Divergent Mechanisms for the Insulin Resistant and Hyperresponsive Glucose Transport in Adipose Cells from Fasted and Refed Rats

Alterations in Both Glucose Transporter Number and Intrinsic Activity

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Abstract

The effects of fasting and refeeding on the glucose transport response to insulin in isolated rat adipose cells have been examined using 3-*O*-methylglucose transport in intact cells and cytochalasin B binding and Western blotting in subcellular membrane fractions. After a 72-h fast, basal glucose transport activity decreases slightly and insulin-stimulated activity decreases > 85%. Following 48 h of fasting, insulin-stimulated glucose transport activity is diminished from 3.9±0.5 to 1.3±0.3 fmol/cell per min (mean±SEM). Similarly, the concentrations of glucose transporters are reduced with fasting in both the plasma membranes from insulin-stimulated cells from 38±5 to 18±3 pmol/mg of membrane protein and the low density microsomes from basal cells from 68±8 to 34±9 pmol/mg of membrane protein. Ad lib. refeeding for 6 d after a 48-h fast results in up to twofold greater maximally insulin-stimulated glucose transport activity compared with the control level (7.1±0.4 vs. 4.5±0.2 fmol/cell per min), before returning to baseline at 10 d. However, the corresponding concentration of glucose transporters in the plasma membranes is restored only to the control level (45±5 vs. 50±5 pmol/mg of membrane protein). Although the concentration of glucose transporters in the low density microsomes of basal cells remains decreased, the total number is restored to the control level due to an increase in low density microsomal protein. Thus, the insulin-resistant glucose transport in adipose cells from fasted rats can be explained by a decreased translocation of glucose transporters to the plasma membrane due to a depleted intracellular pool. In contrast, the insulin hyperresponsive glucose transport observed with refeeding appears to result from (*a*) a restored translocation of glucose transporters to the plasma membrane from a repleted intracellular pool and (*b*) enhanced plasma membrane glucose transporter intrinsic activity.

Presented in part at the 1984 Annual Meeting of the American Diabetes Association and published in abstract form in 1984. (*Diabetes.* 33:71a.) A preliminary experiment was briefly discussed in a recent review in 1985. (*Diabetes Metab. Rev.* 1:203–227.)

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Received for publication 18 August 1987 and in revised form 25 February 1988.

The Journal of Clinical Investigation, Inc.
Volume 82, August 1988, 691–699

Introduction

A classic study by Moore (1) in 1963 in rat adipose tissue demonstrated that fasting decreased and refeeding caused an overshoot in insulin-stimulated glucose uptake. Subsequently, more sophisticated techniques have shown progressive resistance to insulin's stimulatory effects on both glucose transport and metabolism in rat and human adipose cells with fasting, although the mechanisms have not been defined (2–7). In addition, short periods of refeeding after fasting have been reported to restore insulin-stimulated glucose transport activity in rat adipose cells to normal (3). However, the effects of longer periods of refeeding on glucose transport activity have not been studied, although an overshoot in insulin-stimulated glucose metabolism has been demonstrated (4–6, 8).

Studies from this laboratory (9, 10) and independent studies by Kono and co-workers (11, 12) have shown that insulin stimulates glucose transport in isolated rat adipose cells by recruiting functional glucose transporters from a large, membrane-associated intracellular pool to the plasma membrane. In addition, the alterations in insulin-stimulated glucose transport activity associated with several insulin-resistant (13–18) and hyperresponsive (19–23) states have been explained by changes in the number of glucose transporters. Three rat models characterized by insulin resistance at the cellular level, i.e., high fat/low carbohydrate feeding (13), obesity and aging (14), and streptozotocin diabetes (15), are accompanied by a depletion of intracellular glucose transporters in the basal state and a resultant diminished translocation of glucose transporters to the plasma membrane with insulin stimulation. Cellular hyperresponsiveness to insulin has been demonstrated in four rat models: the young, genetically obese Zucker fatty rat (21), experimental chronic hyperinsulinemia (19), physical training (22), and insulin treatment of streptozotocin diabetes (20, 23). All four models are associated with an increased insulin-stimulated translocation of glucose transporters to the plasma membrane from an enlarged intracellular pool. The latter appears to result from a net increase in microsomal membrane protein synthesis. However, the increase in the number of glucose transporters observed with insulin treatment of the diabetic rat is not sufficient to explain the marked overshoot in glucose transport activity suggesting, in addition, a substantial change in glucose transporter intrinsic activity.

This investigation was undertaken to systematically determine the effects of fasting and refeeding on the stimulation of glucose transport activity by insulin in the rat adipose cell, define the mechanisms for the altered glucose transport responses to insulin in the rat adipose cell with fasting and refeeding, and further elucidate the extent to which metabolic factors can influence glucose transporter number and intrinsic

activity. The results indicate that refeeding induces an overshoot in insulin-stimulated glucose transport activity similar to that previously reported for insulin-stimulated glucose metabolism (4–6, 8). In addition, these nutritional alterations appear to affect insulin-stimulated glucose transport by at least two divergent mechanisms. The insulin-resistant glucose transport in adipose cells from fasted animals can be explained by a decreased translocation of glucose transporters to the plasma membrane from a depleted intracellular pool. In contrast, the potentiated glucose transport response to insulin in adipose cells from rats refed after fasting is associated with a restoration of the number of glucose transporters translocated only to the normal level and therefore appears to involve an additional modulation of glucose transporter intrinsic activity. The latter observation demonstrates that glucose transporter intrinsic activity can be altered as a result of physiological manipulations in the absence of a disease state or pharmacological interventions.

Methods

Animals and experimental design. Male Sprague-Dawley rats (CD strain, Charles River Breeding Laboratories, Wilmington, MA) were received at body weights ranging from 130 to 140 g and maintained with ad lib. feeding (standard NIH chow) for several days before initiation of the experimental period. Food was then removed at 8:30 a.m. (8:30 p.m. for a 12-h fast) and the animals were fasted for varying periods up to 3 d. Additional animals were refed ad lib. for varying periods up to 13 d after an initial 2-d fast. Control animals were fed ad lib. without interruption. All animals were killed by cervical dislocation and decapitation between 8 and 10 a.m.

Initially, a series of experiments was performed to establish the time course of the effects of fasting and refeeding on basal and maximally insulin-stimulated glucose transport activity. A second series of experiments was then undertaken with 2 d of fasting and 6 d of refeeding to investigate the mechanisms for the alterations observed.

