Metformin Blocks Downregulation of Cell Surface GLUT4 Caused by Chronic Insulin Treatment of Rat Adipocytes

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Large decreases in insulin-responsive glucose transport occur in rat adipocytes maintained in culture for 24 h in the continuous presence of insulin. After 24 h in culture, an acute treatment with insulin increased 3-*O*-methyl-D-glucose transport by only approximately fivefold. In chronically insulin-treated cells, the transport activity was more severely reduced. The transport activity was only approximately twofold higher than in basal cells. To attribute changes in transport to alterations in cell surface transporters, we labeled the cell surface GLUT4 and GLUT1 transporters with the impermeant photoaffinity label 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis(D-mannos-4-yloxy)-2-propylamine. Cell surface labeling was compared with the labeling obtained in digitonin-permeabilized cells where the normally impermeant reagent had access to the total cellular pool of transporters. Labeling showed that in basal cells the proportions of GLUT4 and GLUT1 at the cell surface were 20 and 22% of the total. After an acute treatment with insulin, the proportions of GLUT4 and GLUT1 at the cell surface were increased to 49 and 37% of the total, respectively. The chronic insulin treatment was associated with a very low proportion of GLUT4 (25% of the total) at the cell surface. The downregulation of GLUT4 observed after chronic insulin treatment was alleviated by metformin, and the proportion of GLUT4 at the cell surface was maintained at 60% of the total. Furthermore, cells that were chronically treated with insulin showed severe resistance to subsequent acute insulin restimulation of transport and GLUT4 recruitment to the cell surface. This effect was also alleviated by inclusion of metformin during the chronic insulin treatment.

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GLUT4 is the glucose transporter isoform that is primarily responsible for insulin-stimulated glucose transport in adipose and muscle cells (1–4). Previous studies in isolated rat adipocytes showed that GLUT4 is the more abundant isoform and constitutes ~90% of the total cellular transporter pool; the remaining portion is GLUT1 (3,5). In unstimulated cells, most of the GLUT4 is efficiently sequestered within the cell, and on insulin stimulation, GLUT4 is rapidly translocated to the cell surface to participate in glucose transport (2,6,7). It is the propensity of GLUT4 to become sequestered within the cell in the basal state that renders adipose and muscle cells uniquely sensitive to insulin. In freshly isolated adipocytes, insulin can increase the cell surface levels of this isoform ~20-fold compared with basal cells (2,5). Loss of the sequestration process for GLUT4 or a decrease in its translocation efficiency or both may therefore contribute to impairment in the insulin responsiveness of glucose transport.

A specific depletion of cellular GLUT4 mRNA and protein has been observed in adipose tissue and adipocytes from fasted and STZ-induced diabetic rats (8–10). Similar results have been reported for adipose tissue from obese and NIDDM patients (11,12). However, cellular depletion of glucose transporters in adipocytes from NIDDM patients cannot always account for the observed deficiency in glucose transport activity (13). Furthermore, Pedersen et al. (14) found no significant changes in GLUT4 mRNA or protein in muscle from obese and NIDDM patients. These observations suggest that insulin resistance in glucose transport may also be caused by a defective translocation mechanism.

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GLUT, glucose transporter; NIDDM, non-insulin-dependent diabetes mellitus; STZ, streptozocin; ATB-BMPA, 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis(D-mannos-4-yloxy)-2-propylamine; DMEM, Dulbecco's modified Eagle's medium; $C_{12}E_9$, nonaoctaethyleneglycol-dodecyl ether; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE; polyacrylamide gel electrophoresis.

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The maintenance of adipose cells in culture has been shown by Garvey et al. (15) to provide a system where insulin resistance can be induced without impairment of insulin binding to its receptor. They found that prolonged exposure of cultured adipose cells to insulin results in subsequent loss of responsiveness to further insulin treatment (15). This effect of chronic insulin treatment occurs without a depletion of the total cellular content of glucose transporters as detected in a cytochalasin B binding assay (15). We have studied the effects of chronic insulin treatment in 3T3-L1 cells and find that GLUT4 is downregulated at the cell surface under these conditions (16). In 3T3-L1 cells also, insulin binding to its receptor is unimpaired and the total cellular content of GLUT4 is not altered by the 24-h chronic insulin treatment (16). The insulin resistance that is induced by prolonged exposure of cultured rat adipocytes and in 3T3-L1 cells to insulin may therefore be caused by an impaired transporter recruitment process.

In this study, we have used the membrane impermeant photolabel ATB-BMPA (17) to label glucose transporters in the primary cultured adipose cell system. Photolabeled transporters have been precipitated with specific antibodies raised against the COOH-terminal region of either GLUT4 or GLUT1. In addition, we have extended the utility of the photolabeling reagent as we now show that we can compare cell surface and total cellular levels of transporters by labeling in the absence and presence, respectively, of digitonin (18). Use of this procedure allows glucose transporter distribution within the cell to be determined without the need for subcellular fractionation and separation of plasma membrane from light microsome membranes.

Using the ATB-BMPA photolabeling approach, we have found that the poor insulin responsiveness of glucose transport after chronic treatment with insulin is associated with a downregulation of the proportion of GLUT4 recruited to the cell surface. We have also examined the effects of the oral antihyperglycemic compound metformin and have shown that this can alleviate the downregulation of cell surface GLUT4.

