Coordinate Regulation of Glucose Transporter Function, Number, and Gene Expression by Insulin and Sulfonylureas in L6 Rat Skeletal Muscle Cells

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Abstract

The extrapancreatic actions of sulfonylureas on the glucose transport system were studied in the L6 line of cultured rat skeletal muscle cells. Insulin (10^{-7} M) increased 2-deoxyglucose uptake in differentiated L6 myotubes by 30–40% after 8 h of incubation. The sulfonylurea tolazamide (0.6 mg/ml, 22 h) had no effect on glucose uptake in the absence of insulin, but increased insulin-stimulated 2-deoxyglucose uptake twofold. The total cellular content of glucose transporters was assessed with a monoclonal anti–transporter antibody by a solid-phase ELISA method. Insulin (8 h) increased the quantity of glucose transporters, with a maximal twofold increase at 10^{-7} M and a dose-response curve similar to that for insulin stimulation of glucose uptake. In spite of its lack of effect on glucose uptake, tolazamide alone (0.6 mg/ml) increased the cellular content of transporters by 70%. The effects of insulin and tolazamide on transporter gene expression were studied with probes derived from Hep G2 glucose transporter cDNA. Insulin increased the transporter mRNA level 1.7-fold, tolazamide increased it 1.5-fold, and the combination of insulin and tolazamide increased transporter mRNA 3-fold. It is concluded that sulfonylureas, together with insulin, enhance glucose uptake in L6 skeletal muscle cells by increasing the number of functioning glucose transport molecules. The long-term regulation of the glucose transport system in skeletal muscle by insulin and sulfonylureas in vivo may involve similar changes in transporter function, number, and gene expression.

Introduction

One of the most important mechanisms through which insulin controls blood glucose concentrations is by the stimulation of glucose uptake in specific insulin-sensitive tissues. Skeletal muscle glucose uptake is a particularly important determinant of glucose homeostasis, since muscle is the largest tissue in the body and a major site of insulin-regulated glucose clearance (1). The uptake of glucose in skeletal muscle and other tissues is mediated by one or more specific carriers, designated glucose transporters (2–4). Multiple mechanisms appear to be involved in the regulation of these glucose transporters, in-

cluding rapid changes in transport activity mediated both by movement of the transporter from intracellular sites to the cell surface (5, 6) and by rapid alterations in intrinsic transport activity (7). In addition, there are more gradually developing responses that involve changes in the abundance of the transporter in cells, resulting from alterations in the rates of transporter synthesis or degradation (7) as well as changes in transporter gene expression (8).

In patients with diabetes mellitus, impaired insulin stimulation of glucose utilization by skeletal muscle is a common feature that contributes to insulin resistance and glucose intolerance (9). Abnormalities in glucose transport in diabetes appear to result both from the acute effects of insulin deficiency and from the loss of more long-term effects of insulin on the total number of glucose transporters in cells. In streptozotocin diabetic rats insulin deficiency is associated with a marked decrease in the total content of glucose transporters in adipocytes (10), and with insulin therapy adipose tissue glucose transporters can be restored to normal (10). Similarly, in skeletal muscle of spontaneously diabetic NOD mice there is a decreased content of glucose transporters (11). It is likely that these changes in number of transporters in diabetic animals result from the loss of insulin effects on transporter synthesis and/or degradation.

In addition to insulin, the oral anti-diabetic sulfonylureas may exert long-term regulatory effects on muscle glucose transport. Actions of sulfonylureas on glucose utilization by peripheral tissues have been demonstrated by in vivo clamp studies in type II diabetes (12) and are further evidenced by the decreased insulin requirements of type I diabetics treated with both sulfonylureas and insulin (13). We have recently observed that sulfonylureas augment insulin-stimulated glucose uptake in cultured L6 skeletal muscle cells (14). Consistent with in vivo findings (15), sulfonylureas had no effect on the rate of glucose transport in L6 cells in the absence of insulin. In the present study our goal was to investigate the effects of both insulin and sulfonylureas on the number and function of glucose transporters and the expression of the glucose transporter gene in L6 cultured skeletal muscle cells in order to determine the mechanism through which insulin and sulfonylureas exert long-term regulatory effects on glucose uptake.