Preparation of isolated adipose cells and measurement of adipose cell size. Immediately after the animals were killed, the whole epididymal fat pads were removed and isolated adipose cells were prepared by the method originally described by Rodbell (24) and subsequently modified by Cushman (25) using crude collagenase (Cooper Biomedical, Malvern, PA). All incubations were carried out in Krebs-Ringer-bicarbonate buffer reduced to 10 mM HCO_3^- and supplemented with 30 mM Hepes (Sigma Chemical Co., St. Louis, MO), pH 7.4, 37°C, containing 1% untreated bovine serum albumin (bovine serum albumin powder, Fractions V, Reheis Chemical Co., Kankakee, IL). Adipose cell size was determined by the osmic acid fixation, Coulter electronic counter method (Method III) described by Hirsch and Gallian (26) for intact tissue fragments, and modified for isolated cell suspensions by Cushman and Salans (27).

Measurement of cellular glucose transport activity. For time course experiments, isolated adipose cells from a minimum of three rats for each experimental group were incubated at 37°C for 30 min in the presence of 0 or 7 nM (1000 μU/ml) insulin (crystalline porcine zinc insulin, courtesy of Dr. Ronald B. Chance, Eli Lilly and Co., Indianapolis, IN). 3-*O*-[^{14}C]methylglucose transport was then assessed by a modification described by Karnieli et al. (28) of the L-arabinose uptake method of Foley et al. (29); 3-*O*-methylglucose was used at a concentration of 0.1 mM. The intracellular water space was assessed as steady-state 3-*O*-methylglucose uptake levels. In experiments designed to determine the Michaelis-Menten constants (K_m) and maximum transport velocities (V_{max}) for 3-*O*-methylglucose transport, cells from a minimum of five rats for each experimental group were incubated at 37°C for 60 min in the presence of 7 nM insulin and 0.5 to 40 mM unlabeled 3-*O*-methylglucose before the measurement of 3-*O*-[^{14}C]-methylglucose transport as just described. For experiments assessing the sensitivity of cells from control and refed rats to the inhibitory effect of cytochalasin B on 3-*O*-methylglucose transport, cells from three to four rats for each experimental group were incubated at 37°C for 30 min with 7 nM insulin and then for 15 min with 0–100 μM cytochalasin B before measurement of 3-*O*-[^{14}C]methylglucose transport as above. For experiments in which subcellular membrane fractions were to be prepared, samples of cells were taken for the determination of 3-*O*-methylglucose transport from larger volume incubations.

Preparation of subcellular membrane fractions, determination of glucose transporter concentration, and assay of plasma membrane glucose transport activity. Isolated adipose cells from a minimum of 27 rats for each experimental group were distributed in two 15-ml samples into 21 ml of incubation medium in each of two 950-ml polypropylene jars and incubated at 37°C for 30 min in the presence of 0 or 700 nM insulin. After removal of samples for the determination of 3-*O*-methylglucose transport, plasma, high density microsomal, and low density microsomal membrane fractions were prepared from the remaining cells by differential ultracentrifugation as previously described (10, 30). Equilibrium D-glucose-inhibitable [^{3}H]cytochalasin B binding was then measured and the concentrations of glucose transporters were calculated (9, 10). In some preparations, glucose transporter cross-reactivity with a rabbit antiserum prepared against the purified human erythrocyte glucose transporter (courtesy of Drs. David C. Sogin and Peter C. Hinkle, Cornell University) was examined also as previously described (31, 32). D-glucose transport into isolated plasma membrane vesicles was measured in some preparations by the method developed by Baldwin et al. (33) as described by Simpson et al. (30).

Membrane protein was determined by the Coomassie Brilliant Blue method (protein assay; Bio-Rad Laboratories, Richmond, CA) described by Bradford (34) and modified by Simpson and Sonne (35) using crystalline bovine serum albumin (Sigma) as the standard. The specific 5′-nucleotidase (36), cytochrome *c* reductase (37), and galactosyltransferase (38) activities of each homogenate and subcellular membrane fraction were also assayed. These marker enzyme activities were used to estimate the relative cross-contaminations and recoveries of plasma membranes, endoplasmic reticulum, and membranes of the Golgi apparatus, respectively (30). Because treatment of adipose cells with insulin had no effect on membrane protein recovery or marker enzyme specific activity, the results from basal and insulin-stimulated cells within each separate experiment were averaged.

Calculations and statistical analyses. The V_{max} and K_m for 3-*O*-methylglucose transport were determined using Eadie-Hofstee plots. The "best fit" curves for the inhibition of 3-*O*-methylglucose transport by cytochalasin B were found using a four parameter logistic model. All calculations were carried out on the Dartmouth Time-Sharing System computer facilities. Comparisons between experimental groups were made using a *t* test of statistical significance and differences were accepted as significant at the $P \leq 0.05$ level.

Results

General characteristics of control, fasted and refed rats. Several physiological parameters were measured sequentially during the 15-d study period. Body weight and adipose cell size decreased progressively with fasting and increased progressively with refeeding, coming close to control levels after 2 wk. Adipose cell intracellular water space, which reflects cytoplasmic mass and correlates with intracellular protein, also decreased with fasting but rapidly surpassed control levels with refeeding and remained slightly elevated at 2 wk. Table I shows these parameters in 2-d fasted and 2-d fasted/6-d refed rats compared with their respective ad lib.-fed control rats. In 2-d fasted

Table I. General Characteristics of Control, 2-d Fasted, and 2-d Fasted/6-d Refed Rats

Parameter	Experimental group			
	Control	Fasted	Control	Refed
Body weight (*g*)	189±3 (7)	149±2* (9)	237±2 (28)	216±2* (27)
Adipose cell size (*μg lipid/cell*)	0.094±0.003 (4)	0.064±0.002* (4)	0.116±0.003 (7)	0.097±0.002* (7)
Adipose cell water space (*pl/cell*)	2.26±0.12 (4)	1.42±0.11* (4)	2.44±0.05 (7)	3.56±0.17* (7)
Adipose cell protein content: (*pg/cell*)				
Homogenate	289±9 (4)	202±9* (4)	350±20 (7)	545±29* (7)
Plasma membranes	26±3	24±2	35±2	33±3
High density microsomes	9±1	7±1	13±2	17±1*
Low density microsomes	15±1	11±1*	20±1	32±3*

Rats were fasted and refed as described in Methods. Body weights were obtained at the time of sacrifice on randomly chosen rats over four and seven separate experiments for fasting and refeeding, respectively. Adipose cell size, intracellular water, and protein content were determined on pooled cells from ≥ 15 rats in each of the four or seven experiments described in Fig. 2. Cell size and intracellular water results are the means±SEM of the mean values obtained from at least duplicate determinations in each of the four or seven experiments. Protein recovery results are the means±SEM of the mean values obtained from triplicate determinations on individual preparations of basal and insulin-stimulated cells in each of the four or seven separate experiments. * Significant difference from corresponding control value. (n) = number of determinations.

rats, body weight is reduced 21%, cell size 32%, and intracellular water 37% compared with control rats of comparable age. With 6 d of refeeding, both body weight and adipose cell size are increased but remain slightly less than the corresponding control levels, while adipose cell intracellular water is increased to 46% greater than control.

