transport was also dose-dependent, with half-maximal stimulation at 5 μg/mL of the agent and full effects at 10 to 20 μg/mL. Total membranes were prepared from control and M3-treated L6 myocytes and myotubes, and glucose transporter (GLUT1 and GLUT4) protein levels were measured by Western blotting. GLUT1 content was increased 2.9- ± 1.3- and 2.8- ± .2-fold by M3 treatment in myocytes and myotubes, respectively. GLUT4 content in myotubes was also increased, but to a lesser extent (1.9- ± .3-fold). In conclusion, the novel antidiabetic agent M3 acts to increase glucose transport into muscle cells in the absence of insulin. This effect appears to occur mainly through an increase in the level of glucose transporter proteins, which could explain, at least in part, its hypoglycemic actions.

AS THE PRIMARY SITE of insulin-mediated glucose disposal, skeletal muscle is of paramount importance in controlling glucose homeostasis.[1,2] Defects in insulin-stimulated skeletal muscle glucose uptake are common to hyperglycemic states such as non–insulin-dependent diabetes mellitus (NIDDM).[3,4] Glucose transport into most tissues is mediated by a family of facilitated-diffusion glucose transport proteins designated GLUT1 to GLUT5.[5] GLUT4 is the major insulin-regulatable transporter isoform and is expressed in cardiac and skeletal muscle and adipose tissue.[6] Impaired glucose transport into adipocytes from insulin-resistant subjects can be explained by a pretranslational decrease in the expression of GLUT4.[7,8] However, in skeletal muscle, decreased glucose transport in NIDDM occurs together with normal GLUT4 content.[9-12]

A number of hyperglycemic agents have been developed to treat multiple sites of insulin resistance. Sulfonylureas improve both insulin secretion and peripherial glucose utilization, although the mechanism of the latter effect is still unknown.[13] Biguanides such as metformin increase peripheral glucose utilization by augmenting glucose uptake, independent of effects on the β cell.[14] A newer class of hypoglycemic agents are the thiazolidinedione derivatives, which include pioglitazone[15] and troglitazone.[16,17] Troglitazone has been shown to potentiate insulin action on peripheral glucose utilization and reduction of hepatic glucose output, yet is free of any influence on β-cell function.[17] Possible cellular mechanisms for the ability of troglitazone to increase glucose uptake were investigated using the mouse-derived cultured skeletal muscle cell line L6[18] as a model of an insulin-responsive tissue. We also tested the effects of a troglitazone metabolite designated M3[19] and found that both agents increased glucose transport in cultured muscle cells through a mechanism that involves increased expression of glucose transport proteins.

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MATERIALS AND METHODS

Materials

Porcine insulin was kindly supplied by Dr Ron Chance of Eli Lilly & Co (Indianapolis, IN). Cell culture materials were purchased from Irvine Scientific (Irvine, CA). Fetal calf serum (FCS) was purchased from Gemini (Calabasas, CA). Bovine serum albumin ([BSA] Cohn fraction V) was supplied by Boehringer Mannheim (Indianapolis, IN). 2-[1,2-^{3}H]-deoxy-D-glucose and L-[1-^{3}H(*N*)]-glucose were purchased from New England Nuclear (Boston, MA). Polyclonal antisera against GLUT1 (RaGLUTRANS) and GLUT4 (RaIRGT) were purchased from East Acres Biologicals (Cambridge, MA). Antirabbit IgG conjugated with horseradish peroxidase and the Enhanced Chemiluminescence Kit were purchased from Amersham (Arlington Heights, IL). All electrophoresis chemicals were obtained from Bio-Rad (Richmond, CA). Pepstatin, leupeptin, phenylmethylsulfonyl fluoride, 2-deoxyglucose, and L-glucose were all purchased from Sigma Chemical (St Louis, MO). M3 and troglitazone were kindly supplied by Dr Hiro Horikoshi (Sankyo, Tokyo, Japan).

