Cellular Basis of Insulin Insensitivity in Large Rat Adipocytes

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ABSTRACT The marked stimulatory effect of insulin on the conversion of 20 mM D-[6-^{14}C]glucose to CO_2, glyceride-glycerol, and fatty acid observed in small rat adipocytes was greatly diminished in large cells from older rats. Similarly, total glucose utilization as estimated by summing the total metabolites accumulated intracellularly plus the release of labeled CO_2 and lactate was substantially lower in large cells in the presence of insulin and 5 mM labeled glucose. However, under conditions of 0.2 mM medium glucose where transport of the hexose into adipocytes is relatively more rate-limiting for subsequent metabolism, large cells actually utilized slightly greater total amounts of glucose than small cells in the presence of insulin. Increments of total glucose utilization due to both submaximal and maximal doses of insulin were similar in large and small cells incubated with a low glucose concentration. Under these conditions, conversion of labeled glucose to CO_2 and fatty acid in response to insulin was somewhat diminished in large cells, while conversion to glyceride-glycerol was enhanced.

The activity of the D-glucose transport system in large and small cells was estimated by monitoring initial rates of 3-*O*-[^{3}H]methylglucose uptake by a rapid filtration method. Transport system activity on a per cell basis was actually severalfold higher in large adipocytes in the basal state as well as in the presence of submaximal and maximal concentrations of insulin compared to small cells. However, the percent stimulation by insulin was less in the large cells. Uptake of 2-deoxyglucose under basal conditions and in response to insulin was also higher in large cells compared to small cells. Analysis of the accumulated label in extracts from fat cells incubated with D-[^{14}C]deoxyglucose revealed the presence of free deoxyglucose, deoxyglucose-6-phosphate, and 6-phosphodeoxygluconate. The levels of these metabolites were significantly higher in large cells compared to small cells indicating hexokinase activity appears not to account for the defective glucose utilization in large cells at high glucose concentrations.

It is concluded that (*a*) possible defects in insulin receptor components, the D-glucose transport system, and the coupling mechanism which links these entities do not significantly contribute to the apparent insulin-insensitivity of large fat cells and (*b*) the principal cellular defect which confers this blunted insulin response to large rat adipocytes involves one or more intracellular enzymes involved in glucose metabolism.

INTRODUCTION

Experiments performed over a decade ago demonstrated a reduced capacity of muscle and adipose tissue in forearms of obese humans to utilize glucose in the presence of insulin in vivo (1). Subsequent studies confirmed the concept that the relatively poor response to insulin characterizes these tissues in several animal models of obesity such as fa/fa rat (2), ob/ob mouse (3), and db/db mouse (4). This relative insulin-insensitive state appears to account for the high blood insulin levels in these animals. The larger size of fat cells from these genetically obese rodents and from obese humans has been implicated in the insulin insensitivity (5). Other factors such as percent carbohydrate in the diet have also been found to dramatically alter the tissue response to insulin action (6).

The use of large fat cells derived from old, large rats (400–600 g) which generally exhibit a smaller increase in glucose metabolism due to insulin than those from young, small rats (125–175 g) as a model system for insulin insensitivity has been widespread (7–9). A major problem, of course, is that one cannot be sure

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whether the differences in insulin responsiveness observed are simply due to age rather than "obesity." Furthermore, caloric intake varies considerably in large and small rats. However, cell size does seem to correlate positively with insulin insensitivity even when fat cells of varying size from the same animal are studied (10). Thus, this model of insulin insensitivity is convenient and useful, although ultimately each model of insulin insensitivity must be examined individually for specific defects.

In spite of intensive investigation it has not been possible to detect the defective cellular site which confers insulin insensitivity to large fat cells. Quantitating the early steps of insulin action on fat cell glucose metabolism has been the major obstacle due to the technical difficulty in monitoring glucose transport system activity in these cells. Recently we succeeded in developing a rapid filtration procedure which monitors initial 3-*O*-methylglucose uptake rates in isolated white fat cells (11). This glucose analogue is not phosphorylated or further metabolized by fat cells and is transported by facilitated diffusion. Uptake of deoxyglucose, which is transported into the cell, phosphorylated by the hexokinase-catalyzed reaction, and converted to some extent to 6-phosphodeoxygluconate (11) but not further significantly metabolized, can also be assayed with this method. Thus possible alterations in either the response of the glucose transport system to insulin or in hexokinase activity can be directly measured in intact cells derived from obese animals or humans.

The aim of the present investigation was to evaluate the activity of the early events of insulin action on the glucose transport system in isolated fat cells from large and small rats. In addition, the directional flow of glucose through various metabolic pathways under conditions of very low versus high medium glucose concentrations was studied as an alternate means of defining the role of glucose transport activity in insulin insensitivity. The results of both approaches indicate that the basic cellular defect which confers apparent insulin insensitivity to large rat adipocytes occurs at the level of intracellular enzyme activity rather than at the membrane insulin effector system.