RESEARCH DESIGN AND METHODS

Collagenase was obtained from Worthington (Freehold, NJ). Monocomponent porcine insulin was a gift from Dr. Ronald Chance (Lilly, Indianapolis, IN). DMEM was from Flow (Irvine, CA). $C_{12}E_9$ was from Boehringer Mannheim (Indianapolis, IN). Phloretin, fraction V BSA, metformin, and protein A-sepharose were from Sigma (St. Louis, MO). 3-*O*-methyl-D[U-^{14}C]Glucose was from Amersham (Arlington Heights, IL). ATB-[2-^{3}H]-BMPA (10 Ci/mmol) was prepared as described by Clark and Holman (17).

Rat adipocytes were prepared from 180 to 200 g male Wistar rats by collagenase digestion of aseptically removed epididymal fat pads as described by Marshall (19). Cells were suspended at 40% cytocrit in an albumin-HEPES buffer (140 mM NaCl, 4.7 mM KCl, 1.25 mM Mg_2SO_4, 2.5 mM $CaCl_2$, 2.5 mM NaH_2PO_4, 10 mM HEPES, and 1% BSA, pH 7.4) at 37°C. In most experiments, isolated adipocytes were maintained in culture as described by Traxinger and Marshall (20) with some modifications. Adipocytes were suspended in sterile DMEM containing 25 mM HEPES, 1% BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin, pH 7.4, at 37°C; 1 ml of 40% cell suspension was added to 40 ml of medium in 100 ml polypropylene containers from Sarstedt (Leicester, U.K.) and maintained at 37°C for 24 h. Where indicated, 500 nM insulin or 0.1–3 mM metformin were present during the culture period. After 24 h in culture, the adipocytes were washed four times at 20°C in albumin-HEPES buffer containing insulin or metformin. The washed cells were resuspended in 40% cytocrit. A proportion of cells cultured in the absence of insulin was acutely stimulated for 30 min at 37°C with 10 nM insulin. In some experiments, cells that were chronically treated with insulin or with insulin and metformin were washed an additional three times at 37°C after treatment in either albumin-HEPES buffer without insulin or in this buffer with 1 mM metformin over a period of 40 min before being rechallenged by a subsequent acute treatment with 10 nM insulin for 30 min at 37°C.

To examine glucose transport after the above-described treatment regimen, we determined the uptake of 50 μM 3-*O*-methyl-D-glucose as described previously for freshly isolated cells (5,21). To photolabel the glucose transporters, 0.2 ml of cells in albumin-HEPES buffer were added to 200 μCi of ATB-[2-^{3}H]-BMPA in 0.1 ml of HEPES buffer without albumin in 35-mm polystyrene dishes and irradiated for 1 min in a Rayonet photochemical reactor as described previously (5). After irradiation, the cells were rapidly washed three times with albumin-HEPES buffer at 18°C and solubilized in 1 ml of detergent buffer containing 2% $C_{12}E_9$ in 5 mM phosphate buffer at pH 7.2 and with 1 μg/ml each of the proteinase inhibitors antipain, aprotinin, leupeptin, and pepstatin A. To estimate changes in total cellular transporters, cells were permeabilized with 0.025% digitonin in the presence of 200 μCi ATB-[2-^{3}H]-BMPA for 10 min at 18°C before irradiation. These cells were directly solubilized in 1 ml of detergent buffer without washing. In both cases, nonsolubilized material was removed by centrifugation at 20,000 *g* for 10 min.

To immunoprecipitate the photolabeled transporters, anti-GLUT4 (15 μl) and anti-GLUT1 (20 μl) antisera, which were prepared as described (5,17), were conjugated to 8 μl of protein A-sepharose by mixing for 2 h in 5 mM phosphate buffer at 0–4°C. The conjugates were washed with 5 mM phosphate buffer to remove excess antibody. The solubilized cells in detergent buffer were then mixed sequentially with anti-GLUT4 and then anti-GLUT1 antisera-protein A conjugates. In each case, the immunopellets were washed four times with 1 ml of the detergent buffer containing 0.2% $C_{12}E_9$. Finally, the labeled transporters were released from the conjugates with electrophoresis buffer containing 10% (wt/vol) SDS, 6 M urea, and 10% (vol/vol) mercaptoethanol. The proteins were separated on 10% polyacrylamide gels in a discontinuous buffer system and gel lanes were then separated and sliced. The radioactivity in the gel slices was extracted as described previously (5,23).