Cell Culture and Treatment

The mouse L6 myocyte cell line was obtained from the American Type Culture Collection (Rockville, MD). Cell culture and differentiation used the procedures described by Klip et al.[20] Cells were initially grown in alpha-minimal essential medium supplemented with 50 μg/mL gentamycin and 10% FCS. Media were changed

every third day. Differentiation of confluent cultures was initiated by changing the supplementation of the media from 10% to 2% FCS. As established by others,[21] differentiation of myocytes into multinucleated myotubes was complete by 3 days after media change. Greater than 90% of the cells became multinucleated under these conditions. Confluent cells maintained in 10% FCS remained growth-arrested with only limited differentiation; 10% to 20% of cells became multinucleated. Agents were added to cells with media change after confluency, and were added again when media were routinely changed every 48 hours. Agents were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide did not exceed 0.1%, which did not influence cell growth or function (not shown). None of the treatment protocols with agents had any effect on extent of differentiation or protein content.

Glucose Transport Assay

The procedure for glucose transport measurement was modified from that described by Klip et al.[20] Cells were grown in 12-well plates. After cultures were treated as described in the text and figure legends, they were rinsed twice with alpha-minimal essential medium supplemented with antibiotics, glucose at the same concentration as for regular culture media, and 0.1% BSA, pH 7.4. Media were added to the cells together with varying concentrations of insulin (0 to 33 pmol/L), and the cells were incubated for 60 to 90 minutes in a 5% CO_2 incubator. An initial period of serum deprivation was not required to reveal insulin responsiveness. Plates were then removed from the incubator, and wells were washed four times with room-temperature reaction buffer: 150 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L $CaCl_2$, 2.5 mmol/L NaH_2PO_4, 10 mmol/L HEPES, and 0.1% BSA, pH 7.4. The transport reaction was started by addition of 10 μL substrate (^{3}H-2-deoxyglucose or ^{3}H-L-glucose 0.1 μCi; final concentration, 0.1 mmol/L) and halted after 5 minutes by aspirating the reaction mixture and rapidly rinsing each well five times with 4°C phosphate-buffered saline. Cells were solubilized by addition of 0.5 mL 0.1N NaOH and incubated with shaking. An aliquot (100 μL) of the suspension was removed for protein analysis using the Bradford assay.[22] After solubilization, 400 μL of the suspension was placed in a scintillation vial and neutralized with 1.0N HCl, and scintillation fluid was added. The value for cell-associated L-glucose was used to correct each sample for the contributions of diffusion and trapping.

Membrane Preparation

Cells for membrane preparation were grown in 100-mm dishes and treated in parallel with cells for transport assay. Total membranes were prepared by the method developed by Walker et al.[23] Briefly, after rinsing with phosphate-buffered saline, cells were scraped from dishes in 3 mL of the following buffer: 250 mmol/L sucrose, 5 mmol/L NaN_3, 2 mmol/L EGTA, 200 μmol/L phenylmethylsulfonyl fluoride, 1 μmol/L leupeptin, 1 μmol/L pepstatin, and 20 mmol/L HEPES, pH 7.4. All steps were performed at 4°C. Cells were collected by centrifugation and homogenized with a Dounce homogenizer. After centrifugation at 750 × *g* for 3 minutes, the pellet was rehomogenized and recentrifuged, and the supernatants were combined. Centrifugation of the supernatant at 190,000 × *g* for 60 minutes produced a total membrane pellet. Membranes were resuspended in homogenization buffer, assayed for protein content, and stored at −70°C before further analysis.

Detection of Glucose Transporter Proteins

Membrane preparations were diluted 1:1 in 2X Lamelli's buffer without β-mercaptoethanol and heated for 5 minutes at 90°C. Boiling was avoided to prevent aggregate formation. Proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and then transferred to nitrocellulose.[24] GLUT1 was identified using a rabbit polyclonal antisera against the rat brain glucose transporter (RaGLUTRANS), which also recognizes human GLUT1. A polyclonal antisera specific for GLUT4 (RaI-RGT) was also used. Immune complexes were detected with horseradish peroxidase–conjugated IgG and enhanced chemiluminescence according to manufacturer's instructions, followed by autoradiography. Quantitation was performed with a scanning laser densitometer (Stratoscan 7000; Stratagene Cloning Systems, San Diego, CA).