METHODS

Isolation of fat cells. Epididymal white adipose tissue was obtained from male Sprague-Dawley rats (120–170 g or 450–600 g) obtained from Charles River Breeding Laboratories, Wilmington, Mass. (CD strain) fed laboratory chow ad lib. (12). For each experiment the tissue from two or three small rats and one large rat was dissected away from the animal and cut into small pieces with scissors. White fat cells were isolated by digestion of about 3 g tissue for 1 h at 37°C with crude bacterial collagenase (*Clostridium histolyticum*, Worthington Biochemical Corp., Freehold, N. J.) at a concentration of 1 mg/ml in Krebs-Ringer phosphate buffer (8 ml) containing 4% albumin. The buffer was prepared fresh daily and adjusted to pH 7.4 with NaOH after addition of bovine serum albumin (Armour Fraction V, Armour Pharmaceutical Co., Chicago, Ill.). The phosphate buffer contained NaCl, 128 mM/$CaCl_2$, 1.4 mM/$MgSO_4$, 1.4 mM/KCl, 5.2 mM/Na_2HPO_4, 10 mM (pH adjusted to pH 7.4 with HCl). At the end of the digestion period the fat cells were filtered through nylon chiffon and washed twice with 6 ml of 2% albumin buffer. The number of fat cells was estimated by counting an aliquot of cells on a microscope slide as described by Gliemann (13).

Assay of hexose transport. Successful assay of initial rates of 3-*O*-methylglucose uptake depended on two major elements. The first is the use of cells which exhibit low basal rates of glucose transport since the time required for hexose equilibration across the cell membrane is inversely related to the rates of hexose entry. The major factors which contribute to this variable are the preparations of collagenase and albumin one uses for digestion and incubation of fat cells as well as the rats themselves. It was necessary to first screen several lots of collagenase and albumin using the conversion of labeled glucose to CO_2 by small fat cells in the presence and absence of insulin as an index of glucose uptake. Best results in the transport assay were obtained using cells prepared with collagenase and albumin preparations which lead to at least 10-fold and preferably 20-fold stimulations by insulin in the glucose to CO_2 assay. The present studies were all performed with lot 236 crude collagenase from Worthington Biochemical Corp. and lot L43405 bovine serum albumin from Metrix Inc. (Aurora, Colo.) which routinely yielded cells which exhibited 20-fold or greater increases in labeled CO_2 production due to insulin when incubated with 0.2 mM D-[1-^{14}C]glucose in 1 ml buffer.

The second important element of the assay was found to be very rapid filtration rates which were attained using glass fiber filters (Whatman GF/C, 5.5 cm diameter. Whatman Inc., Clifton, N. J.) and a Doerr rotary vacuum pump (Doerr Glass Co., Vineland, N. J.). The standard assay procedure involved incubation of about 3×10^5 fat cells in 0.4 ml of Krebs-Ringer phosphate buffer containing 4% albumin to plastic culture tubes (17×100 mm) and incubation at 37°C. Using smaller assay volumes in these experiments was found to be unsuitable due to the large volume of the large fat cells. Deoxyglucose uptake was assayed in 0.1-ml cell suspensions since much more label is taken up by the cells due to phosphorylation of the hexose and less cells could be used. Transport was initiated with the addition of 10 μl of a solution containing 3-*O*-[^{3}H]methylglucose (4–6 μCi/tube) or D-[1-^{14}C]2-deoxyglucose (0.4–0.8 μCi per tube). The labeled hexoses were dissolved in isotonic saline before addition. The tubes were immediately vigorously shaken by hand and incubated at 37°C. At the appropriate times transport was stopped by the addition of 3 ml of ice-cold Krebs-Ringer phosphate buffer containing 0.1% albumin and the cells are poured onto the center of the glass fiber filters under pressure provided by the pump. It was crucial that the filters be first wetted (routinely 10 s before cells are poured) with albumin buffer (0.3–0.5% albumin) and that the cold buffer containing cells and labeled hexose be aspirated through the filter before spreading to its edges and contaminating the glass of the filter apparatus (Millipore Corp., Bedford, Mass.). The cells were immediately washed by rapidly decanting 6 ml of ice-cold Krebs-Ringer phosphate buffer containing 0.1% albumin onto the filters. The total time taken to filter and wash

the cells was less than 15 s. The filters were dried in air at room temperature and immersed in 4 ml of liquid scintillation fluid containing 33% vol/vol Triton X-100 in toluene with 4 g/liter of Omnifluor (New England Nuclear, Boston, Mass.). ^{3}H or ^{14}C labels were determined on each filter paper sample in a Packard Model 3,320 liquid scintillation system. (Packard Instrument Co., Inc., Downers Grove, Ill.)

Net uptake of label is the amount of radioactivity accumulated at a given time minus the radioactivity bound on filters containing cells to which labeled 3-*O*-methylglucose or deoxyglucose and 3 ml of cold buffer were added together. Under the conditions of our experiments, these control values represented about 5–20% of the total radioactivity obtained from 1-min incubations depending on the number of cells used. This method gave highly reproducible results if performed in triplicate. This method was found to be valid for use with large cells since uptake was linear for over 30 s and initial rates of uptake were inhibited by transport inhibitors and unlabeled glucose to similar degrees compared to small cells (not illustrated). The experiments presented in this report were each performed on 2–5 separate days, and the values presented are either the results of representative experiments or the means of the several experiments performed.