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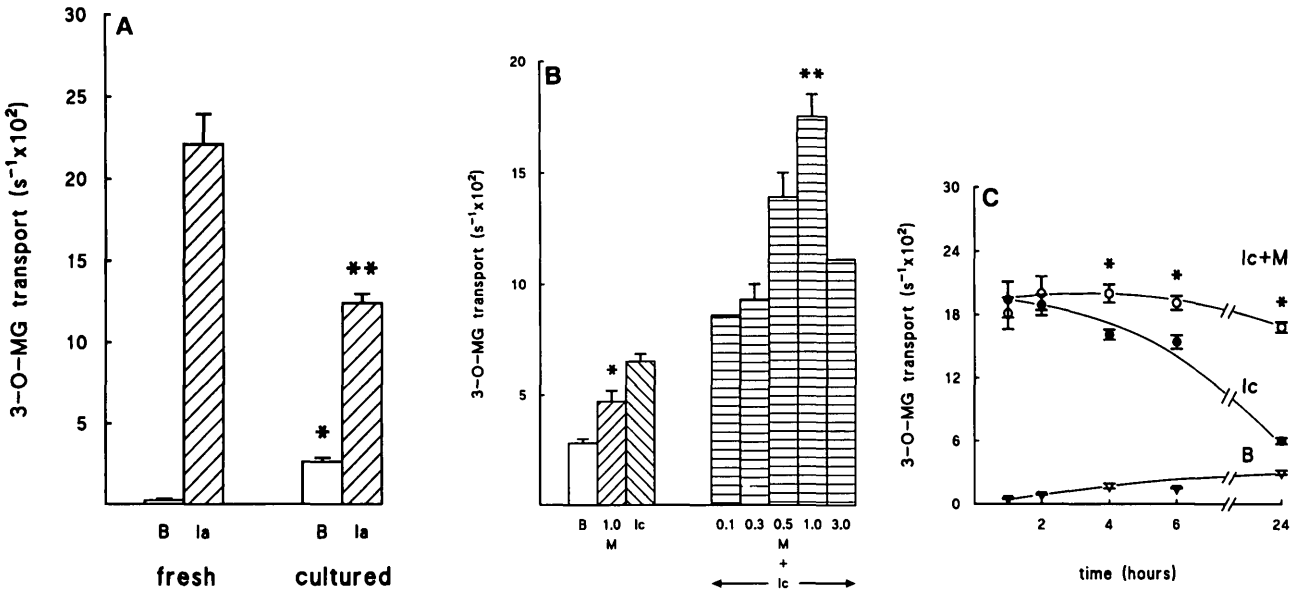

FIG. 1. ***A*: Effect of culturing of isolated rat adipocytes on 3-*O*-methyl-D-glucose transport activity. Freshly isolated rat adipocytes were incubated in absence (*B*) or presence (*Ia*) of 10 nM insulin for 30 min at 37°C, or maintained in culture for 24 h without any additions before the 30-min incubation without (*B*) and with (*Ia*) 10 nM insulin. Cells were then subjected to either 3-*O*-methyl-D-glucose transport assay as described in METHODS. Data are means ± SE of 3–9 experiments. **P* < 0.001 vs. freshly isolated cells, ***P* < 0.001 vs. freshly isolated cells. *B*: Effect of metformin on 3-*O*-methyl-D-glucose transport activity in cultured rat adipocytes treated chronically with insulin. Isolated rat adipocytes were cultured for 24 h at 37°C in the presence of 500 nM insulin (*Ic*) or 500 nM insulin plus 0.1–3.0 mM metformin (*M* + *Ic*) or with 1 mM metformin alone (*M*). A portion of cells was cultured for 24 h without any additions (*B*). Initial rates of 3-*O*-methyl-D-glucose transport were then determined. Data are means ± SE of 3–9 experiments, except for 0.1 and 3.0 mM (*M* + *Ic*), which represent a mean value obtained in 2 separate experiments. **P* < 0.01 vs. *B*, ***P* < 0.001 vs. *Ic*. *C*: Time course for changes in 3-*O*-methyl-D-glucose transport rates during culture of rat adipocytes in the presence of insulin and metformin. Isolated rat adipocytes were maintained in culture for 1–24 h with addition of either 500 nM insulin (*Ic*) or 500 nM insulin plus 1 mM metformin (*IcM*) or without any additions (*B*). At indicated times, 3-*O*-methyl-D-glucose uptake was measured as described in METHODS. Data are from 3–5 experiments. **P* < 0.001 vs. *Ic*.**

RESULTS

Alterations of glucose transport activity after 24-h culture. When freshly isolated rat adipose cells were treated with 10 nM insulin for 30 min (an acute treatment), the rate constant for the uptake of 50 μM 3-*O*-methyl-D-glucose was increased by >60-fold (Fig. 1*A*). However, after 24 h in culture, the basal 3-*O*-methyl-D-glucose uptake was ~10-fold higher than in fresh cells, whereas the acute insulin treatment only increased transport activity 4.7-fold over these elevated basal levels.

When cells were maintained in the continuous presence of 500 nM insulin for 24 h (a chronic treatment), the 3-*O*-methyl-D-glucose transport activity declined further (Fig. 1*B*). However, when the adipose cells were treated with metformin and 500 nM insulin, the 3-*O*-methyl-D-glucose transport activity remained high. Metformin was most effective at 1 mM, and this concentration of metformin maintained the transport activity at a level that was ~3-fold higher than in cells that were chronically treated with insulin alone. Figure 1*B* also shows that 1 mM metformin elevated basal transport activity.

We have examined the time courses for these changes in 3-*O*-methyl-D-glucose transport (Fig. 1*C*). The chronic insulin treatment resulted in a decrease in glucose transport activity with a half-time of ~8 h. The effect of metformin in alleviating this fall was clearly evident in 4 h of incubation ($P < 0.001$).

Associated changes in cell surface glucose transporters. To investigate which of the glucose transporter isoforms was responsible for the poor insulin responsiveness after maintenance of cells in culture, we have photolabeled the cell surface transporters with the membrane impermeant bis-mannose compound ATB-BMPA. The cell surface labeled GLUT4 and GLUT1 were then immunoprecipitated. Figure 2 shows that the rise in the basal transport activity after maintenance of cells in culture for 24 h was associated with an ~4-fold increase in cell surface GLUT4 and an ~9-fold increase in GLUT1. The poor insulin responsiveness of glucose transport activity was associated with a reduction in the maximally insulin-stimulated level of cell surface GLUT4 (which was ~28% lower than in freshly isolated adipocytes).

