Evidence That Erythroid-type Glucose Transporter Intrinsic Activity Is Modulated by Cadmium Treatment of Mouse 3T3-L1 Cells*

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Previous studies suggest that regulation of hexose uptake in Chinese hamster ovary fibroblasts can occur by alterations in glucose transporter intrinsic activity without changes in cell surface transporter number (Harrison, S. A., Buxton, J. M., Helgerson, A. L., MacDonald, R. G., Chlapowski, F. J., Carrathers, A., and Czech, M. P. (1990) J. Biol. Chem. 265, 5793–5801). We tested this hypothesis using 3T3-L1 fibroblasts and adipocytes which exhibit 5–6-fold increases in 2-deoxyglucose or 3-O-methylglucose uptake when exposed to low micromolar concentrations of cadmium for 18 h. Cadmium treatment decreased the apparent $K_M$ of 3T3-L1 fibroblasts for 3-O-methylglucose influx from approximately 28 to 9 mM and increased the apparent $V_{max}$ by 2–3-fold. These fibroblasts lack the skeletal muscle/adipocyte-type (GLUT4) transporter and showed only a small increase in total cellular immunoreactive GLUT1 or GLUT4 in isolated 3T3-L1 adipocytes exposed to cadmium, as assessed by the binding to intact cells of an antibody which recognizes an extracellular epitope.

Insulin enhanced 2-deoxyglucose uptake 2-fold in 3T3-L1 fibroblasts, but did not further stimulate cadmium-activated transport rates. In contrast, insulin stimulated hexose transport 15-fold in 3T3-L1 adipocytes, which express both GLUT1 and GLUT4 proteins, and this effect was fully additive with the 5-fold effect of cadmium. Cadmium had little or no effect on immunoreactive GLUT1 or GLUT4 in isolated 3T3-L1 adipocyte plasma membranes. In contrast, insulin action led to marked recruitment (3-fold) of GLUT4 to the plasma membrane fraction in adipocytes treated with or without cadmium. Taken together, these data are consistent with the hypothesis that cadmium-activated sugar uptake is catalyzed by GLUT1, whereas insulin-stimulated sugar uptake is catalyzed predominantly by GLUT4 in 3T3-L1 adipocytes. Furthermore, the data suggest that the GLUT1 transporter can undergo significant increases in intrinsic catalytic activity in response to cadmium treatment of 3T3-L1 fibroblasts and adipocytes.

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Recent evidence demonstrates that a family of highly conserved proteins are responsible for facilitative glucose transport in mammalian cells (for reviews, see Carruthers, 1990; Bell et al., 1996; Mueckler, 1990). However, little is known concerning the specific physiological roles of these glucose transporter isoforms. Many tissues and individual cell types express more than one glucose transporter isoform (for review, see Carruthers, 1990; Bell et al., 1990; Mueckler, 1996; Kasanicki and Pilch, 1990). It is unclear why these cells require multiple facilitative transporters to regulate glucose homeostasis. Additionally, sugar transport in many cells is regulated by nutritional status, growth factors, ATP, and hormones (Carruthers, 1990; Kasanicki and Pilch, 1990), further complicating attempts to understand the respective roles of individual transport proteins.

Perhaps the system that provides the best experimental model for hormonal regulation of hexose uptake is the isolated rat adipose cell which is dramatically stimulated by acute exposure to insulin (Simpson and Cushman, 1986). Cushman and Wardzala (1986) and Suzuki and Kono (1980) independently developed the hypothesis that insulin regulates glucose transport in isolated rat adipocytes by stimulating the recruitment of glucose transporters from an intracellular pool to the plasma membranes. Their studies assessed glucose transporters using cytochalasin B-binding assays and by reconstitution of transport activity from low density microsomal membranes and plasma membranes harvested from control and insulin-treated adipocytes. More recently, Oka and Czech (1984), Holman and co-workers (1988, 1990) and Calderhead, Liernhard and co-workers (1988, 1990) have confirmed glucose transporter translocation by covalently labeling glucose transporters in control and insulin-treated intact cells. Additionally, Block and co-workers (1988) and Slot et al. (1991) have obtained evidence in support of the recruitment hypothesis by immunocytochemical analysis using electron microscopy.

Although the recruitment hypothesis has become widely accepted, it has not yet been possible to determine whether this model for insulin action can account for the full stimulatory effect of that hormone on glucose transport in adipocytes or muscle. Estimates of glucose transporter recruitment following insulin treatment do not exclude regulation of glucose transporter intrinsic activity. There is also some evidence that insulin-stimulated increases in glucose transport rates can be dissociated from insulin-stimulated recruitment of transporter proteins (Baly and Honk, 1987; Matthias et al., 1988), although more recent work by Jones and Cushman (1989) contradict those studies.

Analysis of this question has been made more complex by the discovery that isolated rat adipocytes (James et al., 1988, 1989; Birnbaum, 1989) and cultured 3T3-L1 adipocytes (James et al., 1989; Kaestner et al., 1989) express both...
GLUT1 and GLUT4 transporter proteins. Thus, even in the extensively studied adipocyte model system, the mechanism(s) of activation of glucose transport and the relative contributions of each transporter isoform to basal and insulin-stimulated uptake are incompletely understood. Several lines of evidence suggest that insulin-stimulated sugar transport is primarily mediated by the GLUT4 transporter in responsive tissues. GLUT4 protein expression appears to be limited to the hexose transporters in isolated rat adipocytes are GLUT4 versus 3-10% GLUT1 (Oka et al., 1988; Zorzano et al., 1989). Pilch and co-workers (Zorzano et al., 1989) have proposed that GLUT1 and GLUT4 proteins reside in distinct populations of intracellular vesicles, and that stimulation of glucose uptake by insulin in rat adipocytes occurs primarily as a result of hormonal regulation of the GLUT4 containing vesicles. Based on their data, it appears that the ratio of plasma membrane transporters:low density microsomal membrane transporters in basal adipocytes is approximately 1:1 for GLUT1 versus 2:1 for GLUT4. Insulin decreases the low density microsomal membrane content of both GLUT1 and GLUT4 similarly, and thus more dramatically increases the GLUT4 numbers in the plasma membrane. Similar data were obtained using differentiated 3T3-L1 cell membrane fractions (Clancy and Czech, 1990; Harrisen et al., 1990b).

Recent data from this laboratory also suggest that insulin-stimulated transport in 3T3-L1 adipocytes is primarily catalyzed by the GLUT4 transporter (Harrison et al., 1990b). In studies, overexpressing human GLUT1 protein elevated basal 2-deoxyglucose uptake almost 3-fold, with no alteration in the absolute rates of insulin-stimulated sugar uptake. Similarly, Calderhead and co-workers (Calderhead et al., 1990) report that 3T3-L1 adipocytes express significantly higher levels of GLUT1 than GLUT4 protein and yet their data indicate that the extent of the insulin-stimulated recruitment of the GLUT4 transporter, but not the GLUT1 transporter, is similar to the insulin-stimulated increase in deoxyglucose uptake rates in those cells. Studies of the kinetics of basal and insulin-stimulated sugar uptake by isolated rat adipocytes have also suggested the presence of two distinct transporter isoforms, only one of which is substantially translocated to the plasma membranes following insulin treatment of those cells (Suzuki, 1988; Whitesell et al., 1989).

While these data suggest a primary role for GLUT4 protein in insulin-stimulated sugar transport, no direct evidence for this hypothesis has been produced. Also, no convenient tools for studying the relative contributions of each transporter in vivo, such as selective activators or inhibitors of individual glucose transport isoforms, have yet been identified. Studies of 3T3-L1 adipocytes performed in this laboratory have strongly suggested a dissociation between activation of sugar uptake and recruitment of glucose transport proteins (Clancy and Czech, 1990; Clancy et al., 1991). The present work reports our findings that chronic exposure of either undifferentiated or differentiated 3T3-L1 cells to low micromolar concentrations of cadmium is associated with a 5-6-fold activation of hexose uptake with little or no increase in glucose transporter protein levels. We further report that under our experimental conditions, cadmium does not alter insulin stimulation of glucose transport in the mouse 3T3-L1 adipocytes. The hypothesis that cadmium treatment activates the GLUT4 transporter is supported by these data.

**EXPERIMENTAL PROCEDURES**

**Materials**—2-Deoxy-d-glucose, cytochalasin B, bovine insulin, cadmium sulfate, and phloretin were purchased from Sigma. Low specific activity, 125I-labeled protein A (8.31 μCi/μg) and 3-0-methyl-14C-glucose (300 μCi/mmol) were purchased from Du Pont/New England Nuclear. 2-Deoxy-d-[2,6-3H]glucose (50-60 Ci/mmol) was purchased from New England Nuclear. 14C-Insulin was a gift from Eli Lilly and Company. 3-furoylc acid, 3-0-methylglucose, 2-deoxy-d-glucose, 2-3H-glucose, [3H]glucose, [3H]forskolin, 3-Iodinated 4-azido-2-propylamine, and [3H]cytochalasine B were purchased from American Type Culture Collection. Permeosamine was a gift from Gift. Ronald Chance, Lilly Research Laboratories, Inc. Rabbit antiserum R495 and R820 were purchased from East Acres Biologicals. [3H]IAPS-forskolin was the generous gift of Dr. Michael Shanahan, S. Illinois University School of Medicine. Tissue culture media, antibiotics, calf serum, and fetal bovine serum were purchased from Gibco.

**Cell Cultures**—3T3-L1 fibroblasts were seeded at 600,000-750,000 cells/100-mm culture plate for the preparation of membrane proteins or 20,000 or 10,000 cells/well in either 12- or 24-well culture plates, respectively, for hexose uptake assays. Fibroblasts were grown to confluence in high glucose DMEM containing 10% calf serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin sulfate (25 ml of media/150 × 25-mm plate, 2 ml/well of 12-well culture plates or 1 ml/well of 24-well culture plates) and maintained in a 5% CO2-humidified atmosphere at 37 °C. This medium was changed every 2-3 days. Fibroblasts were grown to confluence and 1 day later were incubated overnight in the same medium ± the indicated concentrations of cadmium sulfate, prior to measuring sugar uptake or harvesting cell membranes. Cadmium sulfate was not included in starvation or cell wash buffers.

Two days after the fibroblasts achieved confluence, differentiation to adipocytes was induced by normalizing the cells for 2 days in the same volume of high glucose DMEM containing 10% fetal bovine serum, 5 μg/ml of insulin, 0.25 μM dexamethasone, 0.5 nM 3-isobutyl-1-methylxanthine, 50 units/ml of penicillin, and 50 μg/ml of streptomycin sulfate. The cells were then incubated for an additional 2 days in the same medium without 0.25 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine. The cells were then maintained for an additional 6-10 days in high glucose DMEM containing 10% fetal bovine serum prior to measuring sugar uptake or harvesting cell membranes. At this time greater than 95% of the cells appeared to express the adipocyte phenotype. Cadmium induction was performed by overnight incubation in the same medium ± cadmium sulfate, as described above for the adipocytes.

**Assay of 2-Deoxy-D-Glucose Uptake in 3T3-L1 Cells**—Hexose transport assays in 3T3-L1 fibroblasts and adipocytes were performed essentially as described previously by Frost and Lane (1985). In all of the fibroblast studies, cells were plated in 24-well cluster plates as described above and cell numbers ranged from approximately 4-6 × 150 to 8 × 105 cells/well. In all of the adipocyte studies, cells were plated in 12-well cluster plates and differentiated as described above and cell numbers ranged from approximately 5 to 8 × 105 cells/well. Prior to initiating hexose uptake assays, cells were washed three times with phosphate-buffered saline (157 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM NaHPO4, 0.66 mM CaCl2, and 0.49 mM MgCl2, pH 7.4) and incubated in 1 ml (or 0.5 ml) of serum-free DMEM for 1.5-2 h at 37 °C. Next, the cells were washed once with 1 ml (or 0.5 ml) of Krebs-Ringer phosphate buffer (130 mM NaCl, 5 mM KC1, 1.3 mM CaCl2, 1.3 mM MgSO4, and 10 mM NaHPO4, pH 7.4) and incubated in 1 ml (or 0.5 ml) of Krebs-Ringer phosphate buffer ± 100 μM insulin for 20 min at 37 °C. Sugar uptake was initiated by addition of 2-deoxy-D-[2,6-3H]glucose to a final assay concentration of 0.1 μM (1 μCi/ml) for 5 min at 37 °C. Assays were terminated by 2 rapid washes with 1 ml of ice-cold Krebs-Ringer phosphate buffer. Cells were solubilized with 0.4 μl of 0.1% SDS and 1 ml was added to 4 ml of scintillant. Nonspecific deoxyglucose uptake was measured in the presence of 0.2 μM cytochlasain B, and was subtracted from each determination to obtain specific uptake. Cultured fibroblast and adipocyte cell numbers were determined by counting the trypan blue excluding cells harvested from trypsin-treated cell monolayers, using a hemocytometer. The data were normalized to the absolute rates of insulin-stimulated sugar transport, no direct evidence for this hypothesis has been produced. Also, no convenient tools for studying the relative contributions of each transporter in vivo, such as selective activators or inhibitors of individual glucose transport isoforms, have yet been identified. Studies of 3T3-L1 adipocytes performed in this laboratory have strongly suggested a dissociation between activation of sugar uptake and recruitment of glucose transport proteins (Clancy and Czech, 1990; Clancy et al., 1991). The present work reports our findings that chronic exposure of either undifferentiated or differentiated 3T3-L1 cells to low micromolar concentrations of cadmium is associated with a 5-6-fold activation of hexose uptake with little or no increase in glucose transporter protein levels. We further report that under our experimental conditions, cadmium does not alter insulin stimulation of glucose transport in the mouse 3T3-L1 adipocytes. The hypothesis that cadmium treatment activates the GLUT4 transporter is supported by these data.

**Assay of 3-0-Methylglucose Transport in 3T3-L1 Adipocytes**—3-0-Methylglucose transport assays were performed essentially as described above for 2-deoxyglucose uptake assays, with minor modifi-
cations. Cells were then allowed to stand for 10 min at room temperature and sugar transport was initiated by addition of 3-O-methyl-[14C]glucose (2 μCi/ml) to final assay concentrations as indicated in the legends for Figs. 4, 5, and 10, for the indicated times at room temperature. Velocity versus [substrate] curves were determined for 3T3-L1 adipocytes expressed as specific glucose transport rates at 200 mM and from 0.5 to 50 mM for 3T3-L1 adipocytes. Initial rates were determined from time course experiments performed at each concentration of substrate. Assays were terminated by the addition of 1 ml of calcium- and magnesium-free phosphate-buffered saline containing 500 μM phloretin, followed by 4 washes with 1 ml of each of the wash solution at room temperature. Cells were solubilized with 0.4 ml of 0.1% SDS and [14C] was detected in 4 ml of scintillant. Non-specific 3-O-methylglucose influx was measured in the presence of 250 μM phloretin and subtracted from total influx to yield our specific influx values. The inhibitor and the substrate were added simultaneously and no time-dependent non-specific sugar influx was observed under these conditions. Km and Vmax parameters were determined by nonlinear regression analysis of the V versus [S] data, using the equation: y = (m1 + m0)/(m2 + m0), where m0 = [S], m1 = apparent Vmax, and m2 = apparent Km. This analysis assumes that transport exhibits simple hyperbolic kinetics. Standard errors of the mean were calculated from kinetic parameters determined for each experiment.

Equilibrium exchange experiments were performed exactly as described above except that cells were incubated for 30 min at 37°C in Krebs-Ringer phosphate buffer containing 10 mM unlabeled 3-O-methylglucose. Incubations were initiated by the assay with 3-O-methylglucose (2 μCi/ml final concentration). Rates of non-specific sugar exchange were measured for each experimental condition by assaying 3-O-methylglucose exchange under conditions identical to those utilized for measuring total sugar exchange rates, except that 250 μM phloretin was included in the assay buffer. The rate of non-specific sugar uptake was less than 10% of the basal uptake rate measured in the absence of inhibitor.

**Anti-glucose Transporter Antibody Binding to Intact 3T3-L1 Cells** — The measurement of β-antibody interaction to intact cells was described in detail previously (Harrison et al., 1990a). 3T3-L1 cells were plated in 100-mm culture plates (one plate/condition) in serum-free DMEM containing 10% fetal bovine serum and antibiotics as described above. Cells were washed 3 times with phosphate-buffered saline (Buffer A) and incubated in serum-free DMEM for 2 h at 37°C, followed by a 30-min incubation at 37°C in Krebs-Ringer phosphate buffer ± 100 nM insulin. Prior to incubation with β-antibody, the cells were rapidly cooled to ice temperature and all subsequent incubations and washes were performed at ice temperature. The cells were washed with Buffer A and incubated with either rabbit preimmune serum or β-antiserum (1/500 dilution) for 2 h. The cells were then washed 3 times with Buffer A and incubated for 1 h with [125I]Protein A (1/500 dilution of New England Nuclear low specific activity preparation). Cells were then washed twice with Buffer B. The bound [125I]-Protein A was solubilized in 0.1% SDS and the radioactivity determined in a γ counter. Non-specific binding (preimmune) was subtracted from total binding for each cell line. Each assay point was determined in triplicate.

**3T3-L1 Cell Membrane Preparations** — Cells were grown on 150-mm culture plates (one plate/condition) in 25 ml of DMEM containing 10% fetal bovine serum and antibiotics as indicated. Prior to homogenization, the plates were washed and serum starved as described above. Next, the cells were washed once with 10 ml of room temperature phosphate-buffered saline/EDTA/sucrose buffer (1 mM EDTA and 0.25 M sucrose). Total membranes and subcellular membranes from 3T3-L1 cells were prepared as described previously (Harrison et al., 1990b). Total cellular homogenates and each membrane fraction were assayed for activity of the plasma membrane marker enzyme, 5'-nucleotidase. 5'-Nucleotidase assays were performed as described in detail previously (Clancy and Czech, 1990).

**Glucose Transporter Protein Immunoblot Analysis** — 3T3-L1 cell membrane proteins were solubilized in sample buffer at room temperature for 30 min, resolved by SDS-PAGE using 10% polyacrylamide gels, and transferred to nitrocellulose. Protein immunoblot analyses were performed using rabbit anti-GLUT1 COOH-terminal peptide antisemur (R495, 1/1000 dilution) and rabbit anti-GLUT4 COOH-terminal peptide antisemur (R820, 1/500 dilution) and [125I]-Protein A (1/500 dilution) as described previously (Harrison et al., 1990b). [125I]-Protein A was detected by autoradiography and quantitated using an LKB ULTRASCAN XL Enhanced Laser Densitometer.

**Glucose Transporter Protein Photoaffinity Labeling with [125I] IAPS-forskolin** — 3T3-L1 cell membranes prepared as described above were labeled with [125I]IAPS-forskolin, essentially as reported by Wadzinski et al. (1988). The buffer used for the photolabeling experiments contained 50 mM Tris and 4 mM MgCl₂, pH 7.5. Membrane proteins (200 μg) were incubated with the photoaffinity labeled [125I] IAPS-forskolin, in the presence or absence of 500 μM cytochalasin B for 30 min at room temperature in the dark. The protein/ligand mixtures were then diluted to a final volume of 5 ml and photolyzed for 5 s under a high intensity UV lamp. 2-Mercaptoethanol was then added to the reaction mixture (1% final concentration), the membranes were pelleted by centrifugation at 200,000 × g for 1 h, and the membrane proteins were solubilized and resolved by SDS-PAGE as described above. Polycrylamide gels were then dried under vacuum and [125I]-Protein A was detected by autoradiography.

**RESULTS**

Ezaki (1989) reported that acute exposure of isolated rat adipocytes to millimolar concentrations of cadmium was associated with increased rates of sugar uptake and with insulin-like membrane redistributions of rat GLUT1 and GLUT4 proteins. Our recent work involving heterologous expression of human GLUT1 protein under the regulation of a metallothionine gene promoter (Harrison et al., 1990a and 1990b) caused us to examine the effects of exposure to low micromolar concentrations of cadmium sulfate on sugar uptake and mouse glucose transporter protein expression in 3T3-L1 cells. Since metals can be toxic to cells, we first examined the effects of cadmium on the viability of control 3T3-L1 cells. Micromolar concentrations of cadmium were toxic to subconfluent monolayers of 3T3-L1 fibroblasts and this toxicity was not observed when cadmium exposure was initiated 1–2 days after the fibroblasts had achieved a confluent monolayer. At this time, no cell division could be detected during microscopic examination of the fibroblast monolayers. Concentrations of cadmium up to 5 μM for the post-confluent fibroblasts and 10 μM for the adipocytes had no effect on cell numbers, non-specific glucose uptake, or on glucose transporter expression. Thus, the cells to exclude the membrane impermeant dye, trypan blue.

**Cadmium Activation of 2-Deoxyglucose Uptake by 3T3-L1 Fibroblasts and Adipocytes** — In order to examine the effects of chronic cadmium treatment on the rate of sugar uptake, 1 day post-confluent cluster plates of undifferentiated or confluent cluster plates of differentiated 3T3-L1 cells were exposed to 5 or 10 μM cadmium sulfate, respectively, for 0–24 h in culture and assayed for 2-deoxyglucose uptake over 5 min (Fig. 1). In cadmium sulfate-treated cells, the increased sugar transport rates were due to the action of increased cellular cadmium and not sulfate, since treatment of these cells with 25–50 μM zinc sulfate did not alter cellular sugar transport rates (Harrison et al., 1990b). Cadmium dramatically elevated sugar transport in both cell types, with similar time courses of activation. Exposure of the cells to these concentrations of cadmium for longer than 18 h, or exposure of the cells to higher concentrations of cadmium for 18 h resulted in decreasing the numbers of cells counted per well in some experiments (not shown). In order to minimize the variability introduced by cadmium toxicity, all subsequent experiments involved 18-h exposures of the post-confluent fibroblasts or the confluent adipocytes to 5 or 10 μM cadmium, respectively. Under these conditions, cadmium treatment had no effect on fibroblast or adipocyte cell viability.

The preliminary finding that cadmium treatment resulted in large increases in fibroblast sugar uptake rates led us to quantitate more fully the effects of this treatment on hexose...
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FIG. 1. Time course for cadmium activation of 2-deoxyglucose uptake in 3T3-L1 fibroblasts and adipocytes. Cells were grown to confluence and used in the fibroblast studies (24 well plates) or differentiated as described under “Experimental Procedures” for use in the adipocyte studies (12-well plates). One day post-confluent multwell culture plates of fibroblasts or differentiated adipocytes were incubated for various times in growth medium containing 5 and 10 μM cadmium sulfate, respectively. Cells were then incubated in serum-free medium for 2 h, washed with Krebs-Ringer phosphate buffer, and incubated for 20 min in Krebs-Ringer phosphate buffer at 37 °C. 3T3-L1 cells were assayed for 2-deoxyglucose uptake as described in detail under “Experimental Procedures.” Assays were performed in quadruplicate for each experimental condition. Closed and open circles indicate fibroblasts and adipocytes, respectively.

FIG. 2. Cadmium and insulin stimulation of 2-deoxyglucose uptake and δ-antibody binding by 3T3-L1 fibroblasts. One day post-confluent 24-well plates of cells were incubated for 18 h in growth medium containing 0 or 5 μM cadmium sulfate. Cells were then incubated in serum-free medium for 2 h, washed with Krebs-Ringer phosphate buffer, and incubated for 20 min in Krebs-Ringer phosphate buffer plus or minus 100 nM insulin at 37 °C. A, 3T3-L1 fibroblasts were assayed for 2-deoxyglucose uptake at 100 μM substrate as described in detail under “Experimental Procedures.” 2-Deoxyglucose uptake results are averages ± standard error of the mean of 10 experiments. Assays were performed in quadruplicate for each experimental condition. B, the data are also replotted as percent increases in 2-deoxyglucose uptake due to insulin action, over uptake measured in control cells under the same ± cadmium conditions. C, δ-antibody binding to GLUT1 on the extracellular surface of intact cells was performed as described under “Experimental Procedures.” Specific uptake was determined by subtracting appropriate nonspecific control values, as described under “Experimental Procedures.” Open and shaded bars indicate deoxyglucose uptake rates for untreated and 5 μM cadmium-treated fibroblasts, respectively.

uptake and to examine the possible interactions between cadmium and insulin stimulation of deoxyglucose uptake rates. Confluent plates of undifferentiated 3T3-L1 cells were exposed to growth medium ± 5 μM cadmium for 18 h in culture followed by acute treatment with or without 100 nM insulin in order to examine the relative effects of cadmium, insulin, or cadmium plus insulin treatment on mouse fibroblast transporter function (Fig. 2). Cadmium activated basal sugar transport 5-fold, relative to untreated controls under these experimental conditions. Insulin acutely stimulated sugar uptake 2-fold in fibroblasts that were not exposed to cadmium. Interestingly, the elevated rate of sugar uptake in the cadmium-treated fibroblasts was completely insensitive to insulin action.

Previous studies demonstrated that insulin stimulated deoxyglucose uptake in Chinese hamster ovary fibroblasts with no corresponding increase in GLUT1 protein at the cell surface. These studies were performed using an anti-GLUT1 antibody, δ, that binds to an extracellular domain on that transporter (Harrison et al., 1990a). This antibody bound quantitatively to intact human and rat erythrocytes, to intact Chinese hamster ovary fibroblasts expressing human GLUT1, and to right-side-out erythrocyte ghosts, but not to inside-out erythrocyte ghosts. Therefore, the effects of cadmium and insulin on the level of cell surface GLUT1 protein in intact 3T3-L1 cells were also examined by δ-antibody-binding assays. Confluent plates of undifferentiated 3T3-L1 cells were exposed to 5 μM cadmium for 18 h or to insulin for 20 min and assayed for binding of the δ-antibodies as described under “Experimental Procedures.” Unlike the dramatic increase in the rate of 2-deoxyglucose uptake, no increase in cell surface GLUT1 was observed in cadmium-treated (Fig. 2) or in insulin-treated (data not shown, 1.03 ± 0.05, n = 5 experiments) fibroblasts.

Quantitative analyses of cadmium- and insulin-stimulated 2-deoxyglucose uptake rates were also performed using differentiated 3T3-L1 cells. Confluent plates of differentiated 3T3-L1 cells were exposed to growth medium ± 10 μM cadmium for 18 h in culture followed by acute treatment with or without insulin. In order to examine the effects of cadmium on mouse adipocyte glucose transporter function (Fig. 3), Cadmium-treatment of 3T3-L1 adipocytes activated basal sugar uptake 5-fold, relative to untreated controls, under these experimental conditions. In the differentiated 3T3-L1 cells, 20 min treatment with insulin stimulated 2-deoxyglucose uptake 15-fold in adipocytes that were not exposed to cadmium. However, in contrast to what was observed in the fibroblasts, the absolute increment of adipocyte sugar uptake due to insulin in the cadmium-treated adipocytes was the same as in control cells. The facts of the two agents were completely additive in these cells, such that the combined effects of cadmium and insulin on glucose uptake resulted in a 21-fold stimulation over untreated controls (Fig. 3).

Cadmium and Insulin Action on 3-O-Methylglucose Transport Rates in 3T3-L1 Fibroblasts and Adipocytes—Control and cadmium-treated 3T3-L1 fibroblasts were also assayed for 3-O-methyl[1-14C]glucose transport under 0-trans influx conditions, in order to determine whether the effects of cadmium on 2-deoxyglucose uptake represented increased sugar transport rates rather than increased rates of sugar metabolism.

FIG. 3. Cadmium and insulin stimulation of 2-deoxyglucose uptake by 3T3-L1 adipocytes. Cells were grown to confluence and differentiated in 12-well cluster plates as described under “Experimental Procedures” and then incubated for 18 h in growth medium containing 0 or 10 μM cadmium as indicated. Cells were then incubated in serum-free medium for 2 h, washed with Krebs-Ringer phosphate buffer, and incubated for 20 min in Krebs-Ringer phosphate buffer plus or minus 100 nM insulin at 37 °C. 3T3-L1 adipocytes were assayed for 2-deoxyglucose uptake at 100 μM substrate as described in detail under “Experimental Procedures.” 2-Deoxyglucose uptake results are averages ± standard error of the mean of 5 experiments. Assays were performed in quadruplicate for each experimental condition. Specific uptake was determined by subtracting appropriate nonspecific control values, as described under “Experimental Procedures.” Open and shaded bars indicate deoxyglucose uptake rates for untreated and 10 μM cadmium-treated adipocytes, respectively.
Data presented in Fig. 4A demonstrate that the 6-fold activation of basal fibroblast 2-deoxyglucose uptake by cadmium (Fig. 2) is entirely accounted for by the 6-fold increase in the rate of uptake of the non-metabolized glucose analog by 3T3-L1 fibroblasts. Additionally, the 2-fold stimulation by insulin of 3-O-methylglucose transport by 3T3-L1 fibroblasts is identical to the increase observed due to the hormone on 2-deoxyglucose uptake. Similarly, prior exposure to cadmium rendered these fibroblasts insensitive to the acute stimulatory effects of insulin on 3-O-methylglucose transport (Fig. 4A).

When 3-O-methylglucose rates were examined in control and cadmium-treated 3T3-L1 adipocytes (Fig. 4B), the results obtained also confirmed the results obtained with 2-deoxyglucose (Fig. 3). The 12-fold insulin stimulation of the rate of 3-O-methylglucose transport by 3T3-L1 adipocytes is similar to that observed for 2-deoxyglucose uptake. Cadmium activation of 3-O-methylglucose transport into the mouse adipocytes was 11-fold and the combined effects of cadmium and insulin on glucose transport yielded a 21-fold increase in the rate of 3-O-methylglucose transport relative to untreated controls (Fig. 4B).

Previous investigations have demonstrated differences in hexose transport kinetics measured under 0-trans influx and equilibrium exchange conditions in human red cells and in various muscle preparations (for review, see Carruthers, 1990). Therefore, we examined the effects of insulin and cadmium treatments on 3T3-L1 fibroblast and adipocyte 3-O-methylglucose equilibrium exchange rates. Data obtained from 3T3-L1 cell 3-O-methylglucose equilibrium exchange studies (Fig. 5) performed at the relatively high sugar concentration of 10 mM, also demonstrate the stimulatory effects of both insulin and cadmium. Furthermore, these data confirm that the effects of insulin and cadmium on the cultured adipocytes are additive. The data show that the effects of these two agents on adipocyte glucose transporters can be measured under conditions ranging from 50 μM 0-trans sugar influx to 10 mM sugar equilibrium exchange. Thus, hexose influx rates obtained using either glucose analog and 3-O-methylglucose equilibrium exchange rates all indicate that cadmium activation of basal sugar transport and insulin stimulation of sugar transport are completely additive in 3T3-L1 adipocytes.

Effects of Cadmium on Total Cellular GLUT1 and GLUT4 Protein Levels—Total cellular membrane proteins harvested from 3T3-L1 fibroblasts and adipocytes were analyzed by immunoblot analysis using anti-GLUT1 and GLUT4 COOH-terminal peptide antibodies, in order to determine whether the effects of cadmium on glucose transport might be related to increases in immunoreactive glucose transporter protein levels (Fig. 6). Exposure of 3T3-L1 fibroblasts to 5 μM cadmium resulted in a small 1.6-fold (average of 2 experiments) increase in total cellular GLUT1 protein levels. No GLUT4 protein was detected in these fibroblast membranes. Exposure of 3T3-L1 adipocytes to 10 μM cadmium also resulted in a 1.6-fold (average of 2 experiments) increase in cellular GLUT1 protein levels. 10 μM cadmium treatment did not result in any increase in total cellular immunoreactive GLUT4 protein levels in these adipocytes (0.98-fold, average of 3 experiments).

Although little or no change in the amounts of cellular GLUT1 and GLUT4 due to cadmium treatment were observed in comparison with the large effects on hexose transport, it was possible that the expression of another glucose transporter isoform might be increased in these cells. We observed in preliminary experiments that greater than 85% of the cytochalasin B-sensitive deoxyglucose uptake in 3T3-L1 adi-
Then incubated in serum-free medium for 2 h at 37 °C with Krebs-Ringer phosphate buffer, prior to preparation of the membranes. Total 3T3-L1 cell membranes were prepared as described under "Experimental Procedures." 50 μg of membrane proteins were solubilized in sample buffer at room temperature for 30 min, resolved by SDS-PAGE on duplicate 20% acrylamide gels, and transferred electrophoretically to nitrocellulose at 200 mA for 3 h. Nitrocellulose filters were incubated with the indicated antibody reagents, then incubated with 125I-Protein A and the immunoreactive proteins were visualized by autoradiography. Mouse GLUT1 protein was detected using anti-GLUT1 COOH-terminal peptide antiserum (1/1000 dilution). Mouse GLUT4 protein was detected using anti-GLUT4 COOH-terminal peptide antiserum (1/500 dilution). Mouse GLUT1 co-migrated with human erythrocyte GLUT1 (M, = 55,000) and mouse GLUT4 protein exhibited a slightly lower electrophoretic mobility than the GLUT1 protein.

**FIG. 6. Effects of cadmium on GLUT1 and GLUT4 glucose transporter protein levels in 3T3-L1 cells.** Confluent 150-mm plates of 3T3-L1 cells were incubated for 18 h in growth medium containing the indicated concentration of cadmium sulfate. Cells were then incubated in serum-free medium for 2 h at 37 °C and washed with Krebs-Ringer phosphate buffer, prior to preparation of the membranes. Total 3T3-L1 cell membranes were prepared as described under "Experimental Procedures." 50 μg of membrane proteins were solubilized in sample buffer at room temperature for 30 min, resolved by SDS-PAGE on duplicate 20% acrylamide gels, and transferred electrophoretically to nitrocellulose at 200 mA for 3 h. Nitrocellulose filters were incubated with the indicated antibody reagents, then incubated with 125I-Protein A and the immunoreactive proteins were visualized by autoradiography. Mouse GLUT1 protein was detected using anti-GLUT1 COOH-terminal peptide antiserum (1/1000 dilution). Mouse GLUT4 protein was detected using anti-GLUT4 COOH-terminal peptide antiserum (1/500 dilution). Mouse GLUT1 co-migrated with human erythrocyte GLUT1 (M, = 55,000) and mouse GLUT4 protein exhibited a slightly lower electrophoretic mobility than the GLUT1 protein.

**FIG. 7. Effects of cadmium on photoaffinity labeled glucose transporter protein levels in 3T3-L1 cell membranes.** Confluent 150-mm plates of 3T3-L1 cells were incubated for 18 h in growth medium containing the indicated concentration of cadmium sulfate. Cells were then incubated in serum-free medium for 2 h at 37 °C and washed with Krebs-Ringer phosphate buffer, prior to preparation of the membranes. Total 3T3-L1 cell membranes were prepared as described under "Experimental Procedures." 200 μg of total cellular membrane proteins were incubated with the photoaffinity label [35S]IAPS-forskolin, in the presence or absence of 200 μM cytochalsin B for 30 min at room temperature, in the dark. The protein/ligand mixtures were then diluted and photolyzed for 5 s under a high intensity UV lamp. The membranes were pelleted by centrifugation, and membrane proteins were solubilized and resolved by SDS-PAGE as described in the legend to Fig. 4. The polycrylamide gel was then dried under vacuum and [35S]I was detected by autoradiography. Results from a representative experiment are presented and the labeled glucose transporter protein (M, = 55,000) is indicated.

Fibroblasts were also inhibited by forskolin (not illustrated). Therefore, total glucose transporter protein levels in cadmium-treated 3T3-L1 fibroblasts and adipocytes were measured by photoaffinity labeling with [35S]IAPS-forskolin (Fig. 7). A 1.7-fold increase in total transporter protein detected in membranes harvested from cadmium-treated fibroblasts was observed using this method, consistent with the increased GLUT1 protein level detected by protein immunoblot analysis (Fig. 6). These data are representative of 5 experiments. Only a small 1.3-fold increase in total glucose transporter protein levels was detected in membranes from cadmium-treated adipocytes in spite of the large increase in 3-O-methylglucose transport rates exhibited by such cells (Fig. 4B). Although some variability was observed between preparations of adipocyte membrane proteins, in 5 experiments performed, the levels of total [35S]IAPS-forskolin-labeled glucose transporter protein essentially remained unchanged with individual experiments showing slight increases or even decreases following cadmium treatment of the adipocytes.

**Effects of Cadmium on Membrane Distributions of GLUT1 and GLUT4 Proteins in 3T3-L1 Adipocytes**—One potential explanation for the dramatic effect of cadmium on adipocyte 3-O-methylglucose transport rate is that like insulin, cadmium causes a redistribution of glucose transporters from an intracellular pool to the plasma membrane. To test this hypothesis, 3T3-L1 adipocytes were exposed to 10 μM cadmium for 18 h prior to fractionation of the cells and analysis of plasma membrane GLUT4 and GLUT1 transporter proteins. Protein immunoblot analysis of plasma membranes harvested from adipocytes treated with or without cadmium revealed no change in plasma membrane levels of GLUT4 protein due to the metal (Fig. 8A, lanes 1 and 3). However, cadmium treatment did cause a small and variable increase (average of 50%) in plasma membrane GLUT1 protein levels (Fig. 8B, lanes 1 and 3). These increases are similar to those observed for total cellular levels of GLUT1 protein (Fig. 6) and thus appear to be due to increased steady-state GLUT1 protein levels. The increase in transporter protein is small compared to the increase in sugar transport rates measured in cadmium-treated adipocytes (Figs. 3 and 4B).

The additive effects of insulin and cadmium on sugar transport in adipocytes (Figs. 3, 4B, and 5) indicated that cadmium treatment might not interfere with insulin action on glucose transporter membrane distributions. Therefore, we examined the ability of insulin to modulate movement of the glucose transporter proteins to the plasma membrane following treatment of the 3T3-L1 adipocytes with or without cadmium for 18 h. Several pieces of experimental evidence suggest that cadmium treatment of the adipocytes.
treatment of the cells with cadmium does not alter our ability to separate 3T3-L1 adipocyte membranes into plasma membrane and low density microsomal membrane fractions. 1) Plasma membrane fractions harvested from control 3T3-L1 adipocytes were 5-8-fold enriched in 5'-nucleotidase activity, relative to total cellular homogenates. The plasma membrane enrichment in this marker enzyme activity in the plasma membrane fraction is consistent with values published previously for these cultured cells (Clancy and Czech, 1990). An 18-h treatment of 3T3-L1 adipocytes with 10 μM cadmium did not result in any changes in the enrichment of 5'-nucleotidase activity, relative to untreated adipocytes. 2) Treatment of the 3T3-L1 cells with cadmium did not result in any differences in the amounts of protein harvested in total membrane, plasma membrane, or low density microsomal membrane fractions, relative to untreated control adipocytes. 3) As shown in Fig. 8A, no differences in the plasma membrane distributions of mouse GLUT4 were observed to result from treatment of the cells with cadmium.

Preliminary analysis was performed using δ-antibody binding to GLUT1 protein on the surface of intact cells. Insulin-treated 3T3-L1 adipocytes exhibited a small elevation in the level of δ-antibody binding, 1.2 ± 0.05-fold (n = 4 experiments) relative to control cells, whereas cadmium-treated adipocytes exhibited control levels of δ-antibody binding. Deoxyglucose accumulation assays and glucose transporter protein immunoblot analysis of plasma membrane fractions were performed on 3T3-L1 adipocytes exposed to conditions exactly like those assayed by δ-antibody binding (i.e two 1–2-h incubations and numerous washes at 4 °C) and no differences in insulin-stimulated sugar uptake or plasma membrane recruitment were observed to result from these conditions. Thus, our intact cell δ-antibody assay conditions were not insulinomimetic by either of those two criteria.

Protein immunoblot analysis of plasma membranes harvested from adipocytes treated with insulin revealed insulin-stimulated increases of both GLUT4 and GLUT1 proteins in this fraction (Fig. 8, A and B, respectively, lanes 2 and 4) with or without prior exposure to cadmium. The average increases were approximately 1.5-fold for GLUT1 versus 3-fold for GLUT4 in 10 experiments using this methodology. The 1.5-fold increase in plasma membrane GLUT1 levels is in relatively good agreement with the very small increase in δ-antibody binding to insulin-treated adipocytes. Cadmium did not alter the action of insulin to increase plasma membrane levels of those proteins. Thus, consistent with the additive effects of cadmium and insulin on sugar transport rates, the two agents act independently on glucose transporters in intracellular membrane pools in that only insulin stimulates transporter recruitment and cadmium treatment does not interfere with that action of insulin. Therefore, neither increased total glucose transporter protein levels nor increased plasma membrane glucose transporter protein levels can account for the increased transport rates observed in cadmium-treated 3T3-L1 cells.

Additional experiments were performed in order to determine the sensitivity of the δ-antibody-binding assay for measuring cell surface GLUT1 proteins in 3T3-L1 cells. In these studies, δ-antibody binding to intact 3T3-L1 cells was used to measure cell surface 3T3-L1 levels, and these data were compared with GLUT1 levels in plasma membranes and in total cellular membranes (determined by immunoblot analysis) under a variety of conditions (Fig. 9). The resulting data were normalized to the control 3T3-L1 adipocyte values. 3T3-L1 cell types examined included parental 3T3-L1 fibroblasts (L1 fibro), parental 3T3-L1 adipocytes (L1 adip control), pLENGT 10-45 fibroblasts (10-45 fibro), and pLENGT 10-45 adipocytes (10-45 adip). pLENGT transfected fibroblasts and adipocytes express human GLUT1 protein under the influence of a metal-inducible metallothionen gene promoter as described in detail previously (Harrison et al., 1990b). Both the intact cell δ-antibody binding data and the total cellular membrane GLUT1 immunoblot data demonstrate that 3T3-L1 fibroblasts express GLUT1 proteins at approximately 50% of the adipocyte level (Fig. 9). Additionally, untransfected 3T3-L1 adipocytes treated with 1 mM dibutyryl-cAMP for 18 h at 37 °C exhibit an approximately 2-fold increase in isolated plasma membrane and total cellular membrane GLUT1 levels, relative to untreated control adipocytes. Both assays also demonstrate that constitutive levels of combined mouse and human GLUT1 expressed in the 10-45 fibroblasts and adipocytes are approximately 2-fold greater than GLUT1 levels measured in untransfected control fibroblasts and adipocytes, respectively. Similarly, 10-45 adipocytes exposed for 18 h to 10 μM cadmium or 125 μM zinc exhibit approximately 2.7- and 3.4-fold increases, respectively, in δ-antibody binding to intact cells as well as in isolated plasma membrane and total cellular membrane GLUT1 protein, relative to the untreated parental adipocytes. Thus the δ-antibody-binding assay is sensitive to changes in 3T3-L1 cell surface GLUT1 protein levels, as also reflected in isolated plasma membrane immunoreactive GLUT1 levels, over a 7-fold range. Importantly, these data also indicate that under a variety of experimental conditions, increases in total cellular

![Fig. 9. Calibration of the δ-antibody assay in mouse 3T3-L1 cells. Cell surface GLUT1 was assessed by δ-antibody binding to intact cells or by protein immunoblot analysis of 3T3-L1 cell plasma membranes using anti-GLUT1 COOH-terminal peptide antibody. Additionally, total cellular membrane were also analyzed for GLUT1 protein levels by immunoblot analysis. 3T3-L1 cell types analyzed included fibroblasts (L1 fibro), adipocytes (L1 adip), and pLENGT transfected fibroblasts and adipocytes (10-45 fibro and 10-45 adip, respectively), expressing human GLUT1 under the influence of a metal-inducible promoter (Harrison et al., 1990b). Several additional conditions were used to alter cell surface GLUT1 protein levels in these cultured adipocytes. Cells were incubated with 1 mM dibutyryl-cAMP (+dcAMP) for 18 h as described in detail previously (Clancy and Czech, 1990), with 10 μM cadmium (+cadmium) or with 125 μM zinc (+zinc) for 18 h as described in detail previously (Harrison et al., 1990b). Cellular membrane preparations and immunobasws were performed as described under "Experimental Procedures" and the resulting data were normalized to control 3T3-L1 adipocyte values. These comparative data were plotted to assess the ability of the δ-antibody reagent to report changes in cell surface GLUT1 protein levels. The δ-antibody binding data were plotted against either the plasma membrane GLUT1 protein immunoblot data (X, dashed line) or the total cellular membrane GLUT1 protein immunoblot data, (C, solid line). Linear regression analysis of the data determined slopes of 0.1 for both data sets with correlation coefficients of 0.974 and 0.976, respectively, for the plasma membrane and total cellular membrane data.](image-url)
GLUT1 protein levels result in proportional increases in cell surface/plasma membrane GLUT1 levels in 3T3-L1 adipocytes.

**Effects of Cadmium and Insulin on 0-Trans Sugar Influx Kinetic Parameters** — The large increases in sugar transport rates without corresponding increases in total cellular or plasma membrane glucose transporter protein levels suggest the hypothesis that chronic exposure of 3T3-L1 fibroblasts and adipocytes to low micromolar concentrations of cadmium are associated with altered glucose transporter intrinsic activity. In order to test this hypothesis, 0-trans 3-O-methylglucose transport velocity (V) versus substrate concentration ([substrate]) relationships were determined for control and cadmium-treated 3T3-L1 cells under 0-trans influx conditions (Fig. 10). 3-O-Methylglucose transport rates were determined as described under "Experimental Procedures" and initial transport rates were determined by performing transport assays over a time course of 0–300 s for each concentration of substrate. Nonlinear regression analysis of the V versus [substrate] data was used for curve fitting and for calculating the apparent Kₐ and Vₐₐ values.

![Figure 10](attachment:figure10.png)

**Fig. 10. Effects of cadmium on 0-trans 3-O-methylglucose transport kinetic parameters in 3T3-L1 fibroblasts and adipocytes.** Cells were grown to confluence and used for fibroblast studies or differentiated for adipocyte studies, and then prepared for assays of hexose transport as described under "Experimental Procedures." Cells were incubated in serum-free medium for 2 h, washed with Krebs-Ringer phosphate buffer, and incubated for 20 min in Krebs-Ringer phosphate buffer plus or minus 100 nM insulin at 37 °C. Cells were then allowed to stand for 10 min at room temperature and 3-O-methylglucose transport was initiated by addition of the indicated concentrations of substrate. Initial rates were determined by measuring sugar transport from 0 to 300 s at each of the indicated substrate concentrations. Assays were performed in duplicate for each time point. Specific uptake was determined by subtracting appropriate nonspecific control values, as described under "Experimental Procedures." Data from a representative experiment for each cell type are plotted as velocity versus [substrate]. Kinetic parameters determined by nonlinear regression analysis of these V versus [substrate] plots indicate: A, fibroblast apparent Kₐ values of 23.1 and 7.5 mM, and apparent Vₐₐ values of 2.8 × 10⁻⁴ and 5.5 × 10⁻⁴ mmol/s 10⁶ cells for untreated and cadmium-treated fibroblasts, respectively. B, adipocyte apparent Kₐ values of 37.0, 11.6, and 11.6 mM, and apparent Vₐₐ values of 1.7 × 10⁻⁴, 2.0 × 10⁻⁴, and 2.9 × 10⁻⁴ mmol/s 10⁶ cells for untreated, cadmium-treated, and insulin-treated adipocytes, respectively.

3T3-L1 fibroblasts were exposed to growth medium ± 5 μM cadmium for 18 h, serum starved for 2 h, and then assayed for 0-trans sugar transport at concentrations of 3-O-methylglucose ranging from 0.5 to 20 mM. Fig. 10A contains data from a representative plot of the relationship between sugar concentration and transport velocity measured in 3T3-L1 fibroblasts. Average fibroblast kinetic parameters determined from several experiments are presented in Table I. Analysis of these data indicate that cadmium treatment results in an approximately 3-fold decrease in the apparent Kₐ for influx. Apparent Vₐₐ values derived from the data indicate that cadmium treatment increases the Vₐₐ for sugar influx 2–3-fold. Student’s t tests performed on the data obtained from two paired experiments indicate that the change in apparent Kₐ in cadmium-treated cells is statistically significant at p ≤ 0.02. However, the cadmium-stimulated increase in the apparent Vₐₐ for sugar influx which was observed in both experiments is only significant at p ≤ 0.2, due to variability of the sugar influx rates at higher substrate concentrations and to the limited number of experiments performed.

3T3-L1 adipocytes were exposed to growth medium ± 10 μM cadmium for 18 h, serum starved for 2 h prior to a 20-min incubation ± insulin, and assayed for 0-trans sugar influx at concentrations of 3-O-methylglucose ranging from 0.5 to 50 mM. Fig. 10B contains data from a representative plot of the relationship between sugar concentration and transport velocity measured in those cells. Average adipocyte kinetic parameters determined from several experiments are presented in Table I. Analysis of these data indicate that treatment of 3T3-L1 adipocytes with either insulin or cadmium results in a 2-fold decrease in the apparent Kₐ 3-O-methylglucose influx. Apparent Vₐₐ values derived from the data indicate that 18-h cadmium treatment did not alter the Vₐₐ for sugar influx, while acute exposure to insulin increased the adipocyte Vₐₐ approximately 3-fold.

**DISCUSSION**

This paper reports our findings that chronic exposure to low micromolar concentrations of cadmium and acute exposure to insulin are associated with increases in GLUT1 transporter intrinsic activity in 3T3-L1 fibroblasts. The 6-fold stimulation of fibroblast hexose transport rates by cadmium and the 2-fold increase due to insulin action (Figs. 2 and 4A) were not associated with increased cell surface levels of δ-antibody reactive GLUT1 protein (Fig. 2). Careful examination of the sensitivity of the δ-antibody-binding assay revealed that this assay reliably measures 2–7-fold changes in cell surface GLUT1 in 3T3-L1 cells (Fig. 9). Therefore, it is clear that in these cells the cadmium-stimulated 6-fold increases in sugar transport are not due to corresponding increases in cell surface GLUT1 protein levels. Since the δ-antibody binding was not elevated in the cadmium-treated fibroblasts (Fig. 2) exhibiting a 60% increase in total cellular GLUT1 (Fig. 6), it is possible that the assay is not sufficiently sensitive to measure that small and variable change in GLUT1 protein levels. Similarly, the lack of detectable changes in δ-antibody binding in insulin-treated fibroblasts exhibiting a 1.9-fold stimulation of sugar transport by insulin, must be interpreted with caution since 2-fold changes may represent the limit of sensitivity for this assay. However, our δ-antibody binding data are consistent with electron microscopy data reported previously by Blok and co-workers (1988), indicating that insulin does not stimulate GLUT1 recruitment in 3T3-L1 fibroblasts.

We also examined the effects of cadmium on 0-trans hexose influx kinetic parameters. Analysis of fibroblast 0-trans 3-O-
Summary of 3T3-L1 cell methylglucose transport kinetic parameters

Velocity versus [substrate] curves were determined for 0-trans 3-O-methylglucose influx using undifferentiated or differentiated mouse 3T3-L1 cells, as described under "Experimental Procedures." $K_{m}$ and $V_{max}$ parameters were determined by nonlinear regression analysis of the $V$ versus [S] data, using the equation: $y = (m1 \cdot m0)/(m2 + m0)$, where $m0 = [S]$, $m1 = \text{apparent } V_{max}$, and $m2 = \text{the apparent } K_{m}$. This analysis assumes that transport exhibits simple hyperbolic kinetics. Standard errors of the mean were calculated from kinetic parameters determined for each experiment.

| Cell type        | Treatment Description | Apparent $K_{m}$ | Apparent $V_{max}$ |
|------------------|-----------------------|------------------|--------------------|
| 3T3-L1 fibroblasts* | Control (n = 3)       | $28.4 \pm 2.1$   | $1.8 \pm 0.6$      |
|                  | 100 $\mu$M cadmium    | $8.6 \pm 0.4$    | $5.1 \pm 0.7$      |
| 3T3-L1 adipocytes* | Control (n = 8)$^c$   | $23.3 \pm 3.3^c$ | $1.6 \pm 0.3^c$    |
|                  | 10 $\mu$M cadmium (n = 5) | $10.8 \pm 2.3$  | $1.8 \pm 0.1$      |
|                  | 100 $\mu$M insulin (n = 9)$^d$ | $10.9 \pm 1.2^d$ | $4.6 \pm 0.8^d$    |

* Velocity versus [substrate] curves were determined in 3T3-L1 fibroblasts using a range of substrate concentrations from 0.5 to 20 mM.

* Velocity versus [substrate] curves were determined in 3T3-L1 adipocytes using a range of substrate concentrations from 0.5 to 50 mM.

$^c$ These data are compiled from the results of four experiments performed during the course of these studies and from the results of four experiments performed during the course of the studies presented recently by Clancy et al. (1991).

$^d$ These data are compiled from the results of five experiments performed during the course of these studies and from the results of four experiments performed recently by Clancy et al. (1991).

Regulation of Glucose Transporter Intrinsic Activity

methylglucose influx data indicate that cadmium alters both the affinity and the maximal velocity of sugar influx, relative to untreated control cells (Fig. 10A, Table I). Analysis of experiments performed using 40–80 $\mu$M concentrations of substrate yield similar estimations of the apparent $K_{m}$ values for sugar influx in control and cadmium-treated fibroblasts to those presented in Table I. However, for reasons that we do not presently understand, sugar influx rates measured at 40–80 mM 3-O-methylglucose and the resulting $V_{max}$ extrapolations were highly variable. For these reasons, kinetic parameters determined for the fibroblasts represent estimates of the actual $K_{m}$ and $V_{max}$ values based on initial rates measured from 0.5 to 20 mM substrate. Thus, basal fibroblast maximal transport velocity is determined using concentrations of substrate to or below the apparent $K_{m}$ value and the $V_{max}$ value calculated for basal fibroblasts is likely to represent an underestimation of the actual value. Therefore, the calculated fold increase in $V_{max}$ due to cadmium may overestimate that effect. These calculations suggest that the alterations in GLUT1 function elicited by treating cells with cadmium are complex and may cause changes in this transporter’s affinity for hexose sugars as well as an increased catalytic rate.

Similarly, chronic exposure of 3T3-L1 adipocytes to low micromolar concentrations of cadmium are associated with changes in glucose transporter intrinsic activity. Cadmium increases adipocyte 2-deoxyglucose accumulation rates 5-fold when transport is measured at 100 mM substrate (Fig. 3), with little or no increase in plasma membrane levels (Fig. 8) or total cellular levels (Figs. 6 and 7) of either transporter type. Control 3T3-L1 adipocyte 3-O-methylglucose influx rates measured above 50 mM substrate were variable and, therefore, kinetic parameters determined for these adipocytes represent estimates of the actual $K_{m}$ and $V_{max}$ values based on initial rates measured from 0.5 to 50 mM substrate. The apparent $K_{m}$ for substrate decreased 2-fold and the calculated maximal catalytic influx rate was unchanged in cadmium-treated adipocytes, relative to untreated controls (Fig. 10B, Table I). The interpretation of these data are complicated by the presence of two transporter isoforms in adipocytes, GLUT1 and GLUT4. However, it is clear that in 3T3-L1 adipocytes, like 3T3-L1 fibroblasts, cadmium stimulates sugar transport by altering glucose transport intrinsic activity, without recruiting either GLUT1 or GLUT4 transporters to the cell surface.

$V_{max}$ values determined under these experimental conditions are based on computer extrapolations of a data set that only extends to a 50 mM concentration of substrate or ± 2 times the estimated basal adipocyte $K_{m}$ values, and therefore must be interpreted with caution. However, these data clearly demonstrate that a change in $K_{m}$ is associated with insulin and cadmium treatments and that these two treatments result in additive increases in sugar transport rates. Therefore, it is possible that the estimated differences in $V_{max}$ effects account for the observed additivity between cadmium- and insulin-stimulated sugar transport rates in 3T3-L1 adipocytes. Alternatively, the two treatments lower cellular GLUT1 and GLUT4 $K_{m}$ values completely independently and additively and these $K_{m}$ effects account for the increased sugar transport rates measured under our experimental conditions.

At present the exact extent of insulin-stimulated recruitment of GLUT1 protein in mouse 3T3-L1 cells is not certain. Two recently developed cell surface glucose transporter labeling techniques have indicated that 3T3-L1 adipocyte cell surface GLUT1 levels increase 1.6 and 2.6-fold (carbohydrate labeling by Gould et al., and Calderhead and Lienhard, 1988; respectively), 3–3.5-fold (ATB-BMPA labeling by Kozka et al., 1991), and 6.5-fold (ATB-BMPA labeling by Calderhead et al., 1990). It should be noted that all of these data are dependent upon immunoprecipitation of the GLUT1 protein following the convolent labeling and that in every case the GLUT1 proteins were not quantitatively immunoadsorbed. While the GLUT1 proteins recognized by the anti-GLUT1 COOH-terminal peptide antibodies used in these experiments may be representative of all cell surface GLUT1 proteins, it is not yet clear why the antibodies do not precipitate GLUT1 quantitatively. Additionally, with respect to the ATB-BMPA labeling experiments, two other factors require that the observed fold increases reported be interpreted with caution. First, the basal adipocyte labeling of GLUT1 and GLUT4 proteins is very low, resulting in incorporation of very few tritium counts/min into those proteins, and thus, small vari-

S. A. Harrison, B. M. Clancy, A. Pesinio, and M. P. Czech, manuscript in preparation.
Regulation of Glucose Transporter Intrinsic Activity

The regulation of cellular GLUT1 protein levels, and thus, of cellular hexose transport rates by altering GLUT1 gene expression is well documented in 3T3-L1 cells (Torjman et al., 1989; Garcia de Herreros and Birnbaum, 1989; Clancy and Czech, 1990; Harrison et al., 1990b; Kaestner et al., 1991) and in other cultured cell types including various rodent fibroblast cell lines (Birnbaum et al., 1987; Flier et al., 1987; Hiraki et al., 1988), rat L6 skeletal muscle cells (Walker et al., 1989; Koivisto et al., 1991), isolated rat hepatocytes (Rhoads et al., 1988), and isolated rat brain glial and neuronal cells (Walker et al., 1988; Mudd et al., 1990). This type of regulation is distinct from the acute regulation of cell surface GLUT4 and GLUT1 levels by hormonally stimulated recruitment of intracellular transporters to the plasma membrane, without corresponding changes in total cellular transporter numbers.

The small and variable increase in both total cellular and plasma membrane fraction GLUT1 protein levels in cadmium-treated cells are not sufficient to account for the stimulatory effect of cadmium on sugar transport rates. However, this cadmium-induced increase in GLUT1 protein levels is similar in kind to the regulation of cellular GLUT1 observed previously in these 3T3-L1 cells and in many other cultured cell types in response to chronic cellular stress or to transformation.

Insulin stimulation of 3T3-L1 adipocyte hexose influx is associated with both a decreased apparent Km for 3-O-methylglucose and with an increased maximal transport velocity for that substrate (Fig. 10B, Table I). These results are similar to those described recently for isolated rat adipocytes (Suzuki, 1988; Whitesell et al., 1989), even though their kinetic parameters were determined from equilibrium exchange data. Suzuki (1988) found that insulin stimulation is associated with a 2-fold decrease in the apparent Km for 3-O-methylglucose and an 8.5-fold increase in the maximal transport rate for that glucose analog. These findings are consistent with either insulin-stimulated recruitment of a transporter that has a lower Km and a higher catalytic rate than basal adipocyte transporters, or with recruitment and activation of one or more glucose transporter proteins. Unfortunately, the Km and Vmax values have not yet been determined specifically for GLUT1 and GLUT4 proteins expressed in adipocytes, so that these two models for insulin action cannot be distinguished experimentally. Recent evidence suggests that GLUT4 is the primary contributor to insulin-stimulated hexose transport in 3T3-L1 adipocytes and that heterologously expressed human GLUT1 does not contribute to insulin-stimulated transport rates (Harrison et al., 1990b). However, no direct evidence concerning the actual contributions of mouse GLUT4 and GLUT1 to insulin-stimulated sugar transport is available. Therefore, in spite of the good agreement between the insulin-stimulated 3-fold increases in both the Vmax of hexose transport (Table I) and the plasma membrane levels of GLUT4 protein (Fig. 8), interpretation of these data are complicated by the recruitment of both GLUT4 and GLUT1 proteins to the plasma membranes in insulin-treated 3T3-L1 adipocytes.

Since insulin appears to alter the intrinsic activity of fibroblast GLUT1 transporters, it may also act by a similar mechanism on adipocyte GLUT1 transporters. The effect of insulin on fibroblast GLUT1 activity is only 2-fold, suggesting that this effect in adipocytes may also be small and thus, makes only a limited contribution to the overall insulin effect on adipocyte glucose transport rate. This postulate is consistent with our data suggesting that modulation of plasma membrane GLUT4 is the major factor determining insulin-stimulated glucose transport rates in 3T3-L1 adipocytes (Harrison et al., 1990b). While no specific evidence for insulin regulation of adipocyte GLUT1 or GLUT4 glucose transporter intrinsic activities has yet been described, recent studies of the stimulatory effects of cholera toxin and cAMP analogs (Clancy and Czech, 1990) and protein synthesis inhibitors (Clancy et al., 1991) demonstrate a clear dissociation between increased hexose transport rates and transporter recruitment in 3T3-L1 adipocytes. Evidence for modulation of glucose transporter intrinsic activity does exist for the counter-regulatory action of catecholamines on insulin-stimulated sugar transport in adipocytes (Joost et al., 1986, 1987), for regulation of the GLUT1 transporter in human erythrocytes by ATP (Carruthers, 1986a, 1986b; Hebert and Carruthers, 1986), and for insulin stimulation of the Chinese hamster ovary fibroblast GLUT1 transporter (Harrison et al., 1990a). Additionally, we have recently demonstrated that sugar transport by cell surface GLUT1 protein is greater than 80 and 90% suppressed in mouse 3T3-L1 adipocytes, relative to 3T3-L1 fibroblasts and Chinese hamster ovary fibroblasts, respectively (Harrison et al., 1991).

Our findings also suggest the hypothesis that the additivity of the insulin and cadmium effects on hexose transport in 3T3-L1 adipocytes is related to the differential sensitivity of the GLUT1 versus GLUT4 transporter isoforms to these agents. Mouse 3T3-L1 fibroblasts expressing high levels of GLUT1 protein and no detectable levels of GLUT4 protein respond to insulin or cadmium treatments with stimulations of sugar uptake of approximately 2 and 6-fold, respectively, under the same assay conditions. Clearly, fibroblast GLUT1 is more responsive to the actions of cadmium than to those of insulin. Interestingly, the mode of GLUT1 activation by these two agents may be similar because their effects are not additive (Fig. 4A). In contrast, insulin and cadmium act independently to stimulate glucose transport in mouse 3T3-L1 adipocytes, where high levels of both glucose transporter isoforms are expressed. Thus, the effects of these agents on adipocyte glucose transport are fully additive (Figs. 3, 4B, and 5). Treatment of 3T3-L1 adipocytes with cadmium alone results in a large increase in the rate of sugar transport without changing cell surface GLUT4 protein levels and without altering insulin's ability to increase both sugar transport and the recruitment of GLUT4 proteins to the plasma membrane. The relative membrane distributions of adipocyte GLUT1 and GLUT4 proteins are very different (Clancy and Czech, 1990; Harrison et al., 1990b; Piper et al., 1991), with a major portion of the cellular GLUT1 protein (approximately 35-60% of the total recovered in the membrane fractions) located in the plasma membranes of control adipocytes, while most of the GLUT4 protein (approximately 80-95% of the total recovered in the membrane fractions) is located in intracellular membranes of control adipocytes. Thus, cadmium activates sugar transport with a small percentage of total cellular GLUT4 transporters and a larger percentage of the cellular GLUT1 transporters at the cell surface. These data are consistent with the notion that the large effect of cadmium on adipocyte glucose transport is governed primarily by GLUT1 activation, while the effect of insulin is predominantly a GLUT4 mediated process.

It is worth noting that 3T3-L1 low density membrane fractions (or small membrane fractions) have not yet been
sufficiently characterized to demonstrate that they are purely of intracellular origin. Recent data from Lange and Brandt (1990a, 1990b) suggest that these low density "microosomal" membranes may actually be significantly contaminated by a cell surface glucose transporter-enriched microvilli membrane fraction. Interestingly, one of the predictions of the Lange and Brandt hypothesis is that an antibody like 5, that recognizes all cell surface GLUT1 proteins, would not detect an insulin-stimulated increase in cell surface GLUT1 proteins since all of the insulin-responsive transporters would already be sequestered in microvilli structures, on the cell surface. We are currently investigating this possibility experimentally.

We do not know why our data differ from that of Ezaki (1989) which demonstrate an insulinomimetic recruitment of glucose transporter proteins in response to acute exposures of isolated adipocytes to millimolar concentrations of cadmium. However, such different findings are not surprising given the extremely divergent experimental conditions used in the two studies (isolated adipocytes with little GLUT1 versus cultured adipocytes expressing very high levels of GLUT1, and minutes versus hours of exposure to metal concentrations that differ by a factor of 1000).

Figs. 6, 7, and 9 provide interesting information regarding the relative amounts of GLUT1 protein in undifferentiated and differentiated 3T3-L1 cells, relative to the rates of glucose transport observed in these cells. Approximately 1.8-fold more protein per cell is harvested from 3T3-L1 adipocytes than from the fibroblasts under our experimental conditions. Because equal amounts of adipocyte and fibroblast protein were loaded onto each polyacrylamide gel lane, the relative immunoblot and [125I]APS-forskolin labeling intensities observed in Figs. 6 and 7 must be corrected by a factor of 1.8 to normalize these data to a per adipocyte basis. GLUT1 protein levels per cell are thus calculated to increase 2-fold in the adipocytes relative to the fibroblasts. Increased levels of adipocyte [125I]APS-forskolin labeled protein per gel lane (2.7 ± 0.5-fold over fibroblasts, n = 3 experiments) indicate a 5-fold increase (2.7 X 1.8) in total transporter protein per cell associated with differentiation. Thus, the fact that basal glucose transport rates actually decrease upon differentiation (Fig. 4), combined with these relative glucose transporter protein numbers, suggest the presence of a large number of apparently inactive GLUT1 transporters in control 3T3-L1 adipocytes. This observation is consistent with the recent observations of Calderhead et al. (1990) that 3T3-L1 adipocytes express high levels of cell surface GLUT1 protein, and yet maintain relatively slow basal hexose transport rates. Isolated rat adipocytes express very low levels of GLUT1 protein and slow basal transport rates appear to be maintained by the sequestration of rat GLUT4 protein inside those adipocytes (Zorzano et al., 1989). Presumably these characteristically slow basal sugar transport rates underlie the dramatic insulin stimulation of sugar transport in the rat and mouse adipocytes. This report and the work of Clancy et al. (1991) raise the possibility that 3T3-L1 adipocytes regulate basal sugar transport by inhibiting cell surface GLUT1 proteins, perhaps by expressing an inhibitor whose effects are reversed by chronic exposures of the cells to cadmium or protein synthesis inhibitors.

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