Analysis of intracellular deoxyglucose metabolites. Fat cells were incubated in plastic tubes in 0.1 ml of Krebs-Ringer phosphate buffer containing 4% albumin as described for the transport assay. At the appropriate times after addition of labeled deoxyglucose 3 ml of ice-cold Krebs-Ringer buffer was added to the cells and the cells rapidly filtered as described for transport studies except Millipore Corp. NRW (5.5 cm diameter) filters were used instead of glass fiber filters. The filters were extracted three times in 10 ml ice-cold distilled water by vortex mixing the filters in test tubes and the pooled extracts lyophilized to dryness. The extracted material was then resuspended in 1 ml of water and 0.1 ml was chromatographed as described by Renner et al. (14). The paper was dried and 1-cm strips were analyzed in a liquid scintillation system as described for analysis of the glass fiber filters for radioactivity.

Assay of glucose metabolism. For measurement of labeled CO_2 production the fat cells were resuspended in 4% albumin buffer and incubated in plastic culture tubes (17 × 100 mm) at 37°C with shaking. The final incubation mixture volume in each tube varied as indicated in the legends of the individual experiments. The reaction was stopped by addition of 0.2 ml of 0.5 M H_2SO_4. Glucose conversion to CO_2, glyceride-glycerol, and fatty acid was determined as described by Fain et al. (15). The values for each experiment are the averages of duplicate tubes and are based on changes during the incubation period over those of controls incubated without cells. Lactate production was determined by applying 10-μl aliquots of medium from duplicate tubes of fat cell suspensions incubated with labeled glucose to Whatman no. 3 MM paper (Whatman Ltd., England) and developing for 2 h in solvent 36 (3 vol ethylacetate, 1 vol glacial acetic acid, and 1 vol H_2O) as described by Renner et al. (14).

Reagents. Crystalline porcine insulin was a gift of Eli Lilly and Co. (Indianapolis, Ind.) and contained less than 0.005% glucagon by weight. Vitamin K_5 was obtained from Nutritionals Biochemical Corporation (Cleveland, Ohio) and unlabeled hexoses were purchased from Sigma Chemical Company, St. Louis, Mo. 3-*O*-[^{3}H]Methylglucose, D-[1-^{14}C]2-deoxyglucose, D-[1-^{14}C]glucose, and D[6-^{14}C]glucose were obtained from New England Nuclear.

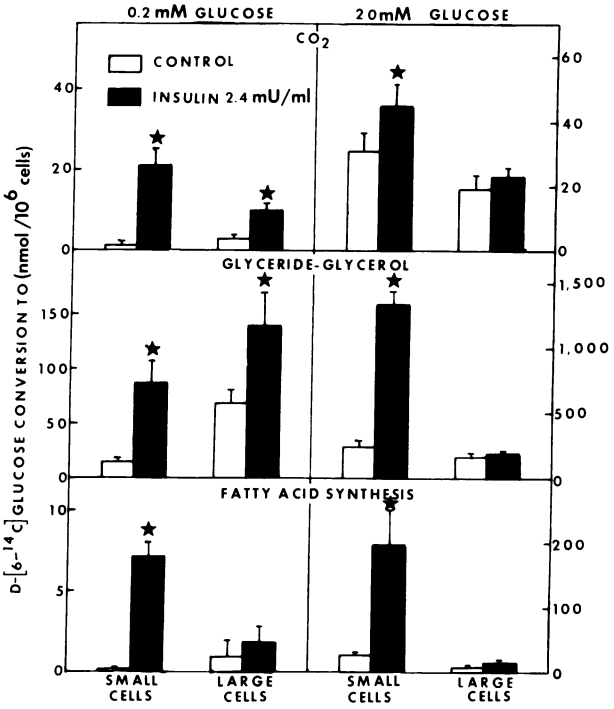

FIGURE 1 Altered D-[6-^{14}C]glucose metabolism in large fat cells in the presence and absence of insulin. Large fat cells from 450–550 g rats and small fat cells from 140–160 g rats were incubated at 37°C in 0.5 ml Krebs-Ringer phosphate buffer containing 4% bovine serum albumin and either 0.2 mM or 20 mM D-[6-^{14}C]glucose. Insulin at 2.4 mU/ml was added at the start and glucose conversion to CO_2, glyceride-glycerol, and fatty acid assayed after a 2-h incubation. The values are the means±standard errors of three paired experiments performed on different days. A star above the insulin values denotes a statistically significant difference ($P < 0.05$) between the insulin value and the control value for the same cells obtained by analysis of the paired differences in three experiments.

