Role of Intracellular Energy in Insulin's Ability to Activate 3-*O*-Methylglucose Transport by Rat Adipocytes†

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ABSTRACT: The energy dependence of insulin's ability to activate adipocyte glucose transport was studied by measuring initial rates of transport using a rapid assay of 3-*O*-methylglucose uptake permitting measurements within 1–4 s of incubation. It was found that a maximally effective insulin level (25 ng/mL) activates glucose transport by increasing V_{max} from 0.4 ± 0.1 to 2.2 ± 0.3 nmol/min per 2×10^5 cells without changing the K_m value (6.6 ± 1.2 mM). The K_i value for D-glucose averaged 8.8 mM and that of 2-deoxyglucose was 3.6 mM. Thus, this transport system displayed stereospecificity for glucose and its analogues; the order of affinity is 2-deoxyglucose > 3-*O*-methylglucose > D-glucose. Dinitrophenol (DNP) and NaCN completely inhibited insulin's effect to activate glucose transport, provided the inhibitor was added before, or at the same time as, insulin. Importantly, these agents had no influence on insulin-activated glucose transport if the cells were preincubated with insulin (40 min) before the inhibitor was added. Additionally, these agents did not inhibit basal rates of transport. Further studies indicated that DNP inhibited the ability of insulin to increase the V_{max} of transport and did not alter the K_m. After an initial lag phase, high concentrations of insulin (100 ng/mL) activate glucose transport gradually with half-maximal and maximal activation at 9 and 20 min, respectively (24 °C). When insulin was added to cells, followed by the subsequent addition of DNP at various intervals (up to 20 min following insulin addition), and the incubation with both agents was continued for an additional 30 min, glucose transport activation was always equal to that degree of activation achieved at the time the DNP was added. Thus, the degree of activation achieved by insulin is "locked in" following DNP addition and further activation is prevented. In conclusion, (1) energy (ATP) is necessary for insulin to activate the glucose transport system but not necessary to maintain the system in a previously activated state or to support basal rates of transport and (2) insulin gradually activates glucose transport sites, and if cells are exposed to energy depleters while this process is ongoing, further coupling between insulin receptors and glucose transport is prevented, and glucose transport is "locked" into a fixed, partially activated state.

Insulin stimulates glucose transport across the adipocyte membrane after binding to specific surface receptors (Czech, 1977). Insulin's ability to activate the glucose transport system is mediated through an increase in the V_{max} of the transport system with no change in the K_m for glucose (Vinten et al., 1976; Olefsky, 1978), and recent studies have indicated that this effect of insulin is dependent on energy (Kono et al., 1977; Chandramouli et al., 1977). We have reported that following a temperature-dependent lag period, insulin receptor complexes can activate glucose transport units, and this process proceeds with a relatively high apparent activation energy (E_A = 24 kcal/mol), again suggesting an energy requirement for this step (Ciaraldi & Olefsky, 1979). However, the mechanism by which insulin receptor occupancy signals an increase in V_{max} of glucose transport is still unknown, as is the specific role of energy in this process. A major obstacle had been the inability to directly and reliably quantitate initial rates of glucose transport, as opposed to assessing end points in glucose metabolism. Recently, Gliemann & Whitesell (1977) have developed a novel method to measure initial rates of 3-*O*-methylglucose transport in rat adipocytes. Using a modification of this method, we have directly studied this transport system and have further explored the role of intracellular energy in insulin's ability to stimulate glucose transport.

Materials and Methods

Materials. Porcine monocomponent insulin was generously supplied by Dr. Ronald Chance of the Eli Lilly and Co. (Indianapolis, IN). D-3-*O*-Methyl[U-^{14}C]glucose and L-[^{14}C]-glucose were purchased from New England Nuclear (Boston, MA), bovine serum albumin (fraction V) was from Armour Pharmaceutical Co. (Phoenix, AZ), collagenase was from Worthington Biochemical Corp. (Freehold, NJ), D-glucose, L-glucose, D-2-deoxyglucose, D-3-*O*-methylglucose, 2,4-dinitrophenol,[1] and NaCN were from Sigma Chemical Co. (St. Louis, MO), phloretin was from Biochemical Laboratories Co. (Redondo Beach, CA), and silicone oil was from Union Carbide (New York, NY).

Preparation of Isolated Adipocytes. All studies used male Sprague–Dawley rats (150–200 g) which were given free access to lab chow. All studies were begun between 8 and 10 a.m. Animals were stunned by a blow to the head and decapitated, and epididymal fat pads were removed. Isolated fat cells were prepared by shaking in a 37 °C water bath for 60 min in Krebs Ringer bicarbonate buffer with 1 mM $CaCl_2$, containing collagenase (3 mg/mL) and albumin (40 mg/mL) according to the method of Rodbell (1964). Cells were then filtered through 250-μm nylon mesh, centrifuged at 400 rpm for 3 min, and washed 2 times in buffer. Adipocyte counts were performed according to a modification of method III of Hirsch & Gallian (1968), in which the cells were fixed in 2% OsO_4 in 0.05 M collidine buffer (made isotonic with NaCl) for 24 h at 37 °C and then taken up in a known volume of 0.154 M NaCl for counting. Counting was performed by using a Model ZB Coulter counter with a 400-μm aperture, and all data were normalized to a cell concentration of 2×10^5 cells/mL.

3-O-Methylglucose Transport. 3-*O*-Methylglucose transport was assayed by a modification of the method of Gliemann

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[1] Abbreviations used: DNP, 2,4-dinitrophenol; ATP, adenosine triphosphate; BSA, bovine serum albumin.

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& Whitesell (1977). The substrate (D-3-*O*-methyl[U-^{14}C]-glucose, 0.2–0.4 μCi) and all other agents were included in a 20-μL volume in 17 × 100 mm plastic test tubes. The hexose concentrations employed are given in the figure legends. The reaction was started by the rapid addition of 50 μL of a concentrated cell suspension containing (1.1–1.9 × 10^5 cells in KRB buffer. The transport reaction was terminated at the desired time by the addition of 11 mL of buffer containing 0.3 mM phloretin. Unless otherwise indicated, all incubations were terminated at 4 s. Control studies demonstrated that phloretin completely inhibits the transport process for at least 8 min. Approximately 1.5 mL of silicone oil was layered over the diluted cell suspension, and the tubes were rapidly centrifuged (2000*g* × 15 s) in a Hereaus Labofuge. The cells coalesce on the surface of the oil and may be collected by sweeping the oil surface with adsorptive material (such as a small piece of pipe cleaner). The adsorptive material plus collected cells are then added to ACS (aqueous liquid scintillant; Amersham Scientific) liquid scintillation cocktail for determination of radioactivity. To correct for extracellular trapping of water, we carried out parallel reactions in which L-[^{14}C]glucose uptake was determined; L-glucose uptake was subtracted from the value for 3-*O*-methylglucose. The extracellular water space in the cell pellet was always <5% of the total intracellular water.