Statistical Analysis

Statistical significance was evaluated using Student's *t* test, and two-tailed *P* values were calculated. Paired analysis was performed for comparisons of short- and long-term drug treatment in the same sets of cells. Significance was accepted at the *P* less than .05 level.

RESULTS

Troglitazone (CS-045) is a member of the thiazolidinedione class of drugs, which enhance insulin sensitivity in animals and man.[16,17] In man, troglitazone is converted to an active metabolite (M3),[19] but the mechanisms of action of either the parent compound, troglitazone, or M3 are poorly understood. Therefore, we have studied the in vitro effects of these agents on glucose transport in the rat L6 muscle cell culture system. Myotubes were incubated with troglitazone or M3 short-term (1 hour) or long-term (72 hours). Long-term exposure of L6 cells to either troglitazone or M3 had no effect on the extent of differentiation into multinucleated myotubes or on protein content. In untreated cells, insulin caused a 132% ± 14% increase in glucose transport. Short-term exposure of L6 cells to troglitazone had no effect on either basal or insulin-stimulated transport activity (Fig 1). Prolonged treatment

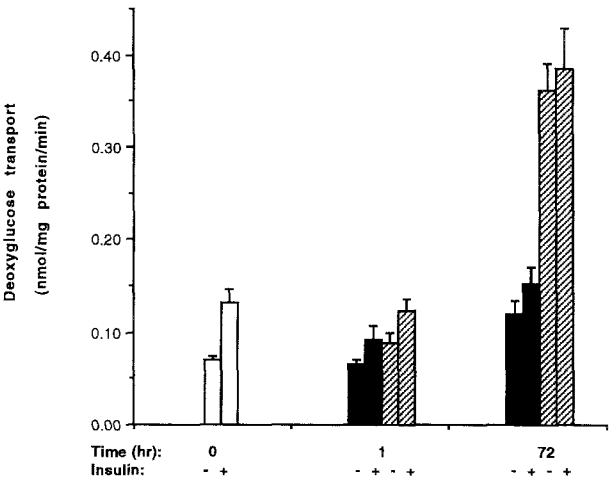

Fig 1. Influence of troglitazone and M3 treatment on glucose transport in L6 myotubes. Confluent, differentiated cells were treated with no additions (□), troglitazone 2 μg/mL (■), or M3 15 μg/mL (▨) for the indicated times before an additional (60-minute) incubation in the absence (−) or presence (+) of 17 nmol/L insulin, followed by glucose transport assay. Results are the mean ± SEM (n = 5).

with troglitazone resulted in a significant increase in basal glucose transport (105% ± 26% over control, $P < .01$). Insulin caused little additional increase in troglitazone-treated cells. M3 had appreciably greater effects. After 1 hour, both basal and insulin-stimulated transport were increased (basal, 48% ± 13% increase, $P < .05$; insulin-stimulated, 58% ± 17% increase, $P < .05$). Prolonged (72 hours) treatment with M3 resulted in a 4.7-fold increase in basal transport, and there was no additional effect of short-term insulin exposure. Since M3 appeared to be the more potent agent, further studies were performed with that compound.

Although L6 cells display insulin-responsive glucose transport at all times, they express the insulin-regulatable transporter GLUT4 only after differentiation into myotubes.[21,25] The possible roles of GLUT1 and GLUT4 in the M3 effect were assessed by treating both confluent, nondifferentiated L6 cells (myocytes maintained in 10% FCS) and differentiated cells with M3 for 72 hours. Results on transport activity are summarized in Fig 2. Myocytes were responsive to insulin (1.6- ± .3-fold increase), but not as responsive as myotubes (2.3- ± .1-fold). Long-term M3 treatment of both myocytes and myotubes led to a marked fivefold increase in basal glucose transport, with little extra effect seen after addition of insulin to M3-treated cells.