Figure 3 shows the results of a series of experiments in which the photolabel has been used to compare both the GLUT4 and GLUT1 levels at the cell surface and in the total cellular transporter pool in basal, acutely insulin-treated, and chronically insulin-treated cells. In addition, changes in levels of GLUT4 and GLUT1 associated with maintenance of high transport activity after metformin treatment have been determined. The total cellular levels of GLUT4 and GLUT1 were determined by photolabeling digitonin-permeabilized cells and were found to be the same in paired experiments that compared basal, acutely insulin-treated, and chronically insulin-treated cells. The proportions of cell surface transporters were determined in 4–13 experiments. The cell surface levels of GLUT4 were 20 ± 3% (basal), 49 ± 5% (acute insulin treatment), and 25 ± 4% (chronic insulin treatment). The surface levels of GLUT1 were 22 ± 4% (basal), 37 ± 5%

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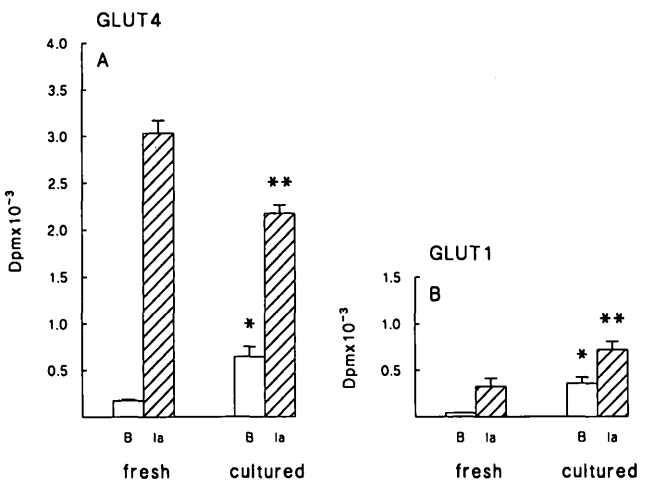

FIG. 2. Effect of culturing of isolated rat adipocytes on cell surface labeling of GLUT4 and GLUT1. Freshly isolated rat adipocytes were incubated in the absence (*B*) or presence (*Ia*) of 10 nM insulin for 30 min at 37°C or maintained in culture for 24 h without any additions before the 30-min incubation without (*B*) or with (*Ia*) 10 nM insulin. Cells were then labeled with 200 μCi of ATB-BMPA and immunoprecipitated with GLUT4 and GLUT1 anti-COOH terminal peptide antibodies and resolved by SDS-PAGE as described in METHODS. Data are means ± SE from 3–14 experiments. *A*: *$P < 0.02$ and **$P < 0.001$, both vs. freshly isolated cells. *B*: *$P < 0.001$ and **$P < 0.05$, both vs. freshly isolated cells.

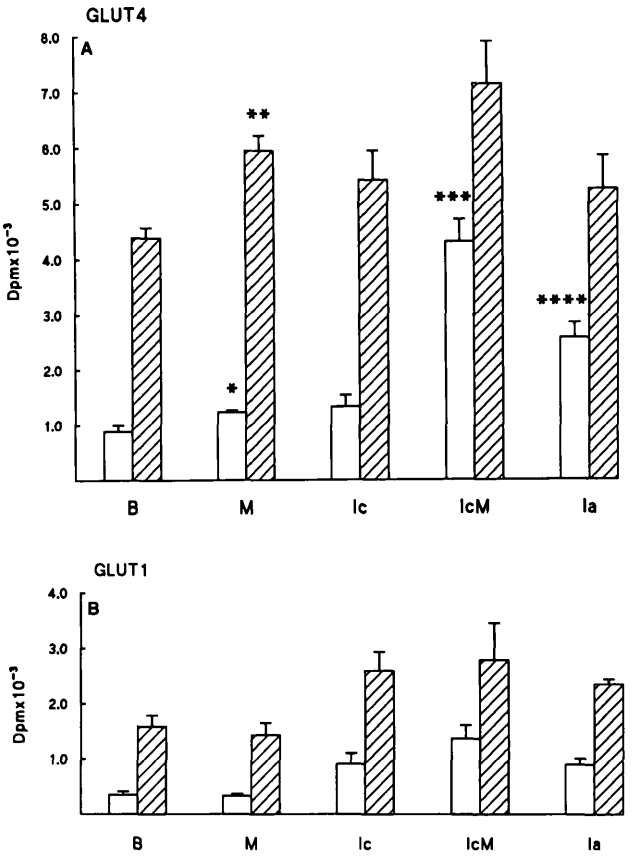

FIG. 3. Effect of acute and chronic treatments with insulin and metformin on cell surface and total cellular labeling of GLUT4 and GLUT1. Isolated rat adipocytes were either cultured for 24 h with no additions (*B*) and then further incubated for 30 min without (*B*) or with (*Ia*) 10 nM insulin, or they were cultured with 500 nM insulin (*Ic*) or with 500 nM insulin plus 1 mM metformin (*Ic + M*). Cells were then photolabeled with 200 μCi of ATB-BMPA in absence (surface labeling; □) or presence of 0.025% digitonin (total labeling; ▨) as described in METHODS. Labeled proteins were immunoprecipitated with anti-GLUT4 and anti-GLUT1 antibodies and resolved on SDS-PAGE as described in METHODS. Data are means ± SE estimated from 4 to 13 separate experiments. *Not significantly different vs. *B*; **$P < 0.002$ vs. *B*; ***$P < 0.001$ vs. *Ic*; ****$P < 0.001$ vs. *Ic*.