Iodination of Insulin. [^{125}I]Insulin was prepared at a specific activity of 100–200 μCi/μg according to the modification by Freychet et al. (1971) of the method of Hunter & Greenwood (1962) as previously described (Olefsky et al., 1974).

Binding Studies. Isolated fat cells were suspended in buffer containing 35 mM Tris, 120 mM NaCl, 1.2 mM $MgSO_4$, 4 mM KCl, 10 mM glucose, 1 mM EDTA, and 1% BSA (Gavin et al., 1973), pH 7.6, and incubated for 15 min with the various energy depleters in plastic flasks in a shaking water bath as previously described (Olefsky, 1975). After 15 min, mixtures of [^{125}I]insulin (0.2 ng/mL) and unlabeled insulin were added to the cells, and the incubation was continued for another 90 min. Less than 10% of the extracellular insulin was degraded under these conditions. Incubations were terminated as described by Gammeltoft & Gliemann (1973) by layering 200-μL aliquots of the cell suspension over 100 μL of silicone oil in plastic microfuge tubes and rapidly centrifuging. Reactions were considered terminated at the start of centrifugation. Tubes were cut through the oil layer, and radioactivity was determined in the tops of the tubes, containing the cell layers.

Nonspecific Binding. For these studies, nonspecific binding is defined as the amount of [^{125}I]insulin remaining in the cell layer in the presence of a large excess (200 μg/mL) of unlabeled insulin (Olefsky, 1975). All values are corrected for nonspecific binding which ranged from 2 to 7% of the total counts bound.

Results

Time Course of 3-O-Methylglucose Uptake in Isolated Adipocytes. When insulin is incubated with cells until binding equilibrium is reached, the ability of adipocytes to accumulate 3-*O*-methylglucose over time can be assessed at a constant level of receptor occupancy. This was done by preincubating 1.67 × 10^{-8} M insulin with cells for 60 min prior to measuring 3-*O*-methylglucose uptake at 24 °C. As seen in Figure 1, accumulation of 3-*O*-methylglucose is more rapid in the insulin treated cells as compared to the basal (no insulin preincubation) cells; the time at which half the maximal amount of uptake occurs is 20 s for basal cells, decreasing to 5 s for

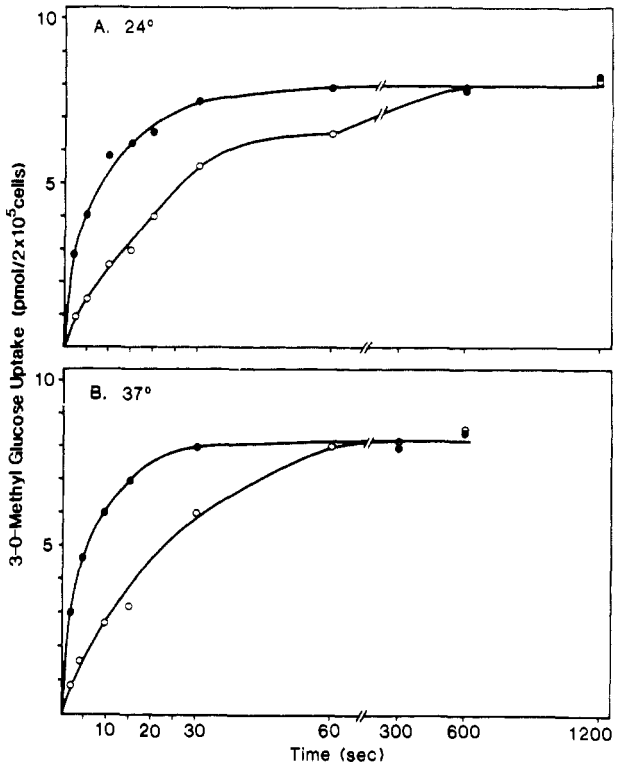

FIGURE 1: (A) Insulin stimulation of 3-*O*-methylglucose uptake by adipocytes. Isolated adipocytes were incubated in the absence (○) or presence of (●) insulin (1.67 × 10^{-8} M) for 60 min at 24 °C in KRB buffer (pH 7.4) containing 1% albumin and 2 mM Ca^{2+}. Uptake was initiated by the addition of 50-μL cells to 20 μL of buffer containing 3 nmol of 3-*O*-methyl[^{14}C]glucose. Uptake was terminated as described under Materials and Methods. By use of L-[^{14}C]glucose as a marker for trapped buffer, all results are corrected for extracellular water space. Values are the means of duplicate determinations of three experiments. (B) Same as in (A) except studies were conducted at 37 °C.

insulin-stimulated cells. Although 3-*O*-methylglucose equilibrates between the intracellular and extracellular space more quickly in insulin-treated cells, both groups of cells attain the same intracellular concentration by 10 min. This indicates that insulin activation of 3-*O*-methylglucose uptake is due to an increase in transport rather than an effect on intracellular water space. It should also be noted that there is no difference between insulin and basal cells with respect to extracellular trapping of 3-*O*-methylglucose in the cell pellet (Table IV).

Studies performed at 37 °C show that transport of 3-*O*-methylglucose in both insulin-treated and basal cells is faster than at 24 °C. However, differences between insulin-treated and basal cells were quantitatively similar (Figure 1B). Both cell groups accumulate the same amount of 3-*O*-methylglucose by 10 min.

Characterization of Insulin's Effects on 3-O-Methylglucose Transport. To determine the concentration of insulin which leads to maximal stimulation of 3-*O*-methylglucose transport, we incubated various concentrations of insulin with cells for 60 min after which 3-*O*-methylglucose uptake was assessed. 3-*O*-Methylglucose transport is maximal in cells preincubated with 6.67 × 10^{-10} M insulin, and a half-maximal response is achieved with 1.33 × 10^{-10} M (data not shown).