Our initial studies (Fig 1) suggested that the effect of M3 to increase glucose transport activity was relatively slow in onset. Therefore, the time course of M3 action on glucose transport was studied in greater detail. Myotubes were treated with M3 (15 μg/mL) for varying periods before assay of basal glucose transport (Fig 3). Significant stimulation of transport was observed after 24 hours of treatment, with maximal effects after 72 hours, which remained stable for an additional 24 hours. Treating cells with M3 for 72 hours after completion of differentiation, a total of 6 days after initiation of the differentiation protocol, led to comparable results (data not shown).

The concentration dependence of the M3 effect on glucose transport was studied after 72 hours of treatment.

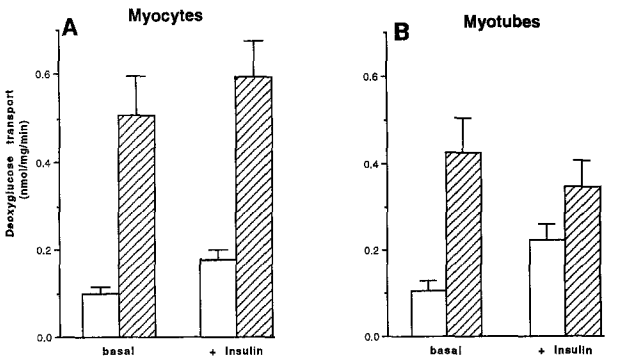

Fig 2. Effect of long-term exposure to M3 on L6 muscle cell glucose transport activity. Confluent cells were cultured in the absence (□) or presence (▨) of M3 15 μg/mL for 72 hours. Cells were then treated for 60 minutes with or without insulin 17 nmol/L before assay of glucose transport. (A) Effects in myocytes (n = 5 to 6); cells maintained in 10% FCS. (B) Effects in myotubes (n = 8); M3 added 24 hours after changing to 2% FCS-containing media. Results are the mean ± SEM.

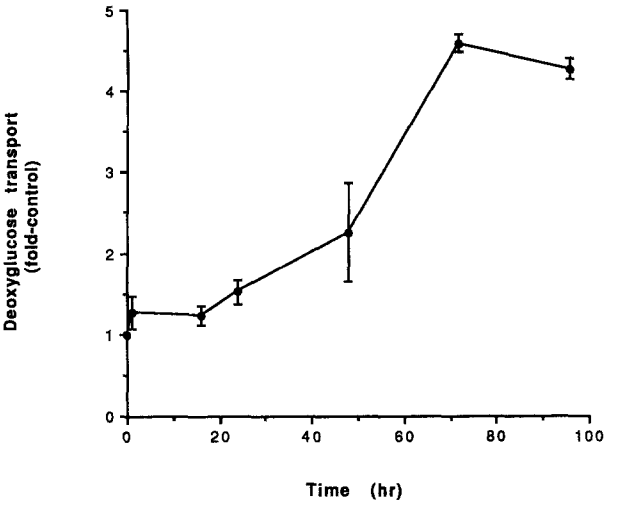

Fig 3. Time course of M3 action on glucose transport activity in L6 myotubes. M3 15 μg/mL was added to confluent, differentiated L6 cells at indicated times before assay. All measurements were made on cells after 4 days of differentiation. Results are expressed as ratio of basal glucose transport activity in treated and untreated (control) cells at each time point, mean ± SEM (n = 3).

Half-maximal effects occurred at 5 μg/mL M3, with maximal effects at 10 to 20 μg/mL (Fig 4). Transport stimulation was somewhat less at higher drug concentrations, but this was not due to decreased cell number or protein content.

One mechanism by which M3 could increase transport activity would be to augment glucose transporter protein expression. This possibility was investigated by preparing total cellular membranes (postnuclear) from control and M3-treated myocytes and myotubes and measuring transporter levels by Western blotting. Representative autoradiograms are presented in Fig 5. GLUT1 is highly abundant in myocytes; the diffuse staining for GLUT4 was variable and barely different from the background. Both GLUT1 and