Methods

Materials. Eagle's MEM was purchased from Gibco Laboratories (Grand Island, NY) and donor calf serum from Flow Laboratories, Inc. (McLean, VA). Sodium tolazamide was a gift from The Upjohn Co. (Kalamazoo, MI) and porcine insulin was from Eli Lilly and Co. (Indianapolis, IN). The MAb against human erythrocyte glucose transporter was provided by Dr. Kurt Isselbacher (Gastrointestinal Unit, Massachusetts General Hospital, Boston, MA) (16). Peroxidase-coupled rabbit anti–mouse Ig and swine anti–rabbit Ig antibodies were from Dako Corp. (Santa Barbara, CA). BSA was from Armour Pharmaceutical Co. (Tarrytown, NY). Nick translation kits were from

Amersham Corp. (Arlington Heights, IL) and [^{14}C]2-deoxyglucose, γ-[^{32}P]dCTP, and γ-[^{32}P]UTP were from NEN Research Products (Boston, MA). The random primed DNA labeling kit was from Boehringer Mannheim Biochemicals (Indianapolis, IN), and T7 RNA polymerase was from Stratagene (San Diego, CA). Polyacrylamide and ammonium persulfate were from Bio-Rad Laboratories (Richmond, CA). Agarose was from Bethesda Research Laboratories (Bethesda, MD) and guanidinium isothiocyanate was from Fluka Chemical Corp. (Ronkonkoma, NY). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. The L6 skeletal muscle cells were cultured as previously described (14). In brief, undifferentiated myoblasts were obtained from frozen stocks every 3 wk to minimize variation in passage and then maintained at low density in MEM supplemented with 10% (vol/vol) donor calf serum. To obtain differentiated myotubes for experiments, myoblasts were plated at a density of 7 × 10^3 cells/cm^2, and the medium was replaced on days 4, 7, 8, 9, 10, 11, and 14 after plating. Cytosine-1-β-D-arabinofuranoside (10^{-3} M) was added on days 8 and 10 to stimulate differentiation and eliminate undifferentiated cells. The resulting nonreplicating myotubes were used for experiments between days 15 and 18 after plating.

2-Deoxyglucose uptake. L6 myotube monolayers were washed three times with glucose-free, serum-free MEM containing 1% BSA, and incubated in the same medium with or without tolazamide for 14 h at 37°C. The medium was then removed and replaced with fresh medium containing the indicated concentrations of insulin and/or tolazamide. After an additional 8 h of incubation the medium was removed and the monolayers were rinsed once with Dulbecco's PBS. The uptake of [^{14}C]2-deoxyglucose was then determined during a 10-min incubation at room temperature as previously described (14). 2-Deoxyglucose uptake was linear with time for at least 15 min in both control and treated cells.

Immunoblotting. L6 cells were grown as described and then scraped from the dishes into an isotonic buffer (0.25 M sucrose, 5 mM CaCl$_2$, 1 mM PMSF, 1 mM aprotinin, and 10 mM Tris-HCl, pH 8.0). After homogenization for three 15-s periods with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY), a total membrane fraction was prepared by the method of Klip et al. (4). Protein content of the membrane preparation was determined by the Lowry method (17), and the volume was adjusted such that the final protein concentration was 2–4 mg/ml. This fraction contained 1.8% of total cellular protein. The membranes were solubilized in Laemmli buffer (18), run on SDS-polyacrylamide slab gels, and electrophoretically transferred to nitrocellulose paper at 0.3 A for 3 h. The blots were incubated with blocking buffer (5% BSA in PBS) and then sequentially with mouse monoclonal anti–glucose transporter antibody, peroxidase-coupled rabbit anti–mouse Ig, and peroxidase-coupled swine anti–rabbit Ig. The blots were washed three times with PBS after incubation with each of the antibodies. Bound antibodies were then visualized by using 4-chloro-1-naphthol as substrate for the peroxidase reaction (19).

Solid-phase ELISA technique. Aliquots of the total membrane extracts from L6 myotubes were suspended in PBS containing 1 mM PMSF and 1 mM aprotinin. Membrane proteins were attached to the surface of 96-well Linbro plates (Flow Laboratories, Inc.) by incubating overnight at 4°C. The relative quantity of the glucose transporter on these membranes was measured by first blocking nonspecific binding with albumin (5% BSA, 1 mM PMSF, and 1 mM aprotinin in 10 mM Hepes, pH 7.4, for 1 h), washing three times with PBS, and incubating overnight at 4°C with monoclonal anti–glucose transporter antibody. The preparations were then sequentially incubated with peroxidase-coupled rabbit anti–mouse Ig and swine anti–rabbit Ig antibodies for 1 h each with three PBS washes between each incubation. The peroxidase reaction was initiated with 3,3-diaminobenzidine tetrahydrochloride as substrate at room temperature and stopped by pipeting 10 μl 8 M H$_2$SO$_4$ into each well. The liquid solution in each well was aspirated and read in a spectrophotometer at 495 nm. The assay was linear over the range of 0.1–10 μg of L6 cell membrane/well. The intraassay coefficient of variation was < 5%, and the interassay coeffi-

cient of variation was ~ 30%. For this reason experimental comparisons were always made with samples analyzed in a single assay, and the data were normalized.