Time course of effects of fasting and refeeding on 3-O-methylglucose transport. Fig. 1 illustrates that fasting and refeeding are accompanied by little absolute change in basal glucose transport activity, but marked and rapid changes in the maximally insulin-stimulated rate. During the 15-d experimental period, both the basal and insulin-stimulated glucose transport activities in adipose cells from the ad lib.-fed control rats remain constant. With fasting, the basal glucose transport activity decreases slightly and with refeeding, it returns to the control level. However, the insulin-stimulated glucose transport activity diminishes rapidly with fasting, with a 30% loss after 12 h and a continued progressive loss > 85% at 3 d. In addition, when refeeding is begun after 2 d of fasting, the insulin-stimulated glucose transport activity rapidly increases until it actually overshoots the control value, peaking at ~ 100% above the control level at 6 d and then returning to the baseline over the next week.

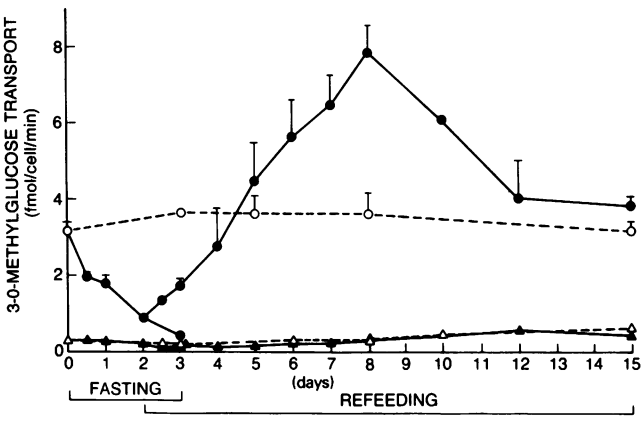

Figure 1. Time course of the effects of fasting or fasting/refeeding on glucose transport activity in the rat adipose cell. Rats were fasted or fasted/refed for sequential periods of time. Isolated cells were prepared from the epididymal fat pads of a minimum of three rats and incubated for 30 min at 37°C in the absence (△, ▲) or presence (○, ●) of 7 nM (1,000 μU/ml) insulin, and 3-*O*-methylglucose transport was measured as described in Methods. Results are the means±SEM of the mean values obtained from quintuplet samples obtained in each of two to eight separate experiments. △, ○, control; ▲, ●, fasted or fasted/refed.

Effects of fasting and refeeding on the subcellular distribution of glucose transporters. Fig. 2, *A* and *B* illustrates the subcellular distributions of glucose transporters in adipose cells from 2-d fasted rats. With fasting, the concentration of glucose transporters in the plasma membranes is unchanged in the basal state, but is markedly decreased in the insulin-stimulated state (Fig. 2 *A*), closely paralleling the decrease in insulin-stimulated glucose transport activity in the intact cell (Fig. 1). In the low density microsomes, fasting is accompanied by a 50% decrease in the concentration of glucose transporters in the basal state and an 83% reduction in the loss of glucose transporters in response to insulin (Fig. 2 *B*).

Fig. 2, *C* and *D* shows the subcellular distributions of glucose transporters in adipose cells from 2-d fasted/6-d refed rats. In the plasma membranes, the concentration of glucose transporters in the basal state is increased 68% with refeeding compared to ad lib. feeding (Fig. 2 *C*). The concentration of glucose transporters in the insulin-stimulated state, however, is restored only to the control level (Fig. 3 *C*), markedly contrasting with the enhanced glucose transport activity observed in the intact cells (Fig. 1). In the low density microsomes, the concentrations of glucose transporters are decreased by 25% in the basal state and 41% in the insulin-stimulated state, with only a 13% reduction in the loss of glucose transporters in response to insulin (Fig. 2 *D*).

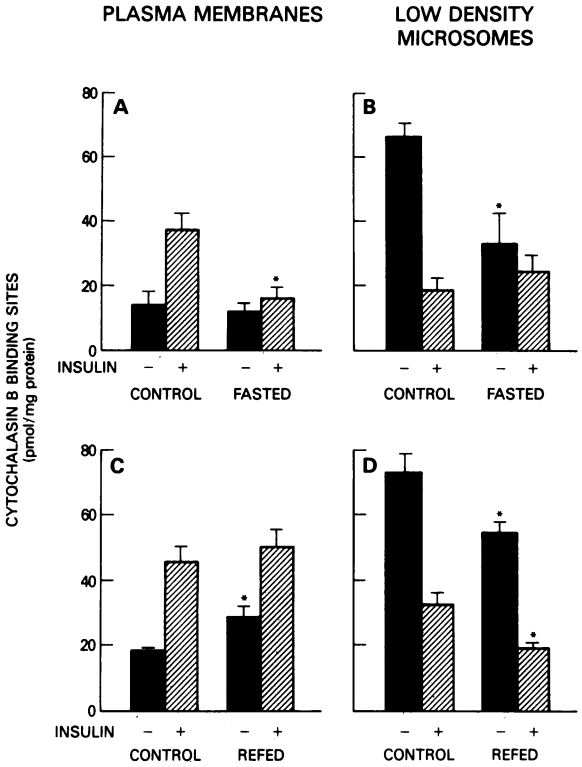

Figure 2. Concentrations of glucose transporters in plasma membranes (*A, C*) and low density microsomes (*B, D*) of basal and maximally insulin-stimulated adipose cells from control, 2-d fasted (*A, B*) and 2-d fasted/6-d refed (*C, D*) rats. Isolated cells were prepared from the epididymal fat pads of a minimum of 15 rats in each group and incubated for 30 min at 37°C in the absence or presence of 700 nM (100,000 μU/ml) insulin, and subcellular membrane fractions were prepared and the concentrations of glucose transporters determined as described in Methods. Results are the means±SEM of the individual values obtained in each of the four and seven separate experiments for fasting and fasting/refeeding, respectively. The following table shows the corresponding 3-*O*-methylglucose transport rates per cell and per unit cellular surface area (means±SEM of the mean values) in the intact cells:

Experimental group	Insulin	fmol/cell/min	amol/μm²/min
Control	−	0.2±0.0	0.02±0.01
	+	3.9±0.5	0.36±0.05
Fasted	−	0.2±0.1	0.02±0.00
	+	1.3±0.3*	0.15±0.04*
Control	−	0.2±0.0	0.02±0.00
	+	4.5±0.2	0.36±0.01
Refed	−	0.3±0.0*	0.03±0.00*
	+	7.1±0.4*	0.65±0.04*

* Significant difference from corresponding control value.