The influence of insulin (1.67 × 10^{-8} M) on transport at a variety of substrate concentrations was assessed by incubating insulin-treated cells with varying concentrations of 3-*O*-methylglucose and measuring uptake after 4 s. When the results are graphed on a Lineweaver–Burk plot (Figure 2), it can be seen that insulin increases the V_{max} of 3-*O*-methyl-

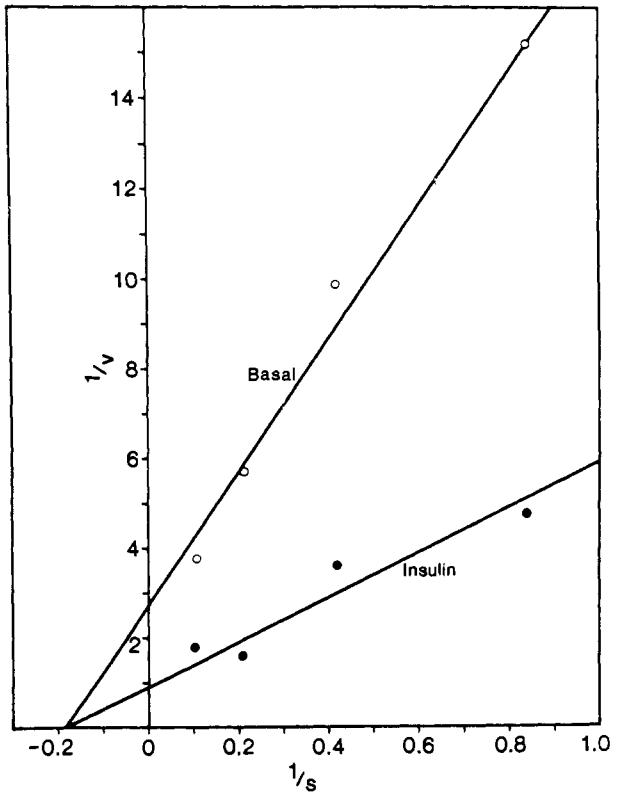

FIGURE 2: Lineweaver–Burk plot of 3-*O*-methylglucose transport. Adipocytes were incubated in the absence (○) or presence (●) of insulin (1.67×10^{-8} M) for 30 min at 24 °C. Uptake was initiated by the addition of 50-μL cells to 20 μL of buffer containing various concentrations of 3-*O*-methylglucose and 0.4 μCi of 3-*O*-methyl-[^{14}C]glucose. The inverse of the rate of 3-*O*-methylglucose transport [pmol/(2×10^5 cells)] is plotted as a function of the inverse of the 3-*O*-methylglucose concentration (mM).

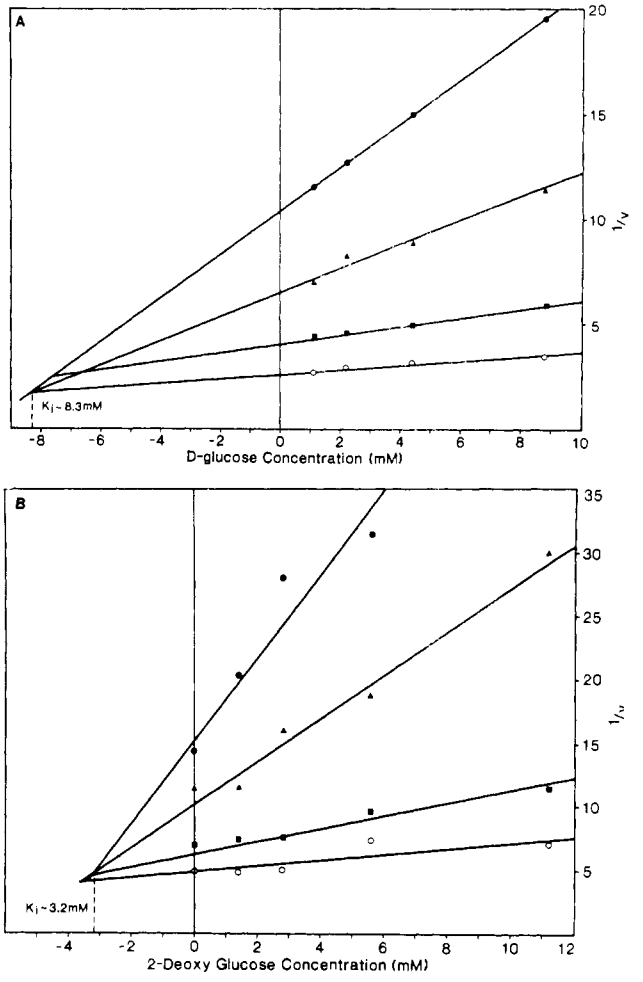

FIGURE 3: Dixon plots of inhibitory effects of D-glucose and 2-deoxyglucose. Reciprocal of 3-*O*-methylglucose transport rates [pmol/(2×10^5 cells)] is plotted as a function of inhibitor concentration. (A) D-Glucose. Adipocytes were incubated with insulin (1.67×10^{-8} M) for 60 min at 24 °C. Uptake was initiated by adding 50 μL cells containing various concentrations of D-glucose plus 3-*O*-methyglucose. The concentrations of D-glucose are as indicated, and the concentrations of 3-*O*-methylglucose were 1.1 (●), 2.2 (▲), 4.4 (■), and 8.8 (○) mM. K_i is determined as the abscissal intercept of the vertical line drawn from the intersections of the lines extrapolated from the data. The K_i was 8.3 mM. (B) 2-Deoxyglucose. Same as in (A). The concentrations of 2-deoxyglucose are indicated, and the concentrations of 3-*O*-methylglucose were 1.4 (●), 2.8 (▲), 5.6 (■), and 11.2 (○) mM. The K_i was 3.2 mM.

glucose transport without affecting the K_m. In five separate experiments the V_{max} of basal vs. insulin-treated cells was (mean ± SE) 0.4 ± 0.1 and 2.2 ± 0.3 nmol/min per 200 000 cells, respectively, and the K_m was 6.6 ± 1.2 mM for both groups of cells.