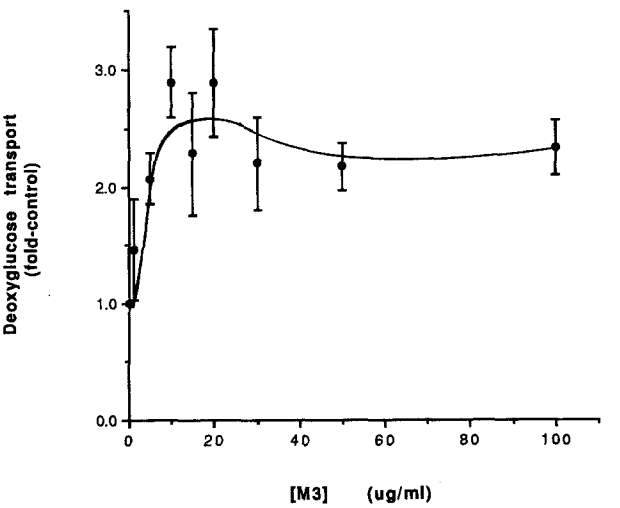

Fig 4. Dose-response of M3 action on glucose transport activity in L6 myotubes. Cells were treated with the indicated concentrations of M3 for 72 hours before transport assay. Results are expressed as ratio of basal glucose transport activity in paired untreated cells, mean ± SEM (n = 3).

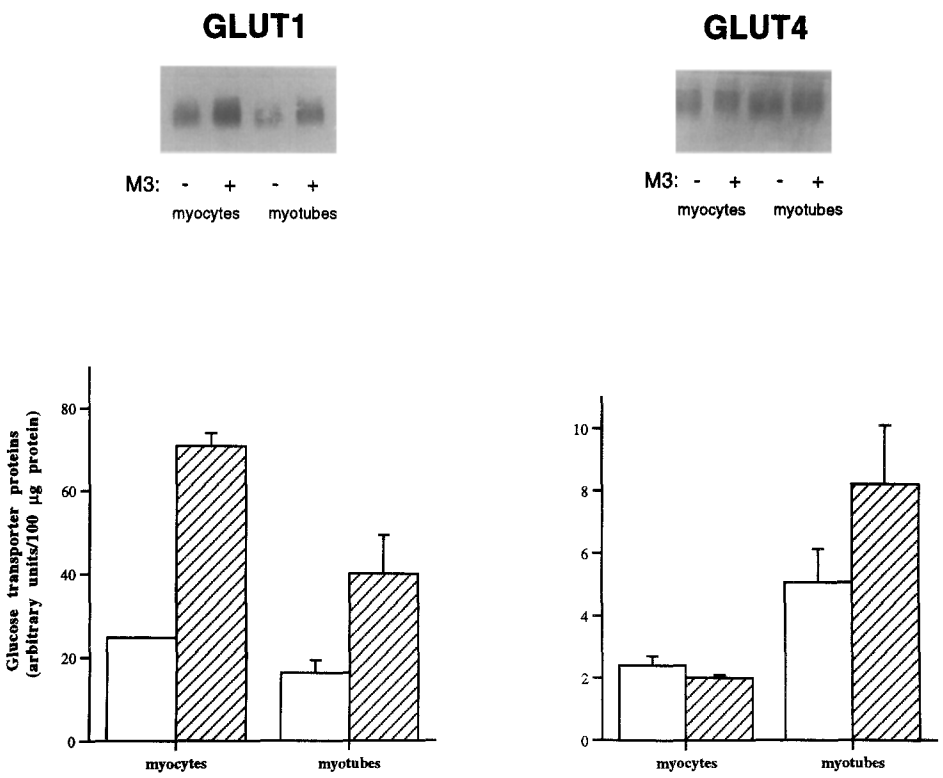

Fig 5. Effect of M3 on glucose transporter expression in L6 myocytes and myotubes. Cells were incubated in the absence (□) or presence (▨) of M3 15 μg/mL for 72 hours before membrane preparation, electrophoresis, and Western blotting. (Top) Representative autoradiograms of membranes probed with antisera against GLUT1 and GLUT4. Equal amounts of membrane protein, from different cells were loaded in each lane (10 μg for GLUT1, 20 μg for GLUT4). (Bottom) Quantitation of glucose transporter expression. Intensity of bands on autoradiograms was determined by scanning densitometry. Results given in arbitrary density units normalized against protein loaded on the gel, mean ± SEM. GLUT1, n = 7 in myotubes, n = 3 in myocytes; GLUT4, n = 6 in myotubes, n = 4 in myocytes.