Slot blot and Northern blot analysis. Total cellular RNA was prepared by the guanidinium isothiocyanate/cesium chloride cushion method (20). Slot blot analysis was performed by applying 2–10 μg of RNA to nitrocellulose filters using a Hybri-slot manifold (Bethesda Research Laboratories). For Northern blot analysis, 10–20-μg samples of RNA were separated on 1.2% formaldehyde agarose gels and then transferred to nylon filters (21). Two Eco RI fragments (450 and 2,400 bp) of the Hep G2 glucose transporter cDNA sequence (2) were labeled with ^{32}P by nick translation, and these cDNA fragments were used for hybridization to the slot blots. A tubulin cDNA fragment was similarly labeled for control slot blot hybridizations. A nearly full-length Hep G2 glucose transporter ^{32}P-labeled anti-sense RNA probe (2,250 bp) made with T7 RNA polymerase was used for hybridization with the Northern blots (21). Northern blots were also probed with a random-primed labeled β-actin cDNA probe. After hybridization and washing under stringent conditions appropriate to either cDNA or anti-sense RNA probes, filters were exposed to Kodak XAR-5 film at -70°C and analyzed by scanning densitometry.

Statistical analysis. Data are presented as mean±SEM, with differences between groups tested by t test. The data on glucose transporter mRNA levels presented in Fig. 7 were analyzed by the Wilcoxon paired-sample test.

Results

Insulin and tolazamide effects on glucose uptake. Glucose transport was studied in differentiated L6 myotubes by measuring the uptake of 2-deoxyglucose. As previously described, under these experimental conditions insulin stimulates glucose uptake in L6 cells maximally after 8–10 h of incubation (14). Insulin alone (10^{-7} M) stimulated glucose uptake by 30–40% after 8 h. When cells were incubated with tolazamide for 14 h and then with insulin together with tolazamide for 8 h, the effect of insulin was increased approximately twofold (Fig. 1).
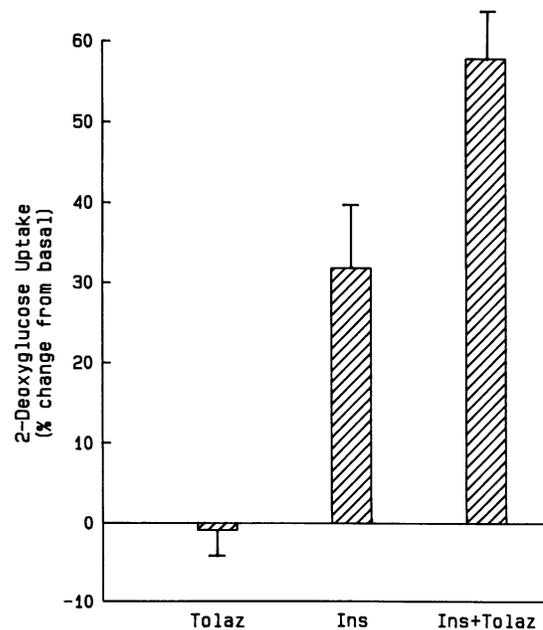

Figure 1. Effects of insulin and tolazamide on 2-deoxyglucose uptake in differentiated L6 myotubes. When indicated, a maximally effective concentration of tolazamide (0.6 mg/ml) was present for 22 h and insulin (10^{-7} M) for 8 h. Basal glucose uptake rate was 0.35 nmol/mg protein per min.

In the absence of insulin, tolazamide alone had no effect on glucose uptake. Since insulin binding has been shown to be unchanged under these conditions (14), it is apparent that the augmentation of insulin-stimulated glucose uptake by tolazamide occurs at a step subsequent to the binding of insulin. The previously published observation that tolazamide increases the maximal efficacy of insulin rather than increasing insulin sensitivity also is consistent with a postbinding mechanism of action (14).