Relatively few glucose transporters are observed in the high density microsomes in adipose cells from either group and in previous studies these have been shown to result primarily from cross-contamination with membranes from the other two subcellular fractions (30). Accordingly, the concentrations of glucose transporters in the high density microsomes from cells of the refed rats are greater than those from cells of the control rats in both the basal (15 vs. 5 pmol/mg of membrane protein, average of two experiments) and insulin-stimulated (19 vs. 10 pmol/mg of membrane protein) states.

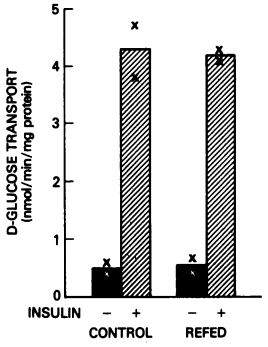

Figure 3. D-Glucose transport in plasma membrane vesicles of basal and maximally insulin-treated adipose cells from control and 2-d fasted/6-d refed rats. Isolated cells were prepared and incubated and subcellular membrane fractions were prepared as described in Fig. 2, and D-glucose uptake was measured as described in Methods. Results are the average of the mean values (*x*) obtained from sextuplet samples in each of two separate experiments.

Differences in the amounts of membrane protein present in the cell or in the recoveries (membrane protein recovered in each fraction divided by homogenate protein) or relative enrichments (specific activity in each fraction divided by specific activity in the fraction for which that enzyme is a marker) of the various membrane fractions may result in changes in the estimated number of glucose transporters per cell when the concentrations per mg of membrane protein are unchanged. For this reason, the protein recoveries were measured for each membrane fraction from adipose cells of the control, fasted, and refed rats (Table I) as well as the percent recoveries and relative enrichments of several specific marker enzyme activities (Tables II and III, respectively): 5′-nucleotidase, characteristic of plasma membranes (36); cytochrome *c* reductase, characteristic of endoplasmic reticulum (37); and galactosyltransferase, characteristic of the Golgi apparatus in many cell types (38), but probably less specific in the rat adipose cell (30, 39).

Table I demonstrates that the recoveries of total homogenate and low density microsomal protein are decreased with fasting, whereas plasma membrane and high density microsomal protein are unchanged. With refeeding, the recoveries of total, and high and low density microsomal protein are increased 56, 31, and 60%, respectively, while plasma membrane protein remains unchanged.

Table II illustrates that fasting is associated with an increased recovery of galactosyltransferase activity in the plasma membranes. Table III further illustrates that fasting is accompanied by an increased relative enrichment of the plasma membranes with galactosyltransferase activity and the low density microsomes with 5′-nucleotidase activity. The actual galactosyltransferase specific activity in the low density microsomes is reduced. Refeeding, on the other hand, is associated with a reduced recovery of 5′-nucleotidase activity in the plasma membranes, and galactosyltransferase activity in the plasma membranes and low density microsomes (Table II). In addition, refeeding is accompanied by an increased relative enrichment of galactosyltransferase in the plasma membranes and high density microsomes, and cytochrome *c* reductase in the plasma membranes (Table III).

Table II. Recovery of Marker Enzyme Activities from Adipose Cells of Control, 2-d Fasted, and 2-d Fasted/6-d Refed Rats

Marker enzyme activity	Experimental group	Membrane fraction: Plasma membranes	High density microsomes	Low density microsomes
		% of homogenate activity		
5′-Nucleotidase	Control	50.7±3.8	5.4±0.7	2.2±0.2
	Fasted	44.2±0.9	5.1±0.6	2.9±0.3
Rotenone-insensitive NADH-cytochrome *c* reductase	Control	17.8±2.4	19.2±0.9	13.8±1.2
	Fasted	20.7±6.4	18.5±5.4	12.4±5.0
UDP-galactose:*N*-acetylglucosamine galactosyltransferase	Control	17.0±1.9	11.6±0.9	29.6±2.3
	Fasted	34.2±5.3*	11.3±2.6	26.6±2.8
5′-Nucleotidase	Control	46.0±4.0	4.4±0.6	1.8±0.3
	Refed	31.0±3.0*	5.9±0.5	1.8±0.8
Rotenone-insensitive NADH-cytochrome *c* reductase	Control	14.2±1.3	14.0±0.7	11.3±0.3
	Refed	11.8±1.2	13.2±1.4	10.7±0.9
UDP-galactose:*N*-acetylglucosamine galactosyltransferase	Control	24.0±1.0	12.3±0.9	35.0±2.0
	Refed	16.0±1.0*	11.1±0.7	25.0±1.0*

Marker enzyme activities were measured in the original homogenates and membrane fractions prepared in the experiments described in Fig. 2 using the procedures described in Methods. Within each experiment, total marker enzyme activities for each membrane fraction from basal and insulin-stimulated cells were expressed as a percentage of the respective homogenate activities and the percent activities for the basal and insulin-stimulated cells were averaged. Results are the means±SEM of the mean values obtained from quadruplicate samples of individual preparations of basal and insulin-stimulated cells in each of three to four separate experiments. * Significant difference from corresponding control value.