GLUT4 are present in differentiated L6 myotubes, with GLUT1 expression decreasing on differentiation, as reported elsewhere.[21,25] Owing to the possible different affinities of the antisera used, it is difficult to measure the relative expressions of GLUT1 and GLUT4. In myocytes, GLUT1 levels are higher after M3 treatment. In myotubes, both GLUT1 and GLUT4 expression are appreciably greater in membranes from M3-treated cells. Quantitation of these results by densitometric scanning (Fig 5B) showed that M3 treatment resulted in 2.9- ± 1.3- ($P < .05$) and 2.7- ± .2-fold ($P < .005$) increases in GLUT1 protein expression in myocytes and myotubes, respectively. There was also a 1.9- ± .3-fold increase in GLUT4 levels ($P < .05$) in myotubes. The background level of GLUT4 in myocytes, which might represent expression in the small portion (10% to 20%) of cells that differentiated spontaneously, was not influenced by M3 treatment.

DISCUSSION

Troglitazone (CS-045) has been shown to be an effective oral antidiabetic agent with hypoglycemic actions in insulin-resistant obese and diabetic animals[16] and in subjects with NIDDM.[17] To investigate mechanisms for this hypoglycemic action, we used cultured muscle cells to determine possible direct effects of troglitazone on glucose transport activity and transporter expression. In addition, we studied the effects of a metabolite of troglitazone, M3, which also has hypoglycemic actions.[19] Both compounds were found to increase glucose transport activity in muscle cells. M3 was the more potent and faster-acting agent and was selected for further study. Under conditions in which glucose transport activity was increased, M3 treatment also resulted in significant increases in levels of GLUT1 and GLUT4 transporter proteins in the total-membrane fraction from myotubes.

Since skeletal muscle is the primary glucose-using, insulin-sensitive tissue and a major contributor to the glucose intolerance present in insulin-resistant states,[1,4] it should also be a focus for evaluation of antidiabetic agents. The mouse-derived L6 cultured muscle cell line has been extensively studied as a model for insulin-responsive skeletal muscle.[18] A major benefit of this cell line for such studies is that, when differentiated, it expresses the insulin-regulatable transporter isoform GLUT4[21] in addition to GLUT1. GLUT1 is the constitutive transporter, mediating most or all of basal glucose transport, whereas GLUT4 contributes little to basal transport but is involved in acute responses to insulin.[5] In L6 cells, GLUT4 is translocated to the plasma membrane in response to insulin,[25] just as occurs with skeletal muscle in vivo.[26,27]

The current results suggest that the M3-mediated increase in glucose transport activity, at least in cultured muscle cells, is a direct one and not necessarily due to potentiation of insulin action. Thus, in the absence of added insulin, glucose transport is markedly increased. The large M3 effect on basal transport suggested that GLUT1 regulation could represent a possible mechanism for the hypoglycemic effects of the drug. One means of testing this hypothesis was to study M3 effects on transport in nondifferentiated L6 cells, which express primarily GLUT1.[21] M3 was similarly effective in myocytes and myotubes, indicating that GLUT4 expression was not essential for stimulation of transport activity. This conclusion was supported by direct measurements of GLUT1 and GLUT4 expression, which

showed that GLUT1 protein was increased nearly threefold by M3 treatment in both differentiated and nondifferentiated cells. Comparing measurements made in intact cells with those in membrane preparations is semiquantitative at best, but the average increase in basal transport activity (fivefold) exceeds the increase in total GLUT1 protein (2.8-fold), although increases in GLUT4 protein could also contribute to the response. It is therefore possible that M3 also increases the concentration of transport proteins (both GLUT1 and GLUT4) on the cell surface by stimulating translocation or enhances transporter intrinsic activity, and these combined actions could result in the larger increase in transport activity.

