Insulin modifies the properties of glucose transporters in rat brown adipose tissue

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The properties of glucose transporters associated with plasma and microsomal membranes have been studied in brown adipose tissue of rats after treatment by saline infusion or hyperinsulinaemic/euglycaemic clamp. In this tissue, insulin produces a 40-fold increase in glucose utilization as measured by the 2-deoxy-D-glucose technique, and therefore a 40-fold increase in the rate-limiting glucose transport. This increase, promoted by insulin, is associated with: (a) translocation of the transporters from a pool associated with the microsomal fraction to the plasma membrane without modification of the total number of transporters; (b) an increase in the Hill coefficient of the plasma-membrane glucose transporters for cytochalasin B from 1.1 to 2.5, indicating the presence of positive co-operativity; (c) a decrease in the K_d (apparent dissociation constant) of the transporters towards cytochalasin B from 148 to 82 nM; (d) no change in the Hill coefficient or K_d for the transporters associated with the microsomal membranes. These data indicate that, in addition to causing translocation of the glucose transporters, insulin modifies their properties and behaviour towards cytochalasin B. This may reflect modifications in their properties and behaviour towards glucose, and by this contribute to bringing about the marked effect of this hormone on glucose transport in brown adipose tissue.

INTRODUCTION

Brown adipose tissue is considered as a tissue specialized in heat production in response to cold (Himms-Hagen, 1976) and, as shown by subsequent data, as a potential regulator of energy balance, due to its capacity to waste energy in cases of overfeeding (Rothwell & Stock, 1979). The main substrate of this tissue was considered to be non-esterified fatty acids. However, more recently, this tissue has been shown to be a potential site of active glucose metabolism, owing to its high rate of lipogenesis with glucose as precursor [reviewed by McCormack (1982) and Sugden *et al.* (1982)], its high pyruvate dehydrogenase activity (McCormack & Denton, 1977) and its high content of glycolytic enzymes (Cooney & Newsholme, 1982; Young *et al.*, 1984).

By using a technique allowing measurements of glucose utilization *in vivo* by specific tissues of rats, glucose utilization by brown adipose tissue was shown to be increased up to 60-fold by insulin (Ferré *et al.*, 1986). This increase in glucose utilization promoted by insulin may to a large extent be the consequence of the action of this hormone on glucose transport. Data obtained *in vitro* (Czech *et al.*, 1974) on brown adipocytes have shown that glucose transport is a rate-limiting step in glucose utilization and that, as with white adipocytes, insulin can increase this process.

In white adipose tissue, the stimulation of glucose transport by insulin, has been shown to result, at least in part, from the translocation of glucose transporters from an intracellular pool to the plasma membrane (Cushman & Wardzala, 1980; Suzuki & Kono, 1980; Karnieli *et al.*, 1981; Ezaki & Kono, 1982; Simpson *et al.*, 1983; Oka & Czech, 1984). This process, however, may not be sufficient

to explain fully insulin's effect, and changes in the intrinsic activity of the transporter have been hypothesized (Hyslop *et al.*, 1985).

In the present study, the effect of insulin on glucose transporters in brown adipose tissue was studied *in vivo* after a euglycaemic/hyperinsulinaemic clamp. Plasma and microsomal membranes were then isolated and their binding characteristics towards cytochalasin B were analysed. The data show that insulin translocates transporters from an intracellular pool to the plasma membrane and, in addition, increases their affinity for cytochalasin B and promotes the appearance of a positive co-operativity amongst transporters. The two last-mentioned properties, measured here toward cytochalasin B, if also present toward glucose, could reflect the changes in the 'intrinsic activity' of the transporters that have been postulated by others (Hyslop *et al.*, 1985; Kahn & Cushman, 1985).

MATERIALS AND METHODS

Normal male rats of the Zucker (*FA/FA*) strain were used throughout the experiments. They were maintained at constant temperature (22 °C) with a fixed (12 h) light cycle. The animals were fed *ad libitum* with a standard laboratory chow (12% water, 17% proteins, 3% lipids, 58.7% carbohydrate, 4.3% cellulose and 5% minerals; UAR, Epinary/Orge, France). At the time of the experiments the animals were 12 weeks old. The animals were studied 5–6 h after food removal. They were anaesthetized with Nembutal (50 mg/kg body wt.). A tracheotomy was performed and a carotid artery (for blood sampling) and a jugular vein (for infusion of glucose and insulin) were catheterized. Body temperature was maintained at 37 °C with a heating blanket.