Effects of insulin and tolazamide on the number of glucose transporters. The long incubation times required for maximal insulin effects (8–10 h) and maximal tolazamide effects (22 h) (14) suggested that either or both of these agents may act by modifying the number of glucose transporters in L6 cells. This possibility was investigated by quantifying glucose transporters with a monoclonal anti–transporter antibody. The antibody was generated with purified human erythrocyte glucose transporter and has previously been shown to inhibit cytochalasin B photoaffinity labeling of the erythrocyte transporter (16). On Western blotting of a crude membrane preparation from L6 cells, the anti–transporter antibody identified a single dominant band with an apparent mol wt of ~ 53,000 (Fig. 2). This band was considered to represent the glucose transporter on the basis of its appropriate molecular weight and the known specificity of the antibody.

A solid phase ELISA method (22) was used to quantify glucose transporters in membrane preparations of L6 cells. After 8 h of incubation with insulin the total quantity of glucose transporters in differentiated L6 cells increased, with a maximal twofold increase over basal at 10^{-7} M (Fig. 3). The half-maximal effect of insulin occurred at ~ 10^{-8} M; thus the dose-response curve was similar to that for insulin stimulation of glucose uptake. Tolazamide also increased the number of glucose transporters in L6 cell membrane preparations. In contrast to glucose uptake activity, which was not altered by tolazamide in the absence of insulin, tolazamide alone increased the amount of glucose transporter protein by 70% at the maximally effective concentration of 0.6 mg/ml (Fig. 4). The dose-response curve for this effect on transporter number was very similar to that for augmentation of insulin-stimulated glucose uptake by tolazamide as noted in our previous study (14).

The combined effects of insulin and tolazamide on the quantity of glucose transporters in L6 cells are illustrated in
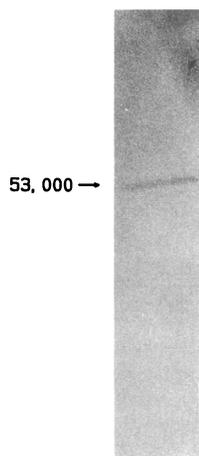

Figure 3. Accumulation of glucose transporters in differentiated L6 myotubes after insulin stimulation. Indicated concentrations of insulin were added to the medium for 8 h. The abundance of transporters in the cell membranes was then quantified by a solid-phase ELISA technique as described in Methods. The data represent mean±SE for two separate experiments in triplicate.

Fig. 5. When added alone, tolazamide and insulin each increased the number of glucose transporters. When the two agents were added together, the mean number of transporters appeared to be greater than with insulin or tolazamide alone, but the effect was less than additive and not statistically increased above the effect of insulin alone. Comparison of the results in Figs. 1 and 5 demonstrates a discordance between the

Figure 2. Immunoblot analysis of glucose transporters in L6 myotubes with monoclonal anti–glucose transporter antibody. A crude membrane preparation from L6 cells was subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose paper. After addition of glucose transporter antibody the transporter was identified by immunoperoxidase staining. A total of 200 μg of the membrane protein was applied in this blot.
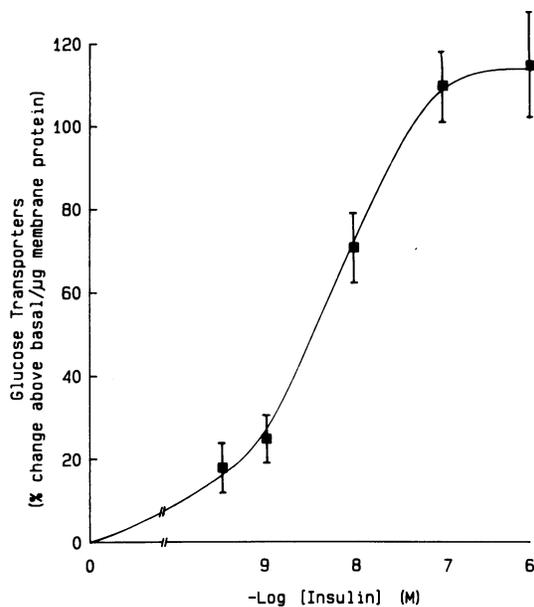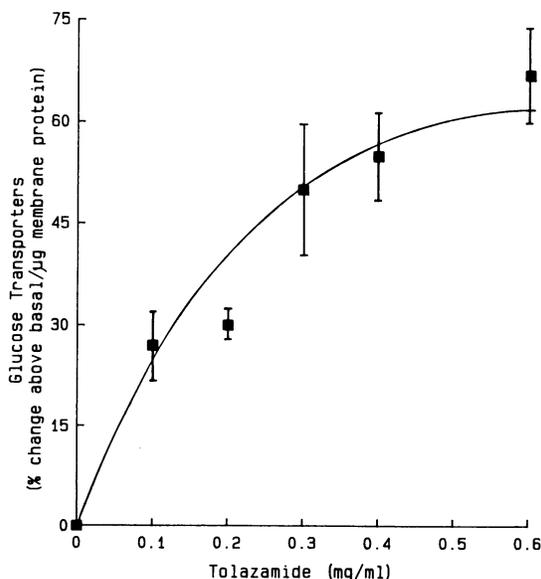