Affinity of Transport System for Other Substrates. The specificity and affinity of the adipocyte glucose transport system was assessed by determining the ability of D-glucose and 2-deoxyglucose to inhibit 3-*O*-methylglucose uptake. At a 3-*O*-methylglucose concentration of 1.1 mM, the hexose concentration which resulted in 50% inhibition of 3-*O*-methylglucose transport was 4.1 and 10.0 mM for 2-deoxyglucose and D-glucose, respectively (data not shown). These values corresponded quite closely to the inhibition constants (K_i) which were determined by analyzing competition data with Dixon plots (Figure 3). In these latter studies, insulin-treated cells were incubated with varying concentrations of substrate (3-*O*-methylglucose) plus varying concentrations of inhibitor (D-glucose or 2-deoxyglucose), and the uptake of 3-*O*-methylglucose was measured. Representative inhibition plots for D-glucose and 2-deoxyglucose are shown in parts A and B of Figure 3, respectively. In three separate experiments the K_i of D-glucose averaged 8.8 ± 0.4 mM and that of 2-deoxyglucose averaged 3.6 ± 0.4 mM. Thus, the order of affinity of the transport system for the hexoses studied is 2-deoxyglucose > 3-*O*-methylglucose > D-glucose.

Effects of DNP on 3-O-Methylglucose Transport. Having characterized certain features of the adipocyte glucose transport system with this rapid assay procedure, we next examined the energy dependence of insulin activation. For the remaining studies 200 ng/mL of insulin was used, since at this concentration insulin binding to receptors is no longer rate limiting with respect to transport (Ciaraldi & Olefsky, 1979), and 4-s 3-*O*-methylglucose uptake measurements were used to quantify initial rates of transport.

DNP, an uncoupler of oxidative phosphorylation (Slater, 1967), rapidly (within 1–2 min) depletes adipocytes of ATP (Kono et al., 1977; Chandramouli et al., 1977; Chandramouli & Carter, 1977), and in our hands 1 mM DNP resulted in a 71% decrease in intracellular ATP content by 2 min (data not shown). The dose response of DNP inhibition of insulin-stimulated glucose transport is shown in Figure 4. When DNP is added to cells prior to, or at the same time as, the addition of insulin (triangles), and transport is assessed 15 min later, activation by insulin is completely prevented by DNP at concentrations > 0.5 mM with a half-maximal effect at 0.2 mM. However, when cells were preincubated with insulin for 45 min prior to the addition of DNP (circles) and transport was measured after 15 min of incubation with DNP, no in-

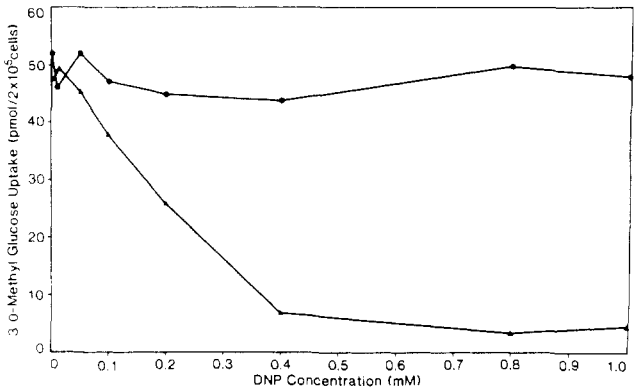

FIGURE 4: Dose response of DNP inhibition of insulin-stimulated 3-*O*-methylglucose uptake. Adipocytes were either incubated with insulin (1.67×10^{-8} M) for 45 min prior to the addition of DNP (●) or were incubated with DNP and insulin added simultaneously (▲). In both cases, uptake was assessed after cells had been exposed to DNP for 15 min. Uptake was initiated by the addition of 50 μL cells to 20 μL of buffer containing 25 nmol of 3-*O*-methyl[^{14}C]glucose and was terminated after 4 s. All procedures were done at 24 °C.

Table I: Effect of Energy Inhibitors on Insulin Binding[a]

additions	unlabeled insulin (ng/mL) 0	10	100	2×10^5
none	2.23	1.06	0.34	0.09
1 mM DNP	2.18	0.97	0.29	0.10
1 mM NaCN	2.30	1.11	0.34	0.08

[a] Cells were incubated with 3.3×10^{-11} M (0.2 ng/mL) [^{125}I]insulin plus various concentrations of unlabeled insulin. Data are expressed as percent of total [^{125}I]insulin bound (see Materials and Methods). Results represent the mean of three experiments.

hibitory effect was observed. Insulin binding was not affected over these concentrations of DNP (Table I).

DNP has no effect on basal rates of 3-*O*-methylglucose transport for up to 25 min (data not shown). A marked fall in transport after 40 min of exposure to DNP occurs, due to cell rupture during centrifugation, with subsequent loss of intracellular water.

In order to adequately evaluate the influence of energy depletion, it was important to determine the time course of activation of 3-*O*-methylglucose transport by insulin and the rapidity with which DNP exerts its inhibitory effects. In Figure 5, transport was measured at the indicated times, after the addition of either insulin (upper curve) or insulin plus DNP (lower curve) at time zero. As can be seen, insulin stimulation is apparent after 1 min and increases each minute until maximal activation is achieved at 10 min.[2] In contrast, DNP totally inhibits any activation. Thus, the full inhibitory effect of DNP on insulin activation of transport occurred within 1 min of the addition of DNP and was maintained throughout the duration of the study.

The next question was whether DNP's ability to inhibit insulin-stimulated 3-*O*-methylglucose transport was related to the process by which insulin activates transport. To determine whether DNP influenced K_m or V_{max}, we performed the experiments presented in Figure 6, in which cells were incubated with either insulin or insulin plus DNP added concurrently. As can be seen, DNP decreases the V_{max} without

[2] This time course of activation is consistent with our earlier work (Ciaraldi & Olefsky, 1979). However, the lag phase prior to the onset of action, which we have previously reported, was not assessed in the experiments depicted in Figure 5, since time points earlier than 1 min were not obtained.