(acute insulin treatment), and 35 ± 7% (chronic insulin treatment).

The rise in transport activity in basal cells that were treated with metformin was associated with an ~40% increase in cell surface GLUT4. Metformin addition to basal cells also resulted in a 36% higher total cellular pool of GLUT4 after 24 h in culture ($P < 0.001$), so that the calculated proportion of GLUT4 at the cell surface was only 20 ± 3% of the total. However, the downregulation of cell surface GLUT4 observed after chronic insulin treatment was alleviated by metformin, and the proportion of GLUT4 at the cell surface was maintained at 60 ± 6% of the total. This proportion of cell surface transporters is similar to that observed in freshly isolated adipose cells. Measurements based on the distribution of cytochalasin B binding sites (24) and on the distribution of immunodetectable GLUT4 (7) have suggested that 40–50% of the transporters are at the surface. Similarly, we have shown here that adipose cells that are maintained without insulin for 24 h and are then acutely treated with insulin distribute 49% of the GLUT4 to the surface. Therefore, metformin addition to chronically insulin-stimulated cells allows the maintenance of normal levels of cell surface GLUT4.

Rechallenge of chronically insulin-treated cells with insulin. We have examined whether the downregulation of cell surface GLUT4 induced by chronic insulin treatment leads to a loss in the ability of the cultured adipocytes to respond to a rechallenge with insulin (Fig. 4). Adipocytes were first treated chronically with either insulin alone or with insulin and metformin and were then washed extensively to remove insulin. The cells were washed over 40 min at 37°C with three changes of either albumin-HEPES buffer without insulin or, in the case of cells that had been chronically treated with metformin, with the albumin-HEPES buffer containing 1 mM metformin. In chronically insulin-treated cells, the washing procedure lowered levels of glucose transport activity and of cell surface transporters below the levels observed in basal cells. Metformin blocked the return to low levels of transport and of cell surface transporters after extensive washing to remove insulin.

Adipose cells that had been subjected to chronic insulin treatment followed by the washing procedure showed a markedly impaired response to a subsequent acute treatment with insulin (Fig. 4). After the treatment, 3-*O*-methyl-D-glucose transport activity was only 21 ± 3% of that found in a normal acute insulin challenge of the cultured cells. This was associated with a failure to retranslocate both GLUT4 and GLUT1 to the cell surface (Fig. 4*B* and *C*). Cells that had been chronically treated with insulin and then rechallenged with insulin showed that GLUT4 and GLUT1 levels that were only 34 ± 6 and 35 ± 11% of those observed in the normal acute insulin

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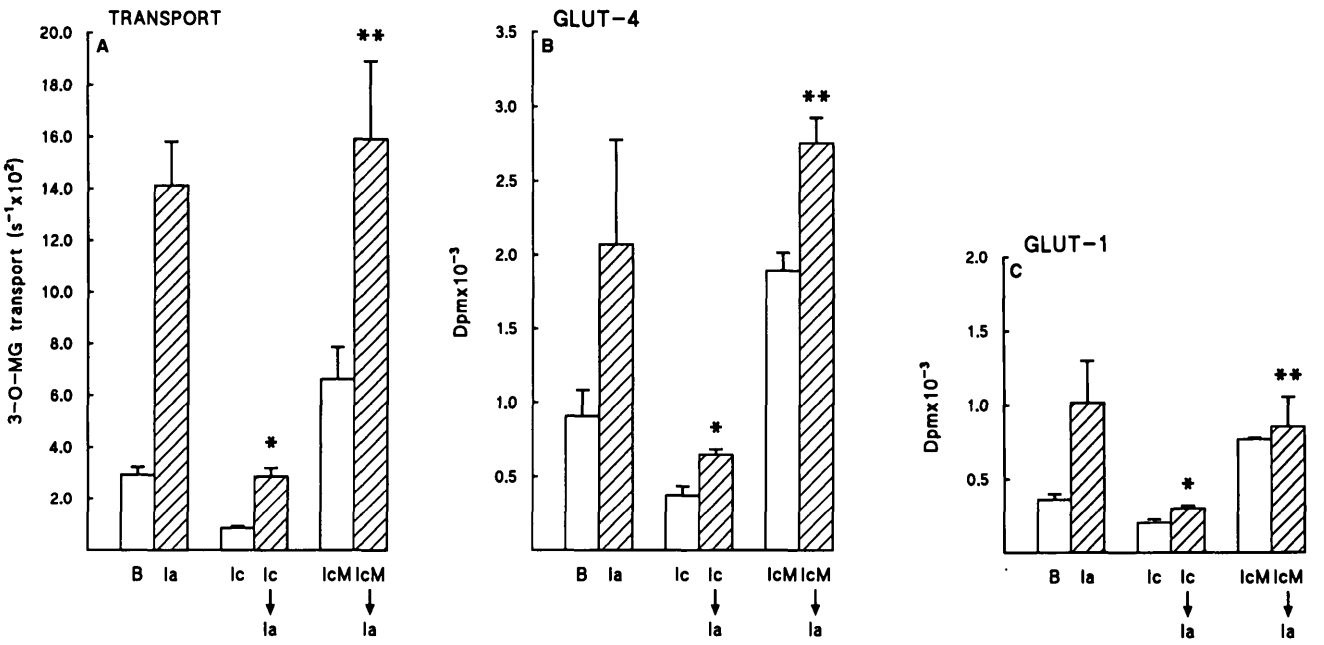