The specific nature of the mechanism by which M3 increases glucose transport activity and transporter expression is not fully revealed by the current studies. Since only total cellular transporter content was measured, it is unknown if transporter distribution may also be influenced. The slow time course of M3 action mitigates against a rapid translocation as the major mechanism, but does not rule out more prolonged changes in distribution. It is currently unknown whether M3 increases transporter synthesis or retards degradation.

Although members of the thiazolidinedione class of antidiabetic agents all increase glucose utilization, they appear to have divergent effects on glucose transport activity and transporter expression. Agents of this class that augmented basal transport in 3T3-L1 or 3T3-F442A adipocytes included englitazone[28,29] and pioglitazone.[30] Pioglitazone also increased GLUT1 protein expression in adipocytes[30] and BC3H-1 myocytes,[31] much as M3 does in L6 muscle cells. In adipocytes, both insulin-stimulated transport and GLUT4 levels were elevated by pioglitazone[30]; insulin-stimulated transport was only modestly elevated by englitazone.[28] Pioglitazone has also been shown to cause differentiation of 3T3-L1 fibroblasts into adipocytes[32]; M3 had no such effect on differentiation in L6 myocytes. The effects of thiazolidinedione agents on skeletal muscle have been previously studied mainly in vivo. Fasting plasma glucose levels in ob/ob mice and insulin-resistant rats were reduced by pioglitazone and ciglitazone,[33-35] suggestive of increased basal glucose disposal into muscle. Ciglitazone and pioglitazone also increased insulin-stimulated transport in perfused hindquarter[34] and soleus muscle,[33,35] respectively. Pioglitazone increased GLUT4 protein in the same studies.[35] There is no information about possible changes in GLUT1 expression in these in vivo studies, whereas the agent was able to increase GLUT1 protein in BC3H-1 muscle cells.[35] The effects of troglitazone and M3 on transport in cultured muscle cells, increasing both basal and insulin-stimulated activities and both GLUT1 and GLUT4 expression, appear to be similar to those of pioglitazone. The advantage of performing the current studies in L6 muscle cells is that it is now possible to separate the direct effects of troglitazone and M3 on glucose transport from the results of reductions in blood glucose and insulin. In addition, the L6 cell system permits analysis of GLUT4 regulation, unlike the case in BC3H-1 cells, which lack this transporter isoform.[31]

Studies with troglitazone in insulin-resistant and obese rodents have shown the drug to be an effective oral hypoglycemic agent that is without direct pancreatic actions.[16] In subjects with NIDDM, troglitazone leads to amelioration of insulin resistance, normalization of elevated rates of hepatic glucose production, and reductions in plasma glucose and insulin levels.[17] In vitro, this agent directly increases glycogen synthase activity in cultured BC3H-1 muscle cells and inhibits glucose output in cultured liver cells.[36] Glucose transport into adipocytes isolated from diabetic rodents is increased after troglitazone treatment,[16] but it is uncertain if this is a direct effect of the drug or a result of the insulin-lowering actions of troglitazone. The current findings suggest that the action of troglitazone or its metabolite to decrease fasting hyperglycemia could be a result of the ability to increase GLUT1 expression and elevate basal glucose transport activity. In addition, increases in GLUT4 expression and insulin-stimulated transport activity would ease insulin resistance, with a compensatory decrease in insulin levels. The troglitazone-mediated increases in transport activity and glycogen synthase, as seen in cultured muscle cells, could combine to greatly increase glucose utilization in muscle.

In summary, troglitazone and an active metabolite have potent effects to increase directly the glucose transport in cultured muscle cells. This stimulation of glucose transport appears to be independent of insulin action, which could account for the ability of the agents to decrease blood glucose levels in the fasting state.[17] The increase in transport activity in cells is due, at least in part, to an increase in the expression of both GLUT1 and GLUT4 transport proteins. The glucose transport– and glycogen synthase–stimulating actions of troglitazone and its metabolite provide multiple pathways to mediate the striking increases in glucose utilization that occur after drug treatment.[17] Troglitazone and M3 differ in many ways from other classes of antidiabetic agents and have considerable promise in the treatment of hyperglycemia.

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