RESULTS

Fig. 1 shows the relative increases in D-[6-^{14}C]glucose conversion to CO_2, glyceride-glycerol, and fatty acid due to insulin in large and small fat cells in the presence of very low (0.2 mM) and high (20 mM) glucose concentrations. Large fat cells exhibited a much lower production of labeled CO_2 as well as fatty acid in the presence of insulin at both glucose concentrations. Surprisingly, at 0.2 mM glucose in the presence of insulin large fat cells converted greater amounts of labeled glucose to glyceride-glycerol. These data indicate that large cells actually accumulated more glucose than small cells in response to insulin at low glucose concentrations but that it was directed into different metabolic routes. The only previous report on large fat cell metabolism at these low glucose concentrations presented only the values for conversion of D-[1-^{14}C]glucose to CO_2 (7). In contrast

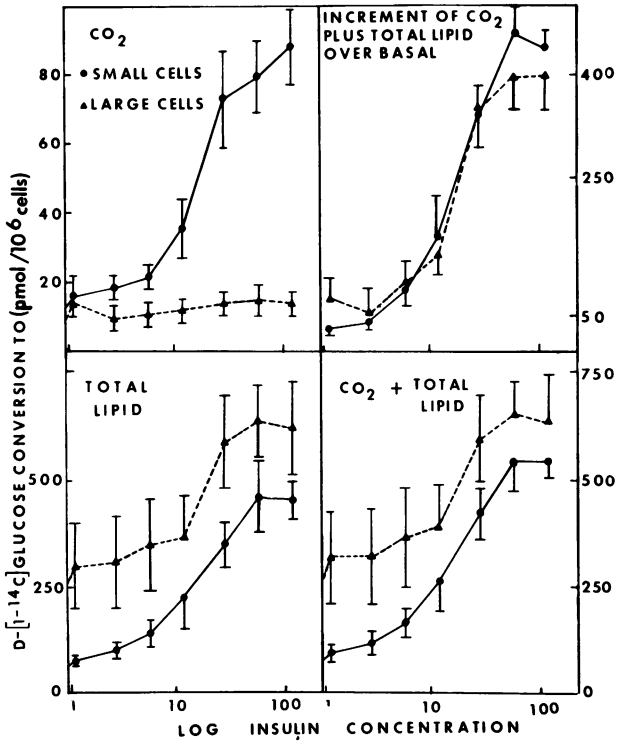

FIGURE 2 The response of glucose metabolism in large and small fat cells to submaximal and maximal doses of insulin. Large fat cells from 500–580 g rats and small fat cells from 140–160 g rats were incubated in 1 ml Krebs-Ringer phosphate buffer at 37°C containing 4% albumin in the presence or absence of the indicated doses of insulin for 15 min. D-[1-^{14}C]glucose was added at a final concentration of 50 μM and its conversion to labeled CO_2 and total lipid was measured over a 30-min incubation period. The values for CO_2, total lipid, and CO_2 plus total lipid represent the nanomoles of glucose incorporated±standard error in four paired experiments performed on different days. The values labeled "increment of CO_2 plus total lipid over basal" represent the increased amount of picomoles of total glucose incorporated due to insulin over the basal values for large and small cells, respectively, in these experiments.

to the results obtained at 0.2 mM glucose, all parameters of glucose metabolism were lower in large cells in the presence of insulin at the high concentration of glucose (Fig. 1). Total glucose utilization by large fat cells in the presence of insulin was only about 20% of that exhibited by small cells under these conditions.

Since the results described above indicated that large fat cells responded to maximal doses of insulin as well as small cells in terms of total metabolism at very low glucose concentrations, it was interesting to test their response to lower doses of the hormone under these conditions. Fig. 2 shows the response of fat cells to concentrations of insulin between 1 and 120 μU/ml in the presence of 50 μM D-[1-^{14}C]glucose. Large cells responded little if at all to insulin with an increase in labeled CO_2 production in these experiments. However, conversion of glucose to total lipid by large fat cells was quite sensitive to low doses of the hormone, and these values were again stimulated to higher levels than exhibited by small cells at maximal hormone concentrations (Fig. 2). When the values for total glucose utilization (CO_2 plus total lipid) were compared for the two cell types it was found that large cells utilized equal or greater amounts of glucose at all concentrations of insulin tested as well as under basal conditions. Furthermore, no significant difference could be detected between large and small cells with respect to the increment in glucose utilization over control levels due to any of the insulin doses used.

In order to monitor the activity of the hexose transport system in large fat cells, 3-*O*-[^{3}H]methylglucose uptake was monitored by a procedure recently described (11, 16) which is very similar to that previously described for brown fat cells (17). The values for 3-*O*-methylglucose uptake over a 15-s period in Fig. 3 represent initial rates of transport since uptake was linear for at least 30 s in small cells (11) as well as large cells (not illustrated). Transport activity per cell was significantly greater in large cells than in small cells in concert with the results obtained on glucose utilization in Fig. 2. Initial rates of 3-*O*-methylglucose uptake in large adipocytes were several times greater in the presence of both submaximal and maximal doses of insulin compared to small cells, although the percent stimulation by insulin was smaller in the large cells. Interestingly, 480 μU/ml insulin was significantly more potent than 120 μU/ml in the large cells while 120 μU/ml insulin was maximal in small cells. No further response was obtained with higher insulin concentrations in large cells (not illustrated). In studies not presented, transport rates were also significantly higher in large than in small cells by insulin when 10 mM 3-*O*-[^{3}H]methylglucose was used. In several experiments accumulation of 3-*O*-methylglucose after equilibration (30 min incubation) was used to estimate total intracellular water space per cell in large and small rat adipocytes. These values were 0.37±0.04 and 0.75±0.10 pl per cell for small and large fat cells, respectively, in 16 experiments. Large adipocytes exhibited a water space which varied from 30 to 200% larger than small fat cells in these experiments. However, this larger intracellular water space does not affect the interpretation of the transport results described in Fig. 3 since initial rates of uptake were assayed. Thus the initial rate of hexose uptake in both large and small fat cells should be equally related to the number of transport systems in the fat cell surface membrane and their activities independent of intracellular water space size.