FIG. 4. Effect of chronic treatment with either insulin or a combination of insulin and metformin on the ability of rat adipocytes to respond to a subsequent acute insulin treatment. Isolated rat adipocytes were cultured for 24 h with either no additions (*B*) or in the presence of 500 nM insulin (*Ic*) or 500 nM insulin plus 1 mM metformin (*IcM*). All cells were then subjected to the same washing procedure with insulin-free buffer (or with buffer containing metformin in the case of *IcM* treatment) over a period of 40 min. Where indicated, a portion of cells from each treatment was then incubated for 30 min with 10 nM insulin (*Ia*, acute insulin restimulation). 3-*O*-methyl-D-glucose transport activity and cell surface levels of GLUT4 and GLUT1 was then assayed as described in METHODS. Data are means ± SE from 3 separate experiments. *A*: *$P < 0.005$ vs. *Ia*, **$P < 0.01$ vs. *Ic* → *Ia*; *B*: *$P < 0.05$ vs. *Ia*, **$P < 0.001$ vs. *Ic* → *Ia*, *C*: *$P < 0.05$ vs. *Ia*, **$P < 0.05$ vs. *Ic* → *Ia*.

treatment. This effect was alleviated by inclusion of metformin during the chronic insulin treatment. In addition, after an acute insulin rechallenge of these metformin-treated cells, the transport activity and maximal levels of cell surface GLUT4 and GLUT1 were 111 ± 14, 146 ± 37, and 89 ± 14% of those obtained in a normal acute insulin treatment.

DISCUSSION

To attribute changes occurring in glucose transport to changes in the cellular distribution of both GLUT4 and GLUT1, we have used the ATB-BMPA photolabeling procedure (5,16,22,23). We have shown previously that the GLUT4 and GLUT1 antibodies immunoprecipitate these isoforms with efficiencies of >80%. In addition, we have shown that GLUT1 and GLUT4 have equal affinity for the photolabel and are labeled with equal efficiency. Thus, the labeling procedure can be used to quantify the relative contributions of these isoforms to the transport activity (25). Because the label will only react with surface-exposed transporters, it can give no information on glucose transporters that are associated with the plasma membrane but are masked by a conformational change (26) or are present in occluded vesicles (27). Inactive glucose transporters of these types would be detectable by Western blotting, but quantification of the levels of inactive but immunodetectable transporters is complicated by the need to obtain purified membrane fractions that are not cross-contaminated by intracellular glucose transporters.

Decreased insulin responsiveness of glucose transport in 24-h cultured cells. In contrast to freshly isolated adipose cells where insulin increases glucose transport activity by >20-fold (5,23), insulin treatment of adipose cells maintained in culture for 24 h resulted in only a 4.7-fold increase in glucose transport. Use of the photolabeling technique showed that the rise in basal glucose transport activity after 24 h in culture was caused by an ~4-fold increase in the cell surface level of GLUT4 and an ~9-fold rise in GLUT1. Acutely insulin-stimulated levels of glucose transport activity in the cultured cells were ~40% lower than in fresh cells. This decreased transport activity was associated with an ~28% lower level of cell surface GLUT4. Thus, the decreased insulin responsiveness of these cells was partly the result of a decrease in cell surface GLUT4. This, in turn, may have decreased because of a decrease in the total cellular content of GLUT4 over the prolonged culture period. Hajduch et al. (28) and S.W. Cushman (unpublished observations) have shown that there is an ~30% decrease in the total cellular membrane content of immunodetectable GLUT4 after incubation of adipose cells in culture for 24 h. The decrease in GLUT4 mRNA is more precipitous and declines by ~50% over 24 h (28).

Because GLUT4 decreases, GLUT1 makes a greater contribution to the transport activity after 24 h in culture. GLUT1 is less effectively sequestered into the intracellular membrane pool and consequently is less responsive to acute insulin treatment. This isoform is typically only elevated 3- to 5-fold above basal levels after acute insulin

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treatment (5,22,23). In addition, the GLUT1 isoform transports 50 μM 3-*O*-methyl-D-glucose (as used here) approximately three times more slowly than GLUT4 because it has a higher K_m (21,25,29). However, because both basal and maximally insulin-stimulated transport activity after 24 h in culture changed by a slightly greater extent than could be accounted for by alterations in either cell surface levels of GLUT4 or GLUT1, it is possible that these discrepancies are caused by a modification in the intrinsic activity of both GLUT1 and GLUT4 during the 24-h culture.

During differentiation of 3T3-L1 cells and probably adipose cells, the ratio of GLUT4 to GLUT1 increases (18,22,30,31), and therefore the decrease in this ratio observed after a 24-h culture is probably a consequence of a de-differentiation of the adipose cells. We do not think that de-differentiation detracts from the utility of this system, because insulin resistance may be a result of de-differentiation of insulin target tissues. Indeed, denervation of muscle results in muscle de-differentiation and also insulin resistance of glucose transport activity (32,33).