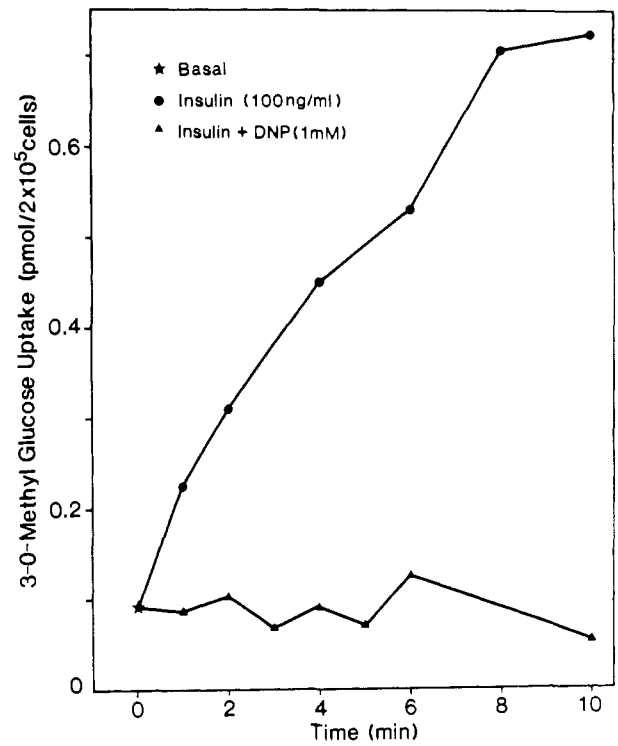

FIGURE 5: Rate of onset of DNP inhibition. Adipocytes were incubated with insulin (3.3×10^{-8} M) in the absence (●) or presence of 1 mM DNP (▲) at 37 °C. Uptake of 3-*O*-methylglucose (2 s) was assessed immediately before (basal) the addition of insulin and at various times after the addition of insulin plus DNP. Results represent the means of duplicate determinations for four experiments. Identical results were seen at 24 °C.

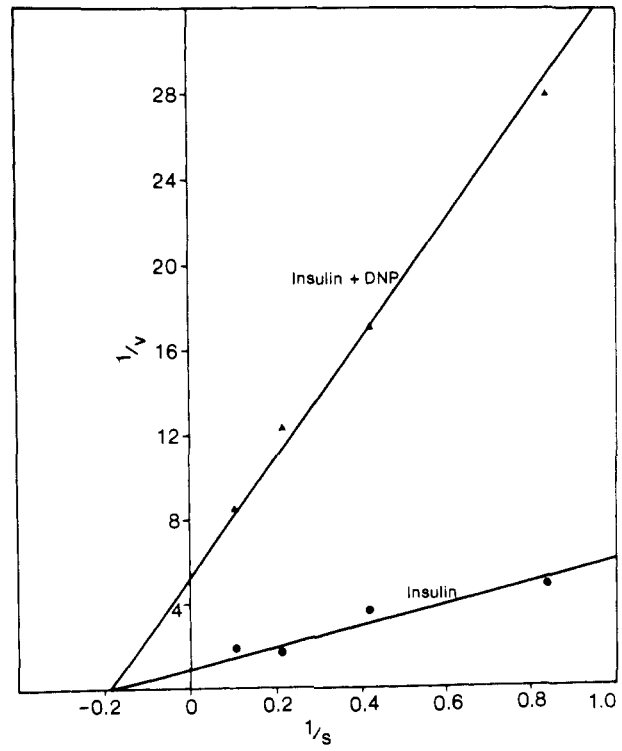

FIGURE 6: Lineweaver–Burk plot of 3-*O*-methylglucose uptake by insulin-treated (●) and insulin plus DNP treated (▲) cells. Cells were incubated with insulin (1.67×10^{-8} M) in the absence or presence of DNP (1 mM) for 30 min at 24 °C.

affecting the K_m. In two separate experiments the average V_{max} in the presence of DNP and insulin was 0.4 ± 0.12 nmol/min per 2×10^5 cells, which was the same as the basal

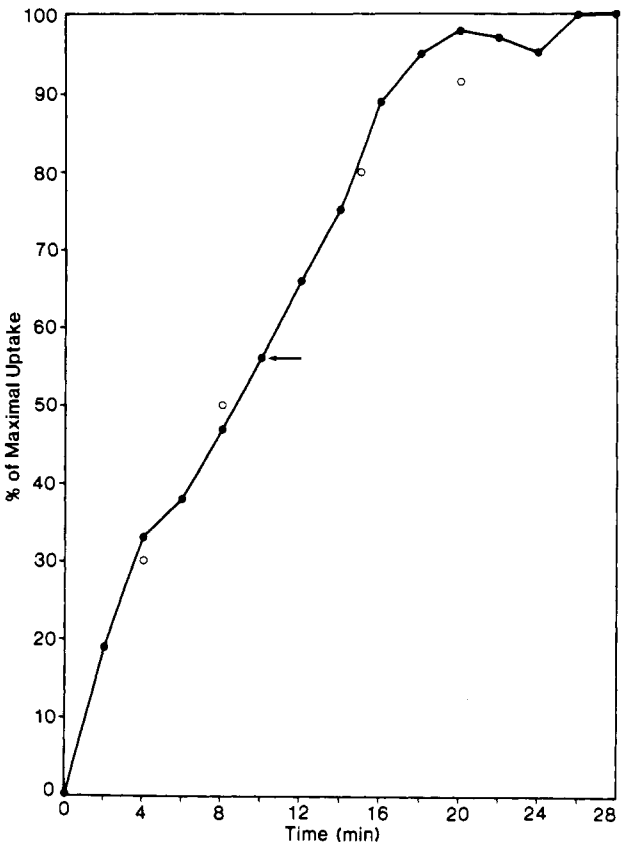

FIGURE 7: Effect of DNP on the time course of insulin activation of 3-*O*-methylglucose transport at 24 °C. In these experiments the inhibitory effect of DNP was studied as a function of the insulin preincubation time. Adipocytes were incubated at 24 °C with insulin (3.3×10^{-8} M) for various times before the addition of DNP (1 mM). All samples were exposed to insulin for 30 min prior to measurement of 3-*O*-methylglucose uptake. Thus, the variable was the length of time the cells were exposed to insulin prior to the addition of DNP. Results represent the means of duplicate determinations for four experiments and are expressed as a percent of control (DNP not added). Open circles represent the time course of insulin action with no DNP added, for comparison.

V_{max}. K_m averaged 5.5 ± 0.8 mM.