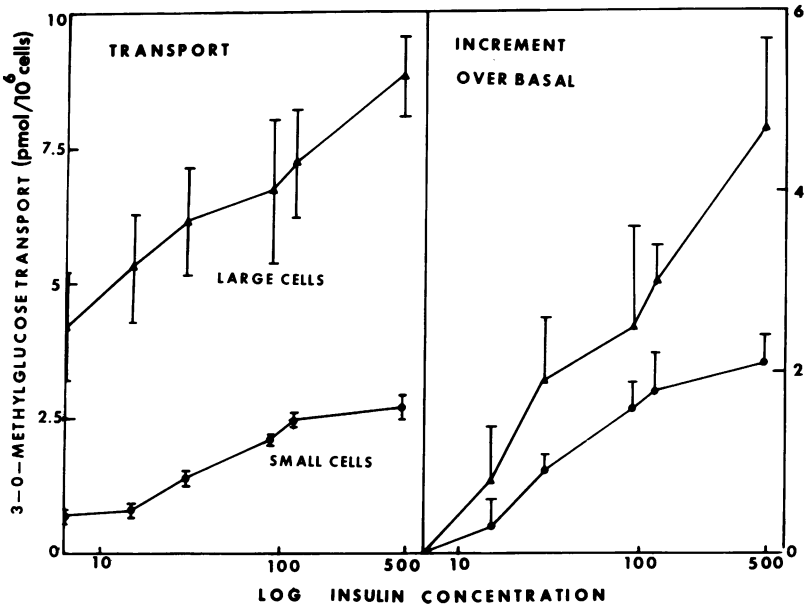

FIGURE 3 Isolated white fat cells from large and small rats were incubated (3×10^6 cells per tube) in 0.4 ml of Krebs-Ringer buffer containing 4% albumin in the presence or absence of the various concentrations of insulin indicated for 15 min. 3-*O*-[^{3}H]methylglucose was then added at a final concentration of 50 μM (4 μCi/tube) and uptake measured at 15 s. The amount of label taken up by simple diffusion in these cells was determined in the presence of the transport inhibitor cytochalasin B (40 μM) and was subtracted from all total uptake values obtained. The values presented in the left panel are the mean net initial uptake rates ±standard error in three paired experiments which are taken to reflect D-glucose transport system activity. The values in the right panel are the mean increments over basal levels due to the various insulin concentrations.

In order to assess the possible involvement of the hexokinase-catalyzed phosphorylation reaction in the reduced capacity of large fat cells to respond to insulin in the presence of high concentrations of glucose, labeled deoxyglucose uptake was monitored. We previously found that in brown fat cells deoxyglucose-6-phosphate is the major intracellular metabolite which accumulates subsequent to incubation with this hexose (11). Table I shows that in the present studies large rat adipocytes actually accumulated higher levels of labeled deoxyglucose metabolites than small cells in the presence or absence of insulin. The percent increase in deoxyglucose uptake due to insulin was not significantly different in the large and small fat cells. Interestingly, vitamin K_5 stimulated deoxyglucose uptake in large cells to a lesser extent than small cells but this effect was not statistically significant. This agent is a potent oxidant of nicotinamide adenine dinucleotide phosphate and nicotinamide adenine dinucleotide and mimics the ability of insulin to activate hexose transport in white fat cells (16). That essentially all the deoxyglucose entered these cells via the hexose transport system or systems is supported by the observation that the potent transport inhibitor dipyridamole (14) blocked deoxyglucose uptake in both types of cells (Table I).

In several experiments the levels of the various individual deoxyglucose metabolites which accumulate intracellularly under these conditions were determined (Fig. 4). In concert with previous findings with Novikoff rat hepatoma cells in culture (14, 18) and brown fat cells (11), white fat cells were found to accumulate deoxyglucose, deoxyglucose-6-phosphate, and 6-phosphodeoxygluconate under the conditions of our experiments. Insulin stimulated the levels of all three metabolites in small fat cells which agrees with the concept that its major effect is at the level of hexose transport into the cell. In both the presence and absence of insulin the levels of these metabolites were uniformly higher in large cells when compared on a per cell basis. When these values are standardized on the basis of intracellular water space the concentrations of metabolites remain slightly higher in large vs. small fat cells (not illustrated).

The increased level of insulin-stimulated 3-*O*-methylglucose transport and deoxyglucose accumulation in large fat cells in the face of a markedly decreased ability to convert glucose to CO_2, glyceride-glycerol, and fatty acid at high glucose concentrations suggested that large fat cells might release large amounts of lactate or pyruvate to the medium which would go undetected in glucose utilization assays used to date in these cells (Figs.