Downregulation of cell surface GLUT4 after chronic insulin treatment. We have shown here that the blunted insulin responsiveness of glucose transport activity that occurs after a chronic insulin treatment of cells in culture is caused by a downregulation of GLUT4 at the cell surface. This effect occurs without an additional decrease in the total cellular content of this isoform below that found without chronic insulin treatment. Instead, the proportion of GLUT4 at the cell surface was reduced to ~25% of the total. Furthermore, cells chronically treated with insulin and then washed to remove insulin are strikingly insensitive to a further challenge with insulin. Because the effect of insulin is normally to redirect the exocytosis and targeting of GLUT4 from the light microsome membrane pool to the plasma membrane (7), the lesion may have resulted from a failure in the exocytosis and re-targeting processes. When chronic insulin treatment was followed by extensive washing, a larger loss of cell surface transporters was observed than in cells that were maintained without insulin. This may have occurred because exocytosis was reduced below that occurring in basal cells.

Effects of metformin on cell surface glucose transporters. The antihyperglycemic effects of metformin have been attributed to increased nonoxidative glucose metabolism (34), diminished intestinal absorption of carbohydrates (35) and to potentiation of the insulin stimulation of glucose uptake activity in diaphragm (36) and GLUT1 translocation in L6 muscle cells in culture (37). Matthaei et al. (38) have reported that a 2-h treatment of freshly isolated adipocytes with metformin increased the level of insulin-stimulated glucose transport activity by 40% and enhanced the plasma membrane recruitment of immunodetectable GLUT4. Those authors also showed that metformin did not significantly increase insulin receptor number, affinity, or tyrosine kinase activity (38). In our cultured cell system, a 2-h treatment with metformin gave an insignificant potentiation of transport activity (Fig. 1*C*). However, longer treatment with metformin, for 4–24 h, resulted in a significant alleviation of the downregulation of cell surface GLUT4 that was associated with insulin-resistant glucose transport. The proportion of GLUT4 at the cell surface after treatment with metformin and insulin was maintained at a level (~60%) that can be regarded as a normal insulin-stimulated level (~50%) in freshly isolated cells (24,7) and in 24-h cultured cells that are acutely treated with insulin (Fig. 3). The alleviation of the downregulation of GLUT4 may be the result of reversal of the lesion in the signaling or insulin-induced retargeting and exocytosis processes, referred to above. Consistent with this possibility, we have shown that the effects of metformin on chronically insulin-treated cells are greater than those caused by metformin alone. The effects of metformin on glucose transporter exocytosis and endocytosis, both in the presence and absence of insulin, are being investigated further.

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REFERENCES

1. Mueckler M: Family of glucose-transporter genes: implications for glucose homeostasis and diabetes. *Diabetes* 39:6–11, 1990
2. Slot JW, Geuze HJ, Gigengack S, Lienhard GE, James DE: Immunolocalization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. *J Cell Biol* 113:123–35, 1991
3. Zorzano A, Wilkinson W, Kotiar G, Thoidis G, Wadzinski BE, Ruoho AE, Pilch PF: 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, 1989
4. Hirshman MF, Goodyear LJ, Wardzala LJ, Horton ED, Horton ES: Identification of an intracellular pool of glucose transporters from basal and insulin-stimulated rat skeletal muscle. *J Biol Chem* 265:987–91, 1990
5. Holman GD, Kozka IJ, Clark AE, Flower CJ, Saltis J, Habberfield AD, Simpson IA, Cushman SW: Cell surface labeling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. *J Biol Chem* 265:18172–79, 1990
6. Bornemann A, Ploug T, Schmalbruch H: Subcellular localization of GLUT4 in nonstimulated and insulin-stimulated soleus muscle of rat. *Diabetes* 41:215–21, 1992
7. Satoh S, Gonzalez-Mulero OM, Clark AE, Kozka IJ, Holman GD, Cushman SW: Recycling of cell-surface GLUT4 glucose transporters in rat adipose cells photolabeled in the insulin-stimulated state (Abstract). *Diabetes* 40 (Suppl. 1):85A, 1991
8. Sivitz WI, DeSautel SL, Kayano T, Bell GI, Pessin JE: Regulation of glucose transporter messenger RNA in insulin-deficient states. *Nature (Lond)* 340:72–74, 1989
9. Berger J, Biswas C, Vicario PP, Strout HV, Saperstein R, Pilch PF: Decreased expression of the insulin-responsive glucose transporter in diabetes and fasting. *Nature (Lond)* 340:70–72, 1989
10. Kahn BB, Charron MJ, Lodish HF, Cushman SW, Flier JS: Differential regulation of two glucose transporters in adipose cells from diabetic and insulin-treated diabetic rats. *J Clin Invest* 84:404–11, 1989
11. Sinha MK, Raineri-Maldonado C, Buchanan C, Pories WJ, Carter-Su C, Pilch PF, Caro JF: Adipose tissue glucose transporters in NIDDM. Decreased levels of muscle/fat isoform. *Diabetes* 40:472–77,1991
12. Garvey WT, Maianu L, Huecksteadt TB, Birnbaum MJ, Molina JM, Ciaraldi TB: Pretranslational supression of a glucose transporter protein causes insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity. *J Clin Invest* 87:1072–81, 1991
13. Garvey WT, Hueckstead TP, Matthaei S, Olefsky JM: Role of glucose transporters in the cellular insulin resistance of type II non-insulin-dependent diabetes mellitus. *J Clin Invest* 81:1528–36, 1988
14. Pedersen O, Bak JF, Andersen PH, Lund S, Moller DE, Flier JS, Kahn BB: Evidence against altered expression of GLUT1 or GLUT4 in skeletal muscle of patients with obesity or NIDDM. *Diabetes* 39:865–70, 1990
15. Garvey WT, Olefsky JM, Matthaeu S, Marshall S: Glucose and