Table III. Relative Marker Enzyme Specific Activities among Membrane Fractions from Adipose Cells of Control, 2-d Fasted, and 2-d Fasted/6-d Refed Rats

Marker enzyme activity	Experimental group	Membrane fraction: Plasma membranes	High density microsomes	Low density microsomes
		% of highest specific activity		
5′-Nucleotidase (*μmol/mg/h*)	Control	100 (1.17±0.22)*	28.8±2.3	6.5±0.7
	Fasted	100 (1.69±0.23)*	32.0±2.3	11.4±0.4‡
Rotenone-insensitive NADH-cytochrome *c* reductase (*μmol/mg/min*)	Control	33.8±2.8	100 (2.44±0.31)*	40.2±3.4
	Fasted	33.3±2.7	100 (2.23±0.26)*	44.4±5.1
UDP-galactose:*N*-acetylglucosamine galactosyltransferase	Control	39.5±4.8	68.7±5.2	100 (99.2±5.3)*
	Fasted	58.0±0.0‡	56.5±2.5	100 (53.0±11.2‡)*
5′-Nucleotidase (*μmol/mg/h*)	Control	100 (0.97±0.03)*	28.3±2.1	6.0±0.7
	Refed	100 (1.02±0.09)*	34.1±4.1	5.0±0.8
Rotenone-insensitive NADH-cytochrome *c* reductase (*μmol/mg/min*)	Control	31.3±4.8	100 (2.56±0.29)*	46.1±6.3
	Refed	49.1±5.8‡	100 (1.76±0.29)*	41.6±4.3
UDP-galactose:*N*-acetylglucosamine galactosyltransferase	Control	36.7±2.1	59.5±3.7	100 (126.8±6.4)*
	Refed	64.3±4.3‡	85.7±5.6‡	100 (111.0±4.7)*

Marker enzyme activities were measured in the membrane fractions prepared in the experiments described in Fig. 2 using the procedures described in Methods. Within each experiment, marker enzyme specific activities were obtained for each membrane fraction prepared from basal and insulin-stimulated cells and expressed as a percentage of the respective highest activity observed. Results are the means±SEM of the average of the basal and insulin-stimulated values obtained in each of three to four separate experiments. * Actual specific activity set at 100% (mean±SEM). ‡ Significant difference from corresponding control value.

Table IV. Kinetic Parameters of 3-O-Methylglucose Transport in Adipose Cells from Control and 2-d Fasted/6-d Refed Rats

Experimental group	Kinetic parameter	
	V_{max}	K_m
	fmol/cell/min	*mM*
Control	148±24	4.78±0.04
Refed	351±33*	4.74±0.14

Isolated adipose cells were prepared from the epididymal fat pads of five rats in each group and incubated for 60 min at 37°C in the presence of 7 nM (1,000 μU/ml) insulin and 0.5–40 mM unlabeled 3-*O*-methylglucose. 3-*O*-methylglucose transport was then determined as described in Methods. Results are the means±SEM of the mean values obtained from triplicate samples in each of three separate experiments.

* Significant difference from control value.

Further studies to elucidate the discrepancy between glucose transport activity in the intact adipose cell and glucose transporter concentration in the plasma membranes with refeeding. Because the increase in the insulin-stimulated concentration of glucose transporters in the plasma membranes of adipose cells from the refed rats is much less than the marked increase in insulin-stimulated glucose transport activity observed in the intact cell, further studies were undertaken to characterize this insulin hyperresponsiveness. For example, a decrease in the K_m indicating an increase in the apparent affinity of the glucose transporter could potentially explain an increase in glucose transport activity in the absence of a corresponding increase in glucose transporter number. However, as shown in Table IV, refeeding is associated with a substantial increase in V_{max} with no change in K_m. The apparent lack of alteration in the interaction of the glucose transporter with its substrate is further indicated by no change in the K_d of the glucose transporter for the binding of cytochalasin B, a potent competitive inhibitor (control, 110±9 nM; fasted/refed, 115±12 nM; mean±SEM), or the K_i of cytochalasin B for insulin-stimulated glucose transport activity in the intact cells (control, 721 nM; fasted/refed, 894 nM; average of two experiments).

Another potential explanation for insulin-hyperresponsiveness is the induction with refeeding of a second glucose transporter not measured by the cytochalasin B binding assay. This is unlikely because cytochalasin B completely inhibits insulin-stimulated glucose transport activity in the intact adipose cells from both the control and refed rats (data not shown). Finally, if this hyperresponsiveness were to represent an increase in glucose transporter intrinsic activity, it could reflect either the presence of an allosteric effector in the cell or a more direct, perhaps covalent, modification of the glucose transporter and/or its microenvironment in the membrane. Fig. 3 illustrates that in contrast to the glucose transport activities observed in the intact cell (Fig. 1), D-glucose transport measured in plasma membrane vesicles prepared from insulin-stimulated cells is unchanged with refeeding compared to the control values, thus correlating closely with the concentrations of glucose transporters. In the basal state, plasma membrane glucose transport is lower than expected relative to the concentrations of glucose transporters, probably reflecting the presence of low density microsomal glucose transporters in the plasma membrane fraction.

To determine whether structural modifications in the glucose transporter might have occurred as a result of fasting and refeeding, membrane fractions from the adipose cells of control and refed rats were probed by Western blotting with an antiserum prepared against the purified human erythrocyte glucose transporter (31, 32). Fig. 4 shows increased antiserum cross-reactivity at $M_r \cong 45{,}000$ in the plasma membranes of

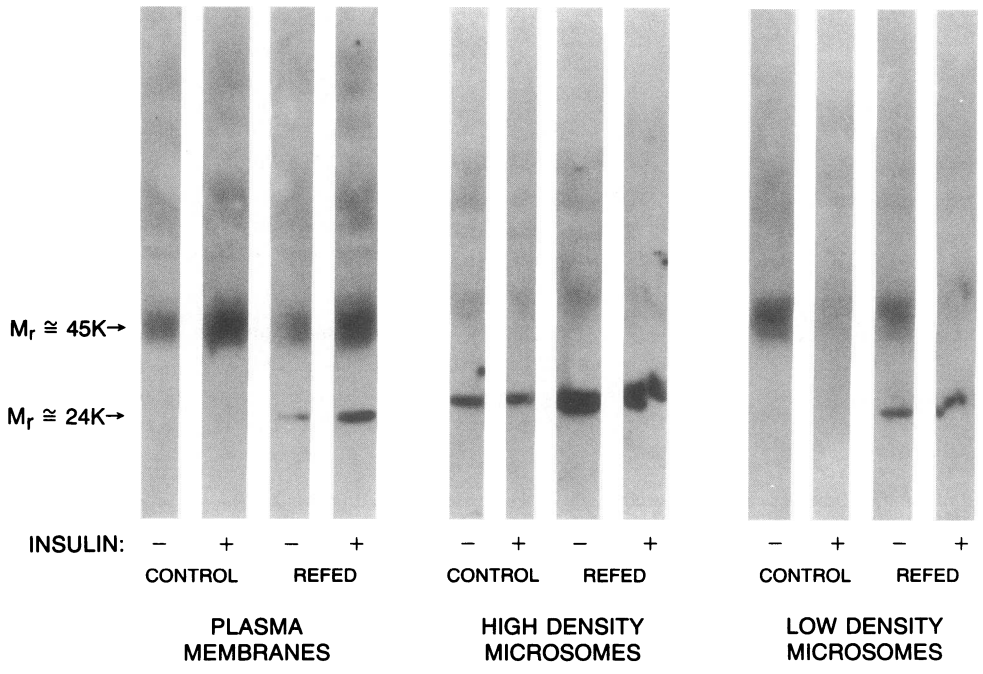

Figure 4. Immunological detection of glucose transporters in subcellular membrane fractions of basal and maximally insulin-stimulated adipose cells from control and 2-d fasted/6-d refed rats. Isolated cells were prepared and incubated and subcellular membrane fractions were prepared as described in Fig. 2, and glucose transporters were assessed by Western blotting using an anti-human erythrocyte glucose transporter antiserum as described in Methods. Each lane was loaded with 100 μg of membrane protein. The Western blot shown is representative of four separate experiments.