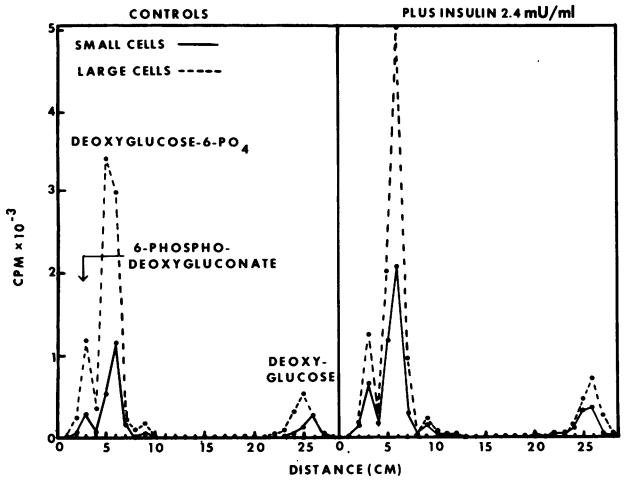

FIGURE 4 Analysis of deoxyglucose metabolites accumulated by large and small rat adipocytes. Large fat cells (500–550 g rats) and small fat cells (140–160 g rats) were incubated (1×10^5 cells/tube) at 37°C in 0.1 ml Krebs-Ringer phosphate buffer containing 4% albumin in the presence or absence of 2.4 mU/ml insulin for 15 min before addition of 0.2 mM D-[1-^{14}C]deoxyglucose. After 2 min the cells were rapidly filtered on NR Millipore Corp. filters, extracted, and the extracts chromatographed as described in Methods. The axis represents the distance the various metabolites migrated during the chromatography. The values are from a representative experiment. Similar results were obtained in the two other similar experiments performed.

1 and 2). Large and small cells were therefore rigorously tested for total glucose consumed by assaying labeled CO_2 production, total intracellular label accumulated, and release of labeled metabolites under the same

TABLE I

The Effect of Insulin, Vitamin K$_5$, and Dipyridamole on Deoxyglucose Uptake by Large and Small Fat Cells

Additions	D-[1-^{14}C]Deoxyglucose uptake	
	Small cells	Large cells
	pmol/10^6 cells	
None	350±96	906±210
Insulin, *2.4 mU/ml*	763±230	2,062±650
Vitamin K$_5$, *50 μM*	728±290	1,394±406
Dipyridamole, *0.2 mM*	23±9	68±30

Isolated white fat cells from large and small rats were incubated (1×10^5 cells/tube) at 37°C in 0.1 ml Krebs-Ringer buffer containing 4% albumin in the absence or presence of insulin (2.4 mU/ml), vitamin K$_5$ (50 μM), or dipyridamole (0.2 mM) for 15 min. D-[1-^{14}C] deoxyglucose was added at a final concentration of 0.2 mM (0.1–0.3 μCi/tube) and uptake measured over a 5-min period. The values presented are the mean pmol of deoxyglucose accumulated by the cells ±standard error of four experiments performed on separate days.

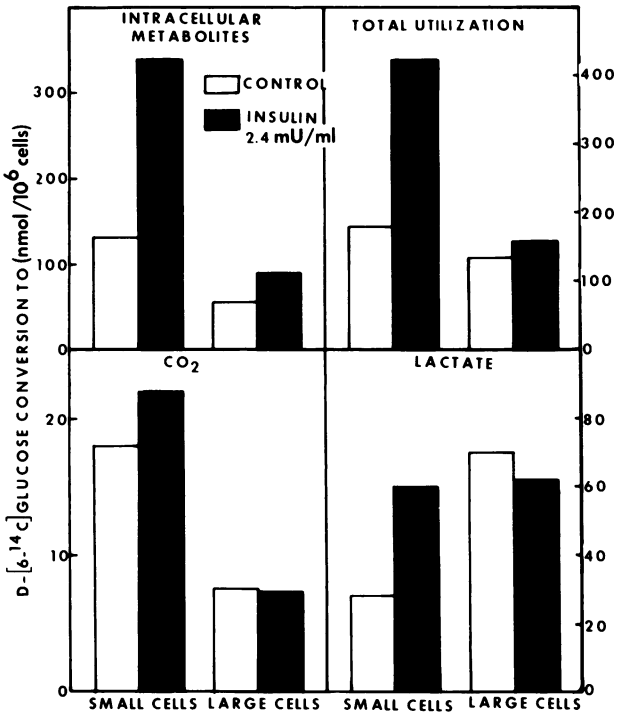

FIGURE 5 Total glucose utilization in large and small fat cells in the presence and absence of insulin. Large fat cells (480–550 g rats) and small cells (140–160 g rats) were incubated (1×10^5 cells/tube) at 37°C in 0.1 ml Krebs-Ringer phosphate buffer containing 4% albumin and 2.4 mU/ml insulin, where indicated, for 1 h. D-[6-^{14}C]glucose at a final concentration of 5 mM was present throughout and total intracellular accumulation of label, labeled CO_2 production, and lactate release into the medium was assayed in separate sets of duplicate tubes. Total intracellular accumulation of label was assayed by the same filtration procedure described for assay of 3-*O*-methylglucose uptake. Lactate was analyzed in medium samples by paper chromatography as described in Methods. The decrease in total glucose utilized by large cells in the presence of insulin in three paired experiments was statistically significant ($P < 0.05$).