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insulin coregulate the glucose transport system in primary cultured adipocytes: a new mechanism of insulin resistance. *J Biol Chem* 262:189–97, 1987
16. Kozka IJ, Clark AE, Holman GD: Chronic treatment with insulin selectively downregulates cell-surface GLUT4 glucose transporters in 3T3-L1 adipocytes. *J Biol Chem* 266:11726–31, 1991
17. Clark AE, Holman GD: Exofacial photolabeling of the human erythrocyte glucose transporter with an azitrifluoroethylbenzoyl-substituted bismannose. *Biochem J* 269:615–22, 1990
18. Yang J, Clark AE, Kozka IJ, Cushman SW, Holman GD: Development of an intracellular pool of glucose transporters in 3T3-L1 cells. *J Biol Chem* 267:10393–99, 1992
19. Marshall S: Kinetics of insulin receptor biosynthesis and membrane insertion: relationship to cellular function. *Diabetes* 32:319–25, 1983
20. Traxinger RR, Marshall S: Recovery of maximal insulin responsiveness and insulin sensitivity after induction of insulin resistance in primary cultured adipocytes. *J Biol Chem* 264:8156–63, 1989
21. Taylor LP, Holman GD: Symmetrical kinetic parameters for 3-*O*-methyl-D-glucose transport in adipocytes in the presence and absence of insulin. *Biochim Biophys Acta* 642:325–35, 1981
22. Calderhead DM, Kitagawa K, Tanner LI, Holman GD, Lienhard GE: Insulin regulation of the two glucose transporters in 3T3-L1 adipocytes. *J Biol Chem* 265:13800–808, 1990
23. Clark AE, Holman GD, and Kozka IJ: Determination of the rates of appearance and loss of glucose transporters at the cell surface of rat adipose cells. *Biochem J* 278:235–41, 1991
24. Simpson IA, Yver DR, Hissin PJ, Wardzala LJ, Karnielli E, Salans LB, Cushman SW: Insulin-stimulated translocation of glucose transporters in isolated rat adipose cells: characterization of subcellular fractions. *Biochim Biophys Acta* 763:393–407, 1983
25. Palfreyman RW, Clark AE, Denton RM, Holman GD, Kozka IJ: Kinetic resolution of the separate GLUT1 and GLUT4 glucose transport activities in 3T3-L1 cells. *Biochem J* 284:275–81, 1992
26. Harrison SA, Clancy BM, Pessino A, Czech MP: Activation of cell surface glucose transporters measured by photoaffinity labeling of insulin-sensitive 3T3-L1 adipocytes. *J Biol Chem* 267:3783–88, 1991
27. Vannucci SJ, Nishimura H, Satoh S, Cushman SW, Holman GD, Simpson IA: Cell surface accessibility of GLUT4 glucose transporters in rat adipose cells: effects of isoproterenol and adenosine. *Biochem J* 288:325–30, 1992
28. Hajduch EJ, Guerre-Millo MC, Hainhault IA, Guichard CM, Lavau MM: Expression of glucose transporters (GLUT1 and GLUT4) in primary cultured rat adipocytes: differential evolution with time and chronic insulin effect. *J Cell Biochem* 49:251–58, 1992
29. Keller K, Strube M, Mueckler M: Functional expression of the human HepG2 and rat adipocyte glucose transporters in Xenopus oocytes: comparison of kinetic parameters. *J Biol Chem* 264:18884–89, 1989
30. Kaestner KH, Christy RJ, McLenithan JC, Braiterman LT, Cornelius P, Pekela PH, Lane MD: Sequence, tissue distribution, and differential expression of mRNA for a putative insulin-responsive glucose transporter in mouse 3T3-L1 adipocytes. *Proc Natl Acad Sci USA* 86:3150–54, 1990
31. Birnbaum MJ: Identification of a novel gene encoding an insulin-responsive glucose transport protein. *Cell* 57:305–15, 1989
32. Burant CF, Lemmon SK, Treutelaar MK, Buse MG: Insulin resistance of denervated rat muscle: a model for impaired receptor function coupling. *Am J Physiol* 247:E657–66, 1984
33. Block NE, Menick DR, Robinson KA, Buse MG: Effect of denervation on the expression of two glucose transporter isoforms in rat hindlimb muscle. *J Clin Invest* 88:1546–52, 1991
34. Widen EIM, Ericksson JG, Groop LC: Metformin normalizes nonoxidative glucose metabolism in insulin-resistant normoglycemic first-degree relatives of patients with NIDDM. *Diabetes* 41:354–58, 1992
35. Schafer G: Biguanides: a review of history, pharmacodynamics, and therapy. *Diabete Metab* 9:148–63, 1983
36. Frayn KN, Adnitt PI: Effects of metformin on glucose uptake by isolated diaphragm from normal and diabetic rats. *Biochem Pharmacol* 21:3153–62, 1972
37. Hundal HS, Rampal T, Reyes R, Leiter LA, Klip A: 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 1992
38. Matthaei S, Hamann A, Klein HH, Benecke H, Kreymann G, Flier JS, Greten H: 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* 40:850–57, 1991

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