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Euglycaemic/hyperinsulinaemic clamps were performed as described previously (Terrettaz & Jeanrenaud, 1983). 2-D-Deoxy[1-³H]glucose (30 μ Ci/rat) was injected as a bolus while the glycaemia was clamped (hyperinsulinaemic/euglycaemic clamp). Blood was sampled via the carotid catheter and analysed for glucose and 2-deoxy[1-³H]glucose as described by Ferré *et al.* (1985). After the last blood sample, the rat was killed, interscapular brown adipose tissue dissected and its 2-deoxy[1-³H]glucose 6-phosphate content determined as described by Ferré *et al.* (1985). The rate of glucose utilization derived from the amount of 2-deoxy-D-[³H]-glucose 6-phosphate was calculated by using a mathematical formula previously justified (Ferré *et al.*, 1985). It implied the determination of a correction factor (referred to as 'lumped constant') used for the possible discrimination of 2-deoxyglucose against glucose. This factor was measured in pieces (30–50 mg) of brown adipose tissue incubated with 5 mM-glucose ([5-³H]-glucose 0.1 μ Ci/ μ mol) and trace amounts of 2-deoxy-[1-¹⁴C]glucose with or without insulin (1 munit/ml) for 60 min. It was not modified by insulin: 0.70 ± 0.08 for control tissues, 0.69 ± 0.02 ($n = 6$) for tissues incubated with insulin. Plasma immunoreactive insulin was measured by radioimmunoassay; charcoal/dextran was used to separate free from bound insulin (Herbert *et al.*, 1965).

For the study of the glucose transporters, brown adipose tissue was collected 60 min after a saline infusion or a hyperinsulinaemic clamp. For one preparation of plasma and microsomal membranes, brown adipose tissue of three or four animals had to be pooled. Brown adipose tissue was transferred into a homogenization medium containing 1 mM-NaHCO₃, 0.5 mM-CaCl₂ and 1.2 mM-MgSO₄, pH 7.5; 1 g of tissue was placed in 10 vol. of the medium and homogenized for 5 s in a Polytron (PTA 10–35 homogenizer; Kinematica G.m.b.H., Lucerne, Switzerland). Plasma and microsomal membrane fractions were prepared by a method previously described for brown adipose tissue (Giacobino, 1979) with 4 ml of 18.5% (w/v) metrizamide. After this, the respective membranes (plasma and microsomal) were immediately washed once in the homogenization buffer to remove metrizamide and centrifuged at 156 000 g for 30 min. The pellets obtained were resuspended in a solution containing 250 mM-sucrose, 10 mM-Tris/HCl and 1 mM-EDTA at pH 7.4, rapidly frozen in liquid nitrogen and stored at -70°C for subsequent use. Final plasma and microsomal membrane protein concentrations ranged from 2 to 5 mg/ml.

The enrichment in the plasma and microsomal membrane fraction was estimated, respectively, by measuring the changes in the specific activity of 5'-nucleotidase (Avruch & Wallach, 1971) and NADPH-cytochrome *c* reductase (Sottocasa *et al.*, 1967). Proteins were measured by the Coomassie Brilliant Blue method, with bovine albumin as standard (Bradford, 1976).

Glucose transporters of brown adipose tissue were measured by the binding of [³H]cytochalasin B to plasma or microsomal membranes as described by Cushman & Wardzala (1980), but with the following modification: [³H]cytochalasin B in 100% ethanol was dried under nitrogen to remove ³H-labelled volatile contaminants and then was reconstituted in the binding-assay buffer containing 255 mM-sucrose, 10 mM-Tris/HCl and 0.2 mM-EDTA at pH 7.4 immediately before use. A