The experiments in Figure 6 indicate that DNP specifically inhibits the mechanism whereby insulin activates 3-*O*-methylglucose transport. To further document this point, we assessed the relationship between the DNP-induced inhibition of insulin activation of transport and the time course of insulin action. Insulin was added to cells at time zero, and DNP (1 mM) was then added to the incubation mixture at various times, up to 28 min, after the addition of insulin. Transport (4 s) was determined, in all cases, 30 min after the addition of insulin. All cell groups were in the presence of DNP for at least 2 min before transport was assessed. Therefore, the variables were the amount of time the cells were exposed to DNP and, more importantly, the length of the incubation with insulin prior to the addition of DNP. The results of four experiments are shown in Figure 7. Data are expressed as a percent of the maximal stimulation in the absence of DNP as a function of the duration of time the cells were exposed to insulin alone (before DNP was added). Activation of 3-*O*-methylglucose transport increases linearly as the duration of DNP-free incubation increases, and maximal activation was seen when cells were incubated with insulin for at least 20 min prior to the addition of DNP. Any greater interval between the addition of insulin and DNP results in no greater stimulation, and once maximal activation is achieved prolongation of the DNP incubation has no effect. Thus, insulin gradually activates 3-*O*-methylglucose transport to maximum rates over a 20-min period, and DNP can only inhibit that proportion of the system which is not activated. For example, when cells are incubated for 10 min without insulin (arrow, Figure 7) and a further 20 min with insulin plus DNP, 55% of maximal activation is observed. Although the cells were in the presence of insulin for a total of 30 min, the subsequently measured 3-*O*-methylglucose transport rate was the same as if the cells had only been incubated with insulin for 10 min.

Table II: Specificity of Inhibitor for Insulin Activation[a]

conditions	3-*O*-methylglucose transport −DNP	3-*O*-methylglucose transport +DNP (1 mM)
basal	100	97
basal plus insulin (1.67×10^{-8} M)	581	102
basal plus antireceptor Ab[b]	608	112
H_2O_2 (0.3 mM)	623	596

[a] All results represent the mean ± SE of triplicate samples from two separate experiments and are expressed as a percent of the basal transport rate. Agents were added at time zero and measurements were made 30 min later. [b] Antireceptor antibody was obtained from the serum of a patient with severe insulin resistance. The antiserum was used at a titer of 1:20 which fully activates the adipocyte glucose transport system.[3]

The time course of activation of 3-*O*-methylglucose transport when insulin alone is added at time zero is given in Figure 7 (open circles) for comparison. Clearly, this curve is superimposable with the experiments in which DNP is added at different times after insulin. This demonstrates that at any point prior to 20 min, a certain portion of the glucose transport system has been converted to the activated state by insulin. If DNP is added at this point, the previously activated portion of the system will be "locked" into the active state and no further activation of the remaining portion of the system will occur. It should be noted that the activation time course at 37 °C (Figure 5, upper curve) is more rapid than the results at 24 °C seen in Figure 7. We have previously documented this temperature dependency of the activation process (Ciaraldi & Olefsky, 1979). The lower temperature was selected for the experiments in Figure 7 because the temporal effects of DNP are more easily quantitated when the activation process is slowed.

Specificity of DNP for the Insulin Activation Mechanism. To more conclusively show that energy depletion specifically inhibits the coupling step(s) between insulin receptor complexes and the glucose transport system, we determined the effects of DNP in the presence of insulin mimickers. H_2O_2 is known to directly stimulate glucose transport, and this effect does not involve the insulin receptor (Livingston et al., 1977). Additionally, we have recently obtained serum from a patient with severe insulin resistance containing an antiinsulin receptor antibody. This antibody inhibits insulin binding but fully activates the adipocyte glucose transport system when added alone, and this is mediated through the insulin receptor.[3] As can be seen in Table II, when DNP (1 mM) is added at the same time as the various mimickers, stimulation by insulin and the antireceptor serum is prevented, whereas the effects of H_2O_2 remain the same. This demonstrates that energy depletion specifically inhibits the insulin action sequence and does not interfere with other mechanisms of transport activation.

Other Inhibitors of Energy Production. Another energy depleter, NaCN, was studied in order to more fully document

[3] D. Baldwin and J. Olefsky, unpublished observations.

Table III: Effect of Inhibitors on Basal and Insulin-Stimulated 3-*O*-Methylglucose Transport[a]

conditions	basal	insulin plus inhibitor added together	inhibitor added after insulin
DNP (1 mM)	100 ± 15	115 ± 6	512 ± 56
DNP (1 mM) after wash		499 ± 56	
NaCN (1 mM)	100 ± 20	120 ± 10	505 ± 30
NaCN (1 mM) after wash		495 ± 42	

[a] All results are expressed as a mean percent (±SD), $n = 4$, of control values observed in the absence of added inhibitor. Measurements were made 20 min after the addition of inhibitor in the basal state, 30 min after the simultaneous addition of insulin (1.67×10^{-8} M) plus inhibitor, and 20 min after the addition of inhibitor to cells which had already been preincubated with insulin (1.67×10^{-8} M) for 20 min. In the washing studies, cells were centrifuged 3 times in KRB buffer containing 1.67×10^{-8} M insulin to remove all extracellular DNP or NaCN. Insulin (1.67×10^{-8} M) was then added, and 3-*O*-methylglucose transport was measured 30 min later. Experiments were conducted at 24 °C.

Table IV: Effect of Inhibitors and Insulin on Intracellular and Extracellular Water Space[a]

conditions	n	intracellular water space (pL/cell)	extracellular water space [nL/(2 × 10^5 cells)]	% of intracellular space[b]
basal	11	1.38 ± 0.16	8.3 ± 1.7	3.0
plus DNP (1 mM)	6	1.28 ± 0.15	8.8 ± 2.2	3.4
plus NaCN (1 mM)	4	1.34 ± 0.12	9.0 ± 2.1	3.4
insulin[c]	15	1.37 ± 0.18	8.5 ± 1.3	3.1
plus DNP (1 mM)	13	1.35 ± 0.22	8.0 ± 1.4	3.0
plus NaCN (1 mM)	4	1.29 ± 0.13	8.8 ± 1.7	3.4

[a] All values represent means ± SD. [b] This value represents the ratio of the extracellular space (L-glucose space) to the intracellular water space (3-*O*-methylglucose space minus the L-glucose space). [c] 1.67×10^{-8} M.

that the observed effects of DNP were due to its ability to deplete cells at ATP. NaCN depletes ATP by inhibiting electron flow across the respiratory chain (Slater, 1967), and, thus, its action differs from that of DNP.