basal cells from the refed rats compared to control, but no differences in the intensity of the cross-reaction at $M_r \cong 45{,}000$ or of the relative molecular mass itself of the reacting protein in the plasma membranes of the insulin-stimulated cells. These results correlate closely with those obtained using the cytochalasin B binding assay. Similarly, antiserum cross-reactivity at $M_r \cong 45{,}000$ concurs with the results of the cytochalasin B binding assay in the high and low density microsomes. However, refeeding is associated with a marked increase in antiserum cross-reactivity at $M_r \cong 24{,}000$, particularly in the high density microsomes, but also in the plasma membranes and low density microsomes. The intensity of this band does not appear to change with insulin treatment of the intact cells nor does it correlate with either cytochalasin B binding (Fig. 2) or plasma membrane glucose transport (Fig. 3). Thus, this band is probably not directly related to the alterations observed in glucose transport activity.

Discussion

This study demonstrates that fasting and refeeding in the rat are associated with rapid, progressive, and reversible adaptations in the glucose transport response to insulin in the adipose cell and that the mechanisms for the changes observed with fasting and refeeding differ. With fasting, glucose transporter concentration in the plasma membranes (Fig. 2) correlates with glucose transport activity in the intact cell (Fig. 1) in both the basal and insulin-stimulated states. Major changes in cell fractionation can be excluded since only minor differences in the recoveries of membrane protein (Table I), and the recoveries and relative specific activities of membrane marker enzymes (Tables II and III, respectively) are observed when comparing cells from the fasted and control rats. Therefore, the insulin resistant glucose transport activity in the intact cell can be fully explained by the decreased concentration of glucose transporters in the plasma membranes in the insulin-stimulated state.

In contrast, in adipose cells from the refed rats, the concentration of glucose transporters in the plasma membranes (Figs. 2 and 4) does not correlate with glucose transport activity in the intact cell (Fig. 1) in either the basal or insulin-stimulated states. Marker enzyme recoveries (Table II) and relative specific activities (Table III), and protein recoveries (Table I) suggest the presence of contaminating protein from the high- and low-density microsomes which preferentially distorts the relatively low value for glucose transporters in the basal state. Additionally, these assays indicate that the recoveries of both plasma membranes and low density microsomes may be decreased in cells from the refed rats (Table II). However, a calculation adjusting for the decreased recovery of plasma membranes would be offset by a similar adjustment for the decreased recovery of low density microsomes. Thus, the changes in marker enzyme relative specific activities and recoveries do not appreciably alter the interpretation of the concentrations of glucose transporters in any of the subcellular membrane fractions, and an effect of refeeding after fasting on plasma membrane glucose transporter intrinsic activity in the insulin-stimulated state must be proposed to explain the marked discrepancy between glucose transport activity in the intact cell and the corresponding concentration of glucose transporters in the plasma membranes. Acute modulation of glucose transporter intrinsic activity by in vitro incubation of insulin-stimulated rat adipose cells with adenylate cyclase stimulators and inhibitors has recently been observed (40–42). In addition, we have also recently reported an apparent chronic regulation of glucose transporter intrinsic activity associated with insulin treatment of diabetic rats (20–23).

Further studies were therefore undertaken to examine the mechanism(s) through which a change in glucose transporter intrinsic activity could take place, with initial consideration given to possible alterations in the interaction of cytochalasin B with the glucose transporter. This proved unlikely since differences between control and refeeding were not apparent in: the sensitivity of the intact adipose cells to cytochalasin B inhibition of glucose transport activity (K_i), the affinity (K_d) of the glucose transporter in the subcellular membrane fractions for cytochalasin B, the affinity of the glucose transporter for its antibody (Fig. 4), or the relative molecular mass of the glucose transporter (Fig. 4).

A highly cross-reactive protein species is seen by immunoblotting, however, at $\sim 24{,}000$ M_r in all membrane fractions from adipose cells of the refed rats (Fig. 4). This band is also observed in membranes from cells of the control rats in some experiments, as is evident here in the high density microsomes (Fig. 4). A species with the same relative molecular mass has been observed in membranes from cells associated with two other states of hyperresponsive glucose transport activity: adipose cells from insulin-treated streptozotocin diabetic rats (20) and fibroblasts transfected with an activated *ras* oncogene (43). Therefore, although this protein is present to some extent in the control state, it appears to be more prevalent in states where glucose transport activity is increased.

The $\sim 24{,}000$ M_r band is present in greatest intensity in the high density microsomes and its distribution roughly parallels those of marker enzyme activities characteristic of the endoplasmic reticulum where glucose transporter concentration is lowest (30). Furthermore, addition of protease inhibitors to cells before homogenization and during membrane preparation has no consistent effect on the relative intensities of the $\sim 45{,}000$ and $\sim 24{,}000$ M_r bands (data not shown), suggesting that the $\sim 24{,}000$ M_r species is not a degradation product of the glucose transporter. Finally, the lack of correlation of the intensity of the $\sim$ 24K band with the cytochalasin B binding results (Fig. 2, *C* and *D*), including those obtained with the high density microsomes (see text), and with the plasma membrane glucose transport results (Fig. 3), indicates that this species does not appear to either bind cytochalasin B or transport glucose, respectively. Therefore, while the identity of this $\sim 24{,}000$ M_r protein is unknown, it is unlikely to explain the insulin hyperresponsive glucose transport activity observed here in the intact cells from refed rats.