stock solution of cytochalasin E was prepared in dimethyl sulphoxide and stored at -20°C until use. The final concentration of cytochalasin E in the assay was 2000 nM and that of dimethyl sulphoxide was less than 0.2%. Cytochalasin B was used at final concentrations in the range 50–500 nM. Incubation of membranes with labelled cytochalasin B was carried out in the dark at 4°C for 1 h. Sucrose was added when glucose was omitted to correct for viscosity. Similar results were obtained when sucrose was replaced with L-glucose (not shown). Duplicate samples (50 μ l) of each of the final membrane suspensions were transferred to small plastic tubes and de-proteinized with 10 μ l of 0.15 M-Ba(OH)₂, immediately (less than 5 s) followed by 10 μ l of 0.15 M-ZnSO₄ solution in order to precipitate proteins. At most 5% of protein remained in the supernatant when either unlabelled glucose or sucrose was used. [¹⁴C]Urea was added for determination of the trapped but unbound [³H]cytochalasin B. The samples were pelleted by centrifugation in a Beckman Microfuge at maximal speed for 10 min. In one series of experiments (see the Results section) the membranes were pelleted at 360 000 g for 1 h, in a Beckman TL100 ultracentrifuge. Then 50 μ l of the supernatant was carefully removed for measurement of free [³H]cytochalasin B concentration, the remaining supernatant being aspirated and discarded. The pellet was obtained by cutting the lower end of the plastic tube with a lancet.

This method of precipitation of bound cytochalasin B was compared with the technique previously reported (Cushman & Wardzala, 1980) and gave comparable results (see the Results section). Scintillation counting was carried out in polyethylene vials containing 3 ml of Aquasol-2 scintillator fluid, supernatants and pellets being counted for 10 min and 30 min respectively, in a

Fig. 1. Glucose-displaceable [³H]cytochalasin B binding sites in rat brown-adipose-tissue plasma membranes obtained by centrifugation or deproteinization

Specific cytochalasin B binding sites (glucose displaceable) were measured in brown-adipose-tissue plasma membranes at six standard concentrations by using a centrifugation (a), or a deproteinization (b), technique to separate bound from free ligand, and the binding curves have been plotted, as described in the Materials and methods section. The same pool of membranes was used for three determinations in duplicate for each of the two techniques. When error bars are not visible, they are contained in the symbol. The total number of binding sites (R_0) was 7.5 ± 0.7 and 6.8 ± 0.7 pmol/mg of protein, Hill coefficient (h) 1.15 ± 0.04 and 1.19 ± 0.05 , and apparent K_d 170 ± 18 and 164 ± 11 nM for centrifugation (a) and deproteinization (b) techniques respectively.

Table 1. Interscapular brown-adipose-tissue glucose utilization and plasma glucose and insulin concentrations in rats after saline infusion or hyperinsulinaemic/euglycaemic clamp

Brown-adipose-tissue glucose utilization was determined as described in the Materials and methods section. Results are means $\pm$ S.E.M. for three (control) and five (hyperinsulinaemic) experiments: * $P < 0.005$ versus control.

	Control	Hyperinsulinaemic
Brown-adipose-tissue glucose utilization (ng/min per mg of tissue)	4.4 $\pm$ 0.3	186.8 $\pm$ 23.3*
Plasma glucose (mmol/l)	7.2 $\pm$ 0.1	6.8 $\pm$ 0.2
Plasma insulin (munits/l)	58 $\pm$ 6.0	632 $\pm$ 69*

LKB/Wallac Rackbeta liquid-scintillation counter. The specific cytochalasin B binding values (i.e. to the glucose transporters) were obtained by determining the differences between the binding values obtained in the presence of unlabelled (500 mM) D-glucose and that obtained in the absence of the hexose, extrapolated by means of linear regression to the same concentration of free cytochalasin B. Sucrose was added when glucose was omitted to correct for viscosity. The specific values were analysed by the Hill (1910) equation. A computer program was developed to find the best fit of the available data to the curve defined by the Hill equation. This enabled determination of values of R_0 (total D-glucose-inhibitable cytochalasin B binding sites; pmol/mg of membrane protein), K_d (apparent dissociation constant; nM), h (Hill coefficient), and plotting of the binding curves (bound versus free). Statistical analysis was carried out by Student's t test for unpaired data.

Chemicals

Labelled compounds and the Aquasol-2 scintillator were from New England Nuclear (Zurich, Switzerland);

metrizamide was from Nyeegard and Co. (Oslo, Norway); protein assay kit was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Other