Table III summarizes the comparative effects of the two inhibitors on basal and insulin-stimulated glucose transport. Neither agent had any effect on basal glucose transport. Both inhibitors greatly impaired activation of transport to 10–20% of control values when added concomitantly with insulin. When DNP or NaCN was added to cells after a 20-min insulin preincubation and transport was assessed 30 min later, no inhibition is seen. Table III also shows that the inhibitory effects of these agents were fully reversible. Thus, adipocytes were incubated with insulin and either DNP or NaCN for 20 min. Following this the cells were washed and resuspended in buffer containing insulin but no inhibitor. After an additional 30 min of incubation transport was determined. Under these conditions maximally activated 3-*O*-methylglucose transport rates were expressed. Therefore, both agents exerted identical effects on the glucose transport system, indicating a common mechanism of action, i.e., inhibition of ATP production with depletion of intracellular ATP content.

Finally, as seen in Table IV, neither agent had any effect on the intracellular or extracellular water space of the cell pellet, alone or in combination with insulin.

Discussion

It is now well-known that the first step in insulin action is binding to specific cell surface receptors (Czech, 1977). However, the subsequent steps by which effector systems are activated following receptor occupancy have not been clearly defined. In the case of glucose transport, a major problem has been the inability to directly quantitate initial rates of transport. Thus, many previous studies have used changes in triglyceride synthesis, glucose oxidation, or 2-deoxyglucose uptake, end points which are at least one step removed from transport, as a measure of insulin's effect on the glucose transport system. Others have been able to measure initial uptake rates of slowly transported sugars such as D-allose (Loten et al., 1977) and L-arabinose (Foley et al., 1978). However, adequate characterization of the glucose transport system cannot be carried out with these sugars because of their very high K_m values (which accounts for their slow rates of transport). 3-*O*-Methylglucose is a glucose analogue which is rapidly transported but not metabolized. It therefore quickly establishes an equilibrium across the cell membrane, and, to use this analogue effectively in assessing changes in rates of glucose transport, one must measure 3-*O*-methylglucose uptake at very early time points. In the current report this has been accomplished by using a modification of the method of Gliemann & Whitesell (1977).

By measuring initial rates of 3-*O*-methylglucose uptake, we have characterized certain features of the glucose transport system in isolated rat adipocytes. In the absence of insulin, transmembrane equilibration with 3-*O*-methylglucose occurs by 10 min with half-maximal uptake by 20 s at 24 °C (Figure 1A). When cells were preincubated with a maximally effective concentration of insulin, the rate of transport was accelerated with half the maximal amount of uptake at 5 s; transmembrane equilibration occurred by 1 min. It is important to note that the intracellular levels of 3-*O*-methylglucose at equilibrium are similar for both insulin-treated and untreated cells (Figure 1 and Table IV). Thus, insulin does not increase 3-*O*-methylglucose transport by increasing intracellular water space.

By increasing the temperature from 24 to 37 °C (Figure 1B), 3-*O*-methylglucose transport is accelerated. At 37 °C untreated cells reach equilibrium by 1 min, with half-maximal uptake at 15 and 3 s in untreated and treated cells, respectively. It should be noted that although raising the temperature increases the rate of flux across the cell membrane, the intracellular levels of 3-*O*-methylglucose at equilibrium are the same at 24 and 37 °C.

Since the rate of uptake of 3-*O*-methylglucose is so rapid, the transport rate is linear for only a brief period of time. Therefore, to accurately assess initial transport rates, measurements of 3-*O*-methylglucose uptake must be made within a few seconds. Under these conditions, insulin increases the transport rate in a dose-dependent manner with a maximal effect at 6.67×10^{-10} M and half-maximal effects at 1.33×10^{-10} M. Lineweaver–Burk analysis of insulin-treated and untreated cells shows that insulin increases V_{max} from 3 to 7 times over that of basal without altering K_m (Figure 2); these results are comparable to other reports (Olefsky, 1978; Vinten et al., 1976; Czech et al., 1974).

The affinity of this system is demonstrated in Figures 2 and 3 which present the K_m value for 3-*O*-methylglucose and the K_i values for D-glucose and 2-deoxyglucose. The affinity of the transport system for the three sugars is 2-deoxyglucose > 3-*O*-methylglucose > D-glucose. Lineweaver–Burk analysis

(data not shown) reveals that D-glucose and 2-deoxyglucose compete with 3-*O*-methylglucose in a competitive manner. In other words, the three substrates interact with the same transport system in a qualitatively similar but quantitatively different fashion.

The K_m for 3-*O*-methylglucose is 6.6 mM while the K_i for 2-deoxyglucose and D-glucose are 3.6 mM and 8.8 mM, respectively. Since glucose transport can be represented by

$$S + C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} SC \xrightarrow{k_3} S + C$$

where S is the substrate glucose and C is carrier (Olefsky, 1978), then $K_i = k_2/k_1$ and $K_m = k_2 + k_3/k_1$. Since, it is most likely that $k_3 \ll k_2$ for the adipocyte glucose transport system (Olefsky, 1978), $K_m = K_i$ for 2-deoxyglucose and D-glucose. The K_m values for 3-*O*-methylglucose and the K_i values for D-glucose and 2-deoxyglucose reported in the current manuscript are essentially identical with those reported for human adipocytes by using the same method to measure hexose transport (Ciaraldi et al., 1979). We have previously reported that the K_i of D-glucose on 2-deoxyglucose uptake was ~2 mM (Olefsky, 1978). However, it is possible that D-glucose interferes with the phosphorylation of 2-deoxyglucose, leading to an underestimate of both uptake and the K_i value and accounting for the difference in results. It is important to note that the K_m of D-glucose (8.8 mM) is well within the physiologic plasma glucose concentration range, indicating that proportionate changes in glucose transport occur following physiologic changes in plasma glucose level. This further indicates that the system we have studied using 3-*O*-methylglucose is the physiologic glucose transport carrier.