Finally, glucose transport in the plasma membrane vesicles from insulin-stimulated adipose cells of the refed rats (Fig. 3) closely correlates with the concentrations of glucose transporters assessed by cytochalasin B binding (Fig. 2 *C*) or Western blotting (Fig. 4). Thus, the apparent alteration in glucose transporter intrinsic activity associated with refeeding is lost with cell disruption. This observation, coupled with the lack of change in relative molecular mass on Western blotting makes a permanent structural change in the glucose transporter unlikely and suggests that the altered intrinsic activity may be due

to a modifying factor in the intact cell. A modifying factor with an inhibitory effect has been suggested previously as a result of changes in glucose transport activity induced by dexamethasone in both rat adipose cells (44) and thymocytes (45).

In previously reported states of altered insulin responsiveness, changes in the concentrations of glucose transporters in the plasma membranes have usually reflected parallel changes in the low density microsomes (13–15). This is evident in cells from the fasted rats (Fig. 2, *A* and *B*), but not in cells from the refed rats where the concentrations of glucose transporters per milligram of membrane protein are the same in the plasma membranes (Fig. 2 *C*) but decrease 40% in the low density microsomes (Fig. 2 *D*). However, the recovery of low density microsomal protein is substantially increased (Table I) and therefore the actual number of glucose transporters in this fraction is equivalent to control. This explains a restoration of insulin responsiveness to the control level from the depressed state associated with fasting, but again does not explain the hyperresponsiveness.

Thus, the refed rat model is similar to the insulin-treated diabetic rat in that reversal of a catabolic state, whether fasting or diabetes, results in restoration of glucose transporter number to approximately normal levels, but a dramatic overshoot in glucose transport activity. This appears to be due to an enhancement of glucose transporter intrinsic activity. In contrast to the insulin-treated diabetic rat, however, the refed rat shows this enhancement in the absence of a disease state, pharmacological doses of insulin, or relative hypoglycemia, all of which have been postulated to play a role in enhanced insulin responsiveness (19, 20).

In summary, fasting and refeeding in rats are accompanied by rapid, progressive, and reversible changes in insulin-stimulated glucose transport activity in the adipose cell. The mechanisms for these alterations differ: the insulin resistant glucose transport seen with fasting can be explained by a reduction in the number of glucose transporters in the plasma membrane resulting from a depleted intracellular pool. Refeeding results in hyper-insulin-responsive glucose transport with only a restoration to normal of the number of glucose transporters in both the plasma membrane and intracellular pool. Therefore, the additional insulin responsiveness appears to be due to enhanced glucose transporter intrinsic activity.

Acknowledgments

The authors wish to thank Dr. Hans G. Joost for critically reading the manuscript, Mary Jane Zarnowski, Dena R. Yver, and Steven Richards for their expert technical assistance, and Bonnie Richards and Denise R. Giles for their patience and expertise in typing the manuscript.

References

1. Moore, R. O. 1963. Influence of fasting and refeeding on response of adipose tissue to insulin. *Am. J. Physiol.* 205:222–224.

2. Olefsky, J. M. 1976. Effects of fasting on insulin binding, glucose transport and glucose oxidation in isolated rat adipocytes. *J. Clin. Invest.* 58:1450–1460.

3. Kasuga, M., Y. Akanuma, Y. Iwamoto, and K. Kosaka. 1977. Effects of fasting and refeeding on insulin receptors and glucose metabolism in rat adipocytes. *Endocrinology.* 100:1384–1390.

4. Owens, J. L., D. Thompson, N. Shah, and M. DiGirolamo. 1979. Effects of fasting and refeeding in the rat on adipocyte metabolic functions and response to insulin. *J. Nutr.* 109:1584–1591.

5. Bjorntorp, P., G. Enzi, M. Karlsson, J. Kral, B. Larsson, L. Sjostrom, and U. Smith. 1980. Effects of refeeding on adipocyte metabolism in the rat. *Int. J. Obes.* 4:11–19.

6. Timmers, K., and J. Knittle. 1980. Effects of undernutrition and refeeding on enzyme activities and rates of glucose catabolism in rat epididymal adipose tissue. *J. Nutr.* 110:1176–1184.

7. Pedersen, O., E. Hjollund, and N. S. Sorensen. 1982. Insulin receptor binding and insulin action in human fat cells: effects of obesity and fasting. *Metab. Clin. Exp.* 31:884–895.

8. Huber, A. M., S. N. Gershoff, and H. N. Antoniades. 1965. The effect of fasting and refeeding on isolated rat adipose tissue activity in the presence of crystalline and "bound" insulin. *Metab. Clin. Exp.* 14:619–624.

9. Wardzala, L. J., S. W. Cushman, and L. B. Salans. 1978. Mechanism of insulin action on glucose transport in the isolated rat adipose cell. *J. Biol. Chem.* 253:8002–8005.

10. Cushman, S. W., and L. J. Wardzala. 1980. Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. Apparent translocation of intracellular transport systems to the plasma membrane. *J. Biol. Chem.* 255:4758–4762.

11. Suzuki, K., and T. Kono. 1980. Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc. Natl. Acad. Sci. USA.* 77:2542–2545.

12. Kono, T., K. Suzuki, L. E. Dansey, R. W. Robinson, and T. L. Blevins. 1981. Energy-dependent and protein synthesis-independent recycling of the insulin-sensitive glucose transport mechanism in fat cells. *J. Biol. Chem.* 256:6400–6407.

13. Hissin, P. J., E. Karnieli, I. A. Simpson, L. B. Salans, and S. W. Cushman. 1982. A possible mechanism of insulin resistance in the rat adipose cell with high fat/low carbohydrate feeding: depletion of intracellular glucose transport systems. *Diabetes.* 31:589–592.

14. Hissin, P. J., J. E. Foley, L. J. Wardzala, E. Karnieli, I. A. Simpson, L. B. Salans, and S. W. Cushman. 1982. Mechanism of insulin-resistant glucose transport in the enlarged adipose cell of the aged, obese rat. Relative depletion of intracellular glucose transport systems. *J. Clin. Invest.* 70:780–790.

15. Karnieli, E., P. J. Hissin, I. A. Simpson, L. B. Salans, and S. W. Cushman. 1981. A possible mechanism of insulin resistance in the rat adipose cell in streptozotocin-induced diabetes mellitus. Depletion of intracellular glucose transport systems. *J. Clin. Invest.* 68:811–814.

16. Horuk, R., M. Rodbell, S. W. Cushman, and L. J. Wardzala. 1983. Proposed mechanism of insulin-resistant glucose transport in the isolated guinea pig adipocyte. Small intracellular pool of glucose transporters. *J. Biol. Chem.* 258:7425–7429.

17. Karnieli, E., B. Chernow, P. J. Hissin, I. A. Simpson, and J. E. Foley. 1986. Insulin stimulates glucose transport in isolated human adipose cells through a translocation of intracellular glucose transporters to the plasma membrane: a preliminary report. *Horm. Metab. Res.* 18:860–861.