Figure 4. Accumulation of glucose transporters in differentiated L6 myotubes after incubation with tolazamide. Indicated concentrations of tolazamide were added to the medium for 22 h. The abundance of transporters in the cell membranes then was measured by a solid-phase ELISA method. The data represent mean±SE for two separate experiments in triplicate.
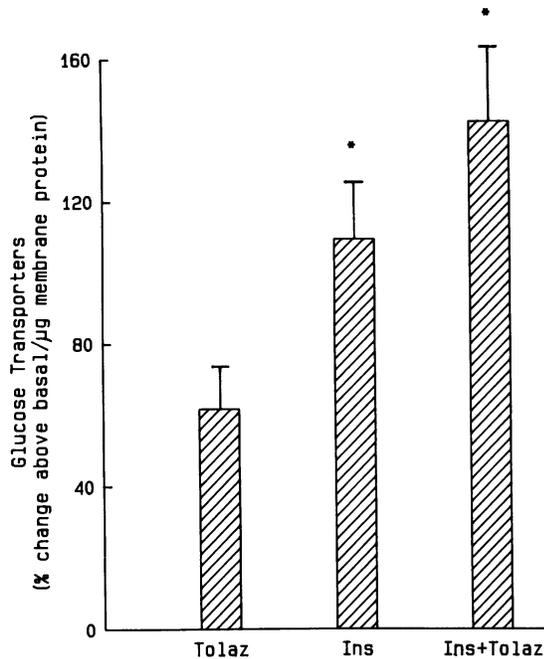
Figure 5. Accumulation of glucose transporters after incubation with insulin and tolazamide in differentiated L6 myotubes. The cells were incubated with insulin (10^{-7} M, 8 h) and/or tolazamide (0.6 mg/ml, 22 h), and then the content of glucose transporters was determined by a solid-phase ELISA method. The data represent mean±SE for three experiments in triplicate. *P vs. control, < 0.005.
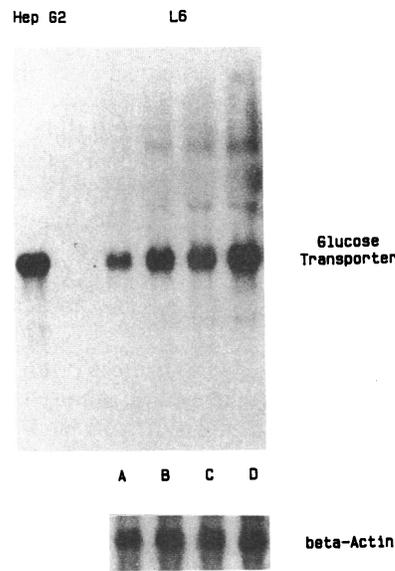

Figure 6. Autoradiograph from Northern blot of total RNA from L6 cells showing induction of glucose transporter mRNA by insulin and tolazamide. In the upper panel, L6 myotube RNA was isolated, electrophoresed (20 µg/lane), transferred, and hybridized under high stringency conditions with a ^{32}P-labeled Hep G2 glucose transporter anti-sense RNA probe as described in Methods. In the lower panel, the same blot was rehybridized with a random-primed labeled β-actin probe. Individual L6 myotube lanes represent (*A*) basal; (*B*) tolazamide, 0.6 mg/ml for 22 h before preparation of RNA; (*C*) insulin, 10^{-7} M for 8 h; and (*D*) tolazamide for 22 h plus insulin for 8 h.

effects of tolazamide alone on glucose uptake and on the number of transporters in L6 cells. The increased content of transporters in the absence of increased glucose uptake suggests that tolazamide may lead to the accumulation of a pool of transporters that are not functional unless insulin is also present.