conditions used to measure deoxyglucose uptake. Fig. 5 shows that lactate production was similar in large and small fat cells incubated with insulin and 5 mM D-[6-^{14}C]glucose. The large cells did convert more D-[6-^{14}C]-glucose to lactate than did small cells in the absence of insulin. No pyruvate or other metabolites were detected in the incubation medium using the chromatography method of Renner et al. (14). Total glucose utilized under these conditions were decreased in large cells compared to small cells very dramatically in the presence of insulin, while a much smaller difference was observed in the absence of hormone.

DISCUSSION

Attempts to identify the cellular locus which confers relative insulin insensitivity to the large fat cells de-

rived from various animal models of obesity must necessarily focus on four general sites: (*a*) the cell-surface insulin receptor, (*b*) the glucose transport system, (*c*) the coupling mechanism which links these two entities and which is activated by insulin-receptor interaction, and (*d*) one or more intracellular enzymes involved in the metabolism of glucose. Previous studies in various laboratories have documented apparent defects in several of these parameters in large rat adipocytes. In an interesting series of reports Roth, Kahn and colleagues have demonstrated that insulin receptor density on the surface membrane of fat cells (19, 20) as well as other cell types (21–23) is greatly diminished in obesity. Olefsky and Reaven have recently reported a diminished degree of insulin binding on a per cell basis to large adipocytes from old rats (24). In contrast, Van Bennett and Cuatrecasas (25) and Amatruda et al. (26) found no significant difference in insulin binding to large vs. small cells in rats and humans, respectively. Furthermore, Cushman and Salans (27) recently found an actual increase in insulin binding per fat cell in large rat adipocytes compared to young, lean controls. Thus it remains an open question whether specific insulin receptors in fat cells of large rats in general are defective or diminished in number.

Livingston et al. (7) recently reported data which indicated that the diminished response to insulin exhibited by large rat adipocytes may be due to a defective "signal(s)" which is generated by insulin-receptor interaction. These workers found that labeled glucose conversions to CO_2 in large cells was refractory to insulin action in spite of an unaltered ability to oxidize very large amounts of glucose under basal conditions. Similarly, DiGirolamo et al. (28) concluded that large fat cells have preserved the capacity to take up and metabolize glucose in response to elevations of glucose in response to elevations of glucose concentration in the medium and that a reduced capacity to metabolize glucose could not explain the defective response to insulin. However, several observations argue quite strongly against this concept. The comparison of only one parameter of glucose metabolism between large and small cells is dangerous since it has long been known that large cells exhibit an altered directional flow of glucose through the various metabolic pathways (29). Furthermore, insulin is known to alter this directional flow independent of its major effect at the level of glucose transport (30). In addition, the data of DiGirolamo et al. (28) showed that in the presence of insulin and 10 mM glucose large rat fat cells actually utilize only about half as much glucose as small fat cells when absolute amounts of glucose converted to CO_2 and total lipid are summed. The recent report of Livingston and Lockwood (31), which appeared while the studies presented in this paper were in progress, showed that 3-*O*-methylglucose transport activity in large rat adipocytes was stimulated to the same extent as occurred in small cells. Thus it seems very unlikely that a defect in coupling mechanism between receptor and transport system can account for insulin insensitivity in these large fat cells.

The data presented in this report clearly demonstrate that the key cellular site which confers the blunted insulin response to large rat adipocytes is a decreased intracellular metabolic enzymic capacity to metabolize glucose. At low concentrations of medium glucose, large fat cells actually utilized equal or greater amounts of glucose in response to insulin compared to small cells (Figs. 1 and 2). Thus the actual defect which blocks glucose utilization at high glucose concentrations must be beyond the step whereby the insulin-effector system triggers the activation of glucose transport in response to submaximal and maximal doses of insulin. Only at the high medium glucose concentrations where transport is relatively less rate-limiting for subsequent glucose metabolism is the diminished response to insulin observed. Thus at physiological glucose concentrations (5 mM), glucose utilization is essentially totally unresponsive to insulin in large cells (Fig. 5). The concept that the insulin effector system may be quite normal in large adipocytes is supported by the fact that the anti-lipolytic action of insulin in unimpaired in these cells (32, 33).

The present results indicate that D-glucose transport system activity in large fat cells is actually greater than that observed in small cells on a per cell basis. The findings with 3-*O*-methylglucose (Fig. 3), deoxyglucose (Table I), and glucose utilization at a low hexose concentration (Fig. 2) are all consistent with this conclusion. In addition, the increment in pmol of hexose entering the large cells due to submaximal and maximal doses of insulin is always equal to or greater than that observed in small cells under these conditions (Figs. 2 and 3, Table I).