We have used this model system to study some of the characteristics whereby insulin activates the glucose transport system. Other investigators have shown that agents which deplete intracellular ATP can impair insulin-stimulated glucose transport (Kono et al., 1977; Chandramouli et al., 1977; Chandramouli & Carter, 1977; Yu & Gould, 1977), and previous work (Ciaraldi & Olefsky, 1979) has indicated that the coupling step between insulin receptors and the glucose transport system has a high activation energy (E_A = 24 kcal/mol), suggesting an energy requirement for activation.

Our data show that DNP, an uncoupler of oxidative phosphorylation, decreases insulin's ability to stimulate 3-*O*-methylglucose transport, without affecting basal transport (Figure 4). The concentration of DNP needed to completely inhibit transport is 0.5 mM. However, DNP must be added before or concomitantly with insulin to exert this inhibitory effect; when DNP is added after a 20-min insulin preincubation, no inhibition is seen even when as much as 1 mM DNP is employed (Figure 4). The inhibitory action of DNP occurs rapidly and the full effect is expressed within 1 min (Figure 5). Thus, for practical purposes, any expected inhibition should be seen immediately.

The finding that DNP inhibits insulin-stimulated, but not basal, 3-*O*-methylglucose transport indicates that this agent is relatively specific for the insulin action mechanism and is not a general depressant of the glucose transport system. Furthermore, since energy depletion does not inhibit transport if cells are preincubated with insulin for a sufficient time to allow full activation to occur, we conclude that the processes whereby insulin activates the glucose transport system and the processes required for maintenance of the activated state represent separate phenomena. Thus, deactivation is not simply a passive occurrence representing the absence of ongoing activation. It should be noted that Kono et al. (1977) found that DNP inhibited glucose transport even when added after insulin. However, these workers preincubated cells with 1 nM insulin for only 5 min, and, as demonstrated in Figure 7 and previous reports (Ciaraldi & Olefsky, 1979; Schoenle et al., 1977; Rozengurt & Stein, 1977), this time period is too brief to allow full insulin activation to occur. It seems likely that this methodologic difference explains the discrepancy between the work of Kono et al. (1977) and the current results.

This effect of DNP is not secondary to a decrease in insulin binding (Table I) or a decrease in intracellular or increase in extracellular water space (Table IV). Furthermore, another known ATP depleter, NaCN (Slater, 1967; Chandramouli & Carter, 1977), had similar effects on both basal and insulin-stimulated rates of 3-*O*-methylglucose transport (Table III). Thus, it is highly unlikely that inhibition by DNP is due to some other unique effect of DNP unrelated to energy depletion. Rather, it is most likely that the absence of ATP, or high-energy phosphate bonds, is specifically involved. It has been previously established that ATP levels return to normal within 3–5 min when the energy inhibitors are removed (Kono et al., 1977; Chandramouli et al., 1977). Consistent with this, we have found that when cells are washed free of DNP or NaCN and allowed to recover, insulin's full stimulatory effects can then be expressed (Table III). Thus, the reversibility of inhibition of insulin activation of the glucose transport system corresponds to the reversibility of ATP depletion.

Figure 2 clearly demonstrates that insulin activates 3-*O*-methylglucose transport by increasing V_{max} with no change in K_m. If the inhibitory effects of DNP involve specific blockade of the insulin activation process, then one would expect this agent to influence V_{max} and not K_m. As Figure 6 shows, this is the case. Thus, DNP completely prevents insulin from augmenting V_{max} while K_m remains the same. We have previously reported kinetic evidence indicating that insulin increases the V_{max} of the glucose transport system by increasing the number of functional transport units (Olefsky, 1978). This is accomplished by converting previously existing, but inactive, units into the active form, and this observation has recently been confirmed by using different methodology (Wardzala et al., 1978). With this in mind, our data are consistent with the concept that this insulin-induced activation process requires ATP, and DNP prevents this step. This concept is strongly supported, and further developed, by the data in Figure 7. When insulin is added for various intervals prior to the addition of DNP, the activity of the glucose transport system is "locked" into the level of activation achieved just prior to the addition of DNP. Since Figure 5 shows that the full inhibitory effect of DNP occurs within 1 min, Figure 7 demonstrates that once DNP is added, further activation of the transport system ceases, but the existing level of activity is maintained. Thus, any fraction of the glucose transport system which is already activated is not susceptible to inhibition by DNP, and the remaining inactive units cannot be activated in the presence of DNP. The functional form of this curve is identical with the time course of insulin stimulation of 3-*O*-methylglucose transport without DNP (Figure 7; Ciaraldi & Olefsky, 1979). Further, after 20 min of insulin preincubation, DNP had no inhibitory effect at all, and this interval corresponds to the time needed for insulin's maximal effect to be expressed. These experiments demonstrate the extremely close correlation between energy depletion and the ongoing process whereby insulin couples to, or activates, the glucose transport system and also show that maintenance of the active state is independent of energy. Theoretically, ATP could be necessary for activation as either an energy source or a phosphate donor. However, since Avruch et al. (1976) have shown that insulin

does not cause phosphorylation of any adipocyte plasma membrane protein, we favor its role as an energy source.

Finally, the specificity of DNP for the insulin activation mechanism was substantiated by examining the effects of energy depletion on glucose transport stimulation by various insulin-like agents. DNP had no effect on a mimicker whose actions are exerted independent of the insulin receptor (H_2O_2) but did prevent transport activation by an antireceptor antibody. Thus, energy depletion specifically inhibits the coupling process between insulin receptors and the glucose transport system.

Our interpretation of these results is that after a brief absolute lag time (Ciaraldi & Olefsky, 1979), insulin gradually activates previously existing but inactive carriers by an ATP-dependent coupling step which is rate limiting. Those carriers which have not yet been activated by insulin at the time DNP is added cannot continue to transport substrate. After a 20-min incubation with insulin, all the potentially active carriers have been recruited, and ATP is not longer required. Thus, the maximal insulin effect is achieved and ATP depletion does not inhibit this state. Consequently, once a carrier is activated, it is no longer dependent on ATP to remain in the active state; the energy-dependent step is like a switch, it activates the carrier but is not needed to maintain activation. In fact, preliminary evidence suggests that ongoing cell metabolism is required to deactivate the system (Ciaraldi & Olefsky, 1980).

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