18. Karnieli, E., A. Barzilai, R. Rafaeloff, and M. Armoni. 1986. Distribution of glucose transporters in membrane fractions isolated from human adipose cells. Relation to cell size. *J. Clin. Invest.* 78:1051–1055.

19. Kahn, B. B., E. S. Horton, and S. W. Cushman. 1987. Mechanism for enhanced glucose transport response to insulin in adipose cells from chronically hyperinsulinemic rats. Increased translocation of glucose transporters from an enlarged intracellular pool. *J. Clin. Invest.* 79:854–859.

20. Kahn, B. B., and S. W. Cushman. 1987. Mechanism for markedly hyperresponsive insulin-stimulated glucose transport activity in adipose cells from insulin-treated streptozotocin diabetic rats. Evidence for increased glucose transporter intrinsic activity. *J. Biol. Chem.* 262:5118–5124.

21. Guerre-Millo, M., M. Lavau, J. S. Horne, and L. J. Wardzala. 1985. Increased number of glucose transport systems in adipocytes

from young, hyperinsulinemic obese Zucker rats. *J. Biol. Chem.* 260:2197–2201.

22. Vinten, J., L. N. Petersen, B. Sonne, and H. Galbo. 1985. Effects of physical training on glucose transporter in fat cell fractions. *Biochim. Biophys. Acta.* 841:223–227.

23. Karnieli, E., M. Armoni, P. Cohen, Y. Kanter, and R. Rafaeloff. 1987. Reversal of insulin resistance in diabetic rat adipocytes by insulin therapy: restoration of pool of glucose transporters and enhancement of glucose transporter activity. *Diabetes.* 36:925–931.

24. Rodbell, M. 1964. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* 239:375–380.

25. Cushman, S. W. 1970. Structure-function relationship in the adipose cell. I. Ultrastructure of the isolated adipose cell. *J. Cell Biol.* 46:326–341.

26. Hirsch, J., and E. Gallian. 1968. Methods for determination of adipose cell size in man and animals. *J. Lipid Res.* 9:110–119.

27. Cushman, S. W., and L. B. Salans. 1978. Determination of adipose cell size and number in suspensions of isolated rat and human adipose cells. *J. Lipid Res.* 19:269–273.

28. Karnieli, E., M. J. Zarnowski, P. J. Hissin, I. A. Simpson, L. B. Salans, and S. W. Cushman. 1981. Insulin-stimulated translocation of glucose transport systems in the isolated rat adipose cell. Time course, reversal, insulin concentration-dependency and relationship to glucose transport activity. *J. Biol. Chem.* 256:4772–4777.

29. Foley, J. E., S. W. Cushman, and L. B. Salans. 1978. Glucose transport in isolated rat adipocytes with measurements of L-arabinose uptake. *Am. J. Physiol.* 234:E112–E119.

30. Simpson, I. A., D. R. Yver, P. J. Hissin, L. J. Wardzala, E. Karnieli, L. B. Salans, and S. W. Cushman. 1983. Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cell: characterization of subcellular fractions. *Biochim. Biophys. Acta.* 763:393–407.

31. Wheeler, T. J., I. A. Simpson, D. C. Sogin, P. C. Hinkle, and S. W. Cushman. 1982. Detection of the rat adipose cell glucose transporter with antibody against the human red cell glucose transporter. *Biochem. Biophys. Res. Commun.* 105:89–95.

32. Lienhard, G. E., H. K. Kin, K. J. Ransome, and J. C. Gorga. 1982. Immunological identification of an insulin-responsive glucose transporter. *Biochem. Biophys. Res. Commun.* 105:1150–1156.

33. Baldwin, J. M., J. C. Gorga, and G. E. Lienhard. 1981. The monosaccharide transporter of the human erythrocyte. Transport activity upon reconstitution. *J. Biol. Chem.* 256:3685–3689.

34. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein, utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

35. Simpson, I. A., and O. Sonne. 1982. A simple, rapid and sensitive method for measuring protein concentration in subcellular membrane fractions prepared by sucrose density centrifugation. *Anal. Biochem.* 119:424–427.

36. Avruch, J., and D. F. Hoelzl-Wallach. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. *Biochim. Biophys. Acta.* 233:334–347.

37. Dallner, G., P. Siekevitz, and G. E. Palade. 1966. Biogenesis of endoplasmic reticulum membranes. II. Synthesis of constitutive microsomal enzymes in developing rat hepatocyte. *J. Cell Biol.* 30:97–117.

38. Fleischer, B. 1974. Isolation and characterization of Golgi apparatus and membranes from rat liver. *Methods Enzymol.* 31:180–191.

39. Smith, M. M., F. W. Robinson, T. Watanabe, and T. Kono. 1984. Partial characterization of the glucose transport activity in the Golgi-rich fraction of fat cells. *Biochim. Biophys. Acta.* 775:121–128.

40. Smith, U., M. Kuroda, and I. A. Simpson. 1984. Counterregulation of insulin-stimulated glucose transport by catecholamines in the isolated rat adipose cell. *J. Biol. Chem.* 259:8758–8763.

41. Joost, H. G., T. W. Weber, S. W. Cushman, and I. A. Simpson. 1986. Insulin-stimulated glucose transport in rat adipose cells. Modulation of transporter intrinsic activity by isoproterenol and adenosine. *J. Biol. Chem.* 261:10033–10036.

42. Kuroda, M., R. C. Honnor, S. W. Cushman, C. Londos, and I. A. Simpson. 1987. Regulation of insulin-stimulated glucose transport in the isolated rat adipocyte. cAMP-independent effects of lipolytic and antilipolytic agents. *J. Biol. Chem.* 262:245–253.

43. Flier, J. S., M. M. Mueckler, P. Usher, and H. F. Lodish. 1987. Elevated levels of glucose transport and transporter messenger RNA are induced by *ras* or *src* oncogenes. *Science (Wash. DC).* 235:1492–1495.

44. Carter-Su, C., and K. Okamoto. 1985. Inhibition of hexose transport in adipocytes by dexamethasone: role of protein synthesis. *Am. J. Physiol.* 248:E215–E223.

45. Mosher, K. M., D. A. Young, and A. Munck. 1971. Evidence for irreversible actinomycin D-sensitive, and temperature-sensitive step following binding of cortisol to glucocorticoid receptors and preceding effects on glucose metabolism in rat thymus cells. *J. Biol. Chem.* 246:654–659.