Effect of insulin and/or tolazamide on transporter gene expression. To gain further insight into the mechanism responsible for the changes in transporter number, the expression of the glucose transporter gene in L6 cells was studied using Hep G2 cell glucose transporter cDNA or RNA probes. A specific increase in the 2.8-kb glucose transporter mRNA was observed on Northern blots after 8 h of incubation with insulin (Fig. 6). Tolazamide-treated cells also demonstrated elevated levels of transporter mRNA compared with the basal state. Combined treatment with insulin and tolazamide induced significantly higher levels of transporter mRNA than were evoked by either insulin or tolazamide alone. To quantitate the change in mRNA levels, six experiments that used either Northern blot or slot blot methodology were analyzed by densitometry and the results were pooled (Fig. 7). Insulin alone increased the glucose transporter mRNA level by 1.7-fold, and tolazamide alone increased it by 1.5-fold. When given together, insulin and tolazamide caused a threefold increase in the transporter mRNA level. The same RNA preparations were also probed with cDNA for tubulin or β-actin. As shown with the β-actin probe in Fig. 6, neither insulin nor tolazamide significantly altered levels of these cytoskeletal mRNAs.

Discussion

Glucose transport in skeletal muscle is an important determinant of peripheral glucose clearance and total body glucose homeostasis. Consistent with its function as a rate-limiting step in glucose metabolism, the uptake of glucose by muscle is regulated by multiple physiologic and pharmacologic factors, including insulin (23, 24), glucose (8), exercise (23, 24), and drugs such as sulfonylureas (14). Increased glucose uptake in response to these stimuli appears to occur through several distinct mechanisms that can lead either to rapid alterations in the cellular distribution or intrinsic activity of glucose transporters, or to more gradually developing changes in the total number of transporters (25). The goal of this study was to investigate the molecular mechanisms of the increase in glucose uptake that develops after several hours of exposure to insulin and sulfonylureas.

The L6 rat skeletal muscle cells used for these studies are a continuous line of undifferentiated myoblasts that readily fuse in culture into multinucleated myotubes with many characteristics of skeletal muscle (26–28). Previous studies have
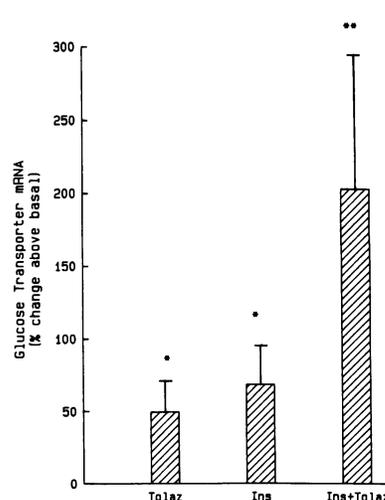

Figure 7. Quantification of the induction of glucose transporter mRNA by insulin and tolazamide. Six hybridization experiments with Hep G2 glucose transporter probes were analyzed by laser densitometry and the results were pooled. The data shown represent mean±SE. *Significantly different from basal, $P < 0.05$. **Significantly different from basal, tolazamide alone, and insulin alone, $P < 0.05$.

shown that the rate of glucose uptake by L6 cells is regulated by both insulin and sulfonylureas (14). Insulin has been shown to have effects on glucose uptake that develop gradually over a period of 8–10 h and are blocked by the protein synthesis inhibitor cycloheximide (29). The effects of the sulfonylurea tolazamide on glucose uptake in L6 cells are not evident in the absence of insulin, but when both insulin and tolazamide are present tolazamide augments the stimulatory effects of insulin. This response is similar to previously described effects of sulfonylureas on glucose uptake in isolated rat diaphragm, in which tolbutamide increased glucose uptake only when insulin was also present (15).

Our previous work on the effects of sulfonylureas in L6 cells has shown that insulin binding is unaltered, suggesting a postbinding mechanism of action (14). Similarly, insulin binding has been shown to be unaffected by sulfonylureas in skeletal muscle (15), adipocytes (30), and hepatocytes (31). Since the effects of both insulin and sulfonylureas on glucose uptake in L6 cells require several hours of incubation and are inhibited by cycloheximide (14), we considered that these agents may be exerting their actions by stimulating transporter synthesis and increasing the cellular content of transporters.

A MAb directed against the human erythrocyte glucose transporter was obtained, shown to crossreact with the L6 skeletal muscle cell transporter, and used to develop a quantitative immunoassay. Using this assay, the total content of glucose transporters in L6 cells was shown to increase after 8 h of incubation with insulin with a maximal effect and dose-response curve that was concordant with the effects of insulin on 2-deoxyglucose uptake under the same conditions. A similar effect of insulin on glucose uptake and the total tissue content of glucose transporters has been inferred from studies on adipose tissue from chronically hyperinsulinemic rats (10). Since insulin administration in vivo is associated with a decrease in plasma glucose concentration, however, it has not been possible to unequivocally separate the effects of changes in insulin and glucose levels. The increase in transporter content in the L6 cells occurred in the absence of changes in glucose concentration and thus is clearly a regulatory response to insulin.

Although the sulfonylurea tolazamide had no effect on glucose uptake activity when administered in the absence of insulin, tolazamide alone caused a dose-related increase in the total number of glucose transporters in L6 cells. When both insulin and tolazamide were added to the culture medium, the total content of transporters in L6 cells did not increase significantly in comparison with the effect of either insulin or tolazamide alone. Under the same conditions, the maximum stimulatory effect of insulin on glucose uptake was increased by approximately twofold. The discordance between the effects of tolazamide on transporter number and function could potentially be explained by the accumulation of a pool of functionally inactive transporters in sulfonylurea-treated cells that is activated or translocated on exposure to insulin. In future studies on the glucose transport system in cells treated with sulfonylureas and/or insulin it will be important to explore this and other potential molecular regulatory mechanisms.

The observation that the effects of insulin and tolazamide are inhibited by cycloheximide and associated with an increase in the total cellular content of glucose transporters is most consistent with a mechanism that involves the stimulation of glucose transporter synthesis by both of these regulators. To further explore this possibility we determined the effects of insulin and tolazamide on glucose transporter mRNA levels in L6 cells. On Northern blots, a single dominant 2.8-kb transporter message was detected in L6 cells, as has been described in other tissues and cell types (3, 21). Both insulin and tolazamide led to an increase in the abundance of this mRNA species. When the two agents were added together there was a further increase in transporter mRNA abundance. The correlation of changes in glucose transporter content of L6 cells and the levels of transporter mRNA strongly suggest that these agents cause accumulation of transporters by stimulating their synthesis. Further studies will be required to determine whether the increased abundance of transporter mRNA results from changes in transcription rate, mRNA stability, or both.

The present observation that tolazamide and insulin cause an increased abundance of glucose transporter mRNA should be put into the larger context of our emerging understanding of the regulation of this gene. In cultured fibroblasts, certain activated oncogenes (ras and src) potently induce transporter gene expression along with increases in the rate of glucose transport (32, 33). Since both ras and src have normal cellular homologues that are thought to be involved in signal transduction pathways, these results suggest a role for such normal pathways in regulation of transporter gene expression. Recent studies have shown that other regulatory factors, such as phorbol esters (32), are also potent inducers of transporter gene expression. As further knowledge about the signalling pathways involved in this regulation develops it should be possible to gain additional insight into the molecular mechanism by which sulfonylureas and insulin alter transporter gene expression and function.

A further issue that needs to be addressed is the increasing evidence for heterogeneity in glucose transporter species at the genetic level. There is now known to be a Na^+-glucose cotransporter (34), a transporter limited to liver and certain other tissues (35), and possibly several other transporters (36) in addition to the Hep G2-brain capillary glucose transporter species (2, 3). Using the Hep G2 probe we have detected glucose transporter mRNA at high stringency in the L6 cultured rat muscle cells as well as in rat and human muscle (21). However, it is possible that additional transporter species will be found to exist in muscle and to play an important role in regulated glucose uptake.

In diabetes mellitus it has been hypothesized that insulin deficiency leads to a decreased intracellular pool of glucose transporters and that this contributes to insulin resistance (25). The stimulatory effect of insulin on transporter content and mRNA levels in L6 cells suggests that insulin resistance in diabetic muscle may result at least in part from decreased glucose transporter synthesis. The coordinate regulatory actions of insulin and sulfonylureas which result in increased functioning glucose transporter number may diminish postreceptor insulin resistance and thus represent a molecular mechanism to explain the therapeutic benefits of sulfonylurea effects in peripheral tissues.

Acknowledgments

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