In experiments not illustrated, the intracellular water space (3-*O*-methylglucose equilibration space) in large cells was on the average twice as great as small cells and usually only about 30–200% greater in 16 separate experiments performed on different days. These data agree with those of Livingston and Lockwood (31), but are at variance with the findings of DiGirolamo and Owens (34) who reported an approximately fivefold increase in intracellular water space in large cells. The basis for this variation in results is not presently known. It is important to note that the 3-*O*-methylglucose transport rates reported here are assayed during the linear portion of the 3-*O*-methylglucose uptake curve and thus represent initial velocities of transport (11). These values are theoretically independent of variability in intracellular water space among cell types since the intra-

cellular concentration of 3-*O*-methylglucose is very low and the transmembrane hexose concentration gradient remains very high after a 15-s incubation period.

The present findings which indicate that basal hexose transport activity is greater in large than in small adipocytes agree with the increased rates of deoxyglucose uptake in large cells reported by Livingston and Lockwood (31). However, these workers reported no difference in deoxyglucose uptake rates between the cell types in the presence of insulin and also found that 3-*O*-methylglucose transport rates were similar in large and small fat cells in the presence or absence of insulin. It seems probable that the 3-*O*-methylglucose transport rates reported by Livingston and Lockwood are underestimates since they did not terminate their transport assay with cold buffer as in the present studies, but instead centrifuged cells through silicone oil at 24°C for 15 s. Efflux of 3-*O*-methylglucose from fat cells occurs extremely rapidly at 24°C and could lead to significant loss of label from the fat cells under these conditions. In any case, both our results and those of Livingston and Lockwood (31) do lead to the same general conclusion that the severely blunted response to insulin observed in large cells cannot be due to a decreased capacity of these cells to transport glucose across the surface membrane. Similarly, both studies using deoxyglucose indicate that the findings of Bernstein (35) and Bernstein and Kipnis (36) who found decreased hexokinase II in homogenates of large rat adipocytes do not appear to account for this blunted insulin response of large cells. It has also been found that vitamin K_5 stimulated large fat cell 3-*O*-methylglucose transport as well as insulin (not illustrated) which is similar to our previous findings with small fat cells (16).

Although total 3-*O*-methylglucose entry in large fat cells in the presence or absence of various insulin concentrations was always greater than in small cells, the percent stimulation by insulin was less. In addition, in large cells 480 μU/ml insulin stimulated hexose uptake to a greater degree than 120 μU/ml which did not occur in small cells (Fig. 3). This is consistent with the observations of Olefsky and Reaven (24) in rat adipocytes and of Roth, Kahn and colleagues in ob/ob mouse and human cells (19–23) which indicate insulin receptors are decreased in cells from obese subjects. However, the data do not prove a causal relationship between insulin receptor defects and this decreased percent stimulation of transport activity by insulin and further work will be necessary to unequivocally document this point. It should be noted that a significant difference in the ability of large and small cells to degrade insulin under the conditions of those experiments might alter the magnitude of differences in response to low doses of the hormone. Even if the decreased percent stimulation does reflect a receptor deficiency, it is apparent that this defect has little physiological consequence since the total glucose utilization of large cells is always equal to or greater than small cells in these experiments at low glucose concentrations. Again the data effectively argue for the concept that it is an inadequate intracellular enzyme or enzymes which play the major role in restricting the large fat cell's ability to utilize high levels of glucose in the presence of insulin.

Comparison of the data presented in Figs. 1, 2, and 3, and Table I underscores the difficulty in relying on glucose metabolism parameters for estimating insulin effector activity. Under conditions where insulin failed to stimulate D-[1-^{14}C]glucose to CO_2 in large cells these cells readily responded to the hormone with an increased labeled triglyceride formation (Fig. 2). It is clear from these studies that measurement of glucose metabolism per se is inadequate to document differences in insulin effector activity. However, these kinds of measurements become useful when performed in conjunction with hexose transport experiments. The lack of strict parallelism between the measurement of glucose metabolism and direct measurement of the hexose transport system activity in these studies lends further strength to the concept previously suggested (11) that transport may not be solely and strictly rate-limiting for metabolism in fat cells. This appears especially true in the case of large fat cells which exhibit much higher rates of membrane transport activity in the presence of insulin than small cells. For in spite of this increased transport activity they utilize glucose at rates nearly the same as small cells at very low glucose concentrations, indicating some restriction in metabolism by intracellular enzymes even under these conditions (Fig. 2). In light of these data it would appear that intracellular free glucose accumulates to a greater extent in large cells than in small cells as a result of a block in glucose metabolism. This lowers the transmembrane glucose gradient in the large cells which in turn decreases net influx of glucose into the cells. This general scheme predicts that the expression of the defective response to insulin in large cells should be most apparent under conditions of high glucose concentrations where the enzymatic capacity to utilize glucose becomes relatively more rate-limiting than glucose transport system activity. The results presented in this paper validate this prediction completely. Further experiments will be necessary to determine which enzyme or enzymes contribute most to defective metabolism in large cells. The studies presented in Fig. 3 suggest that hexokinase may not be involved, but this interpretation must be considered preliminary at present since deoxyglucose and not glucose was used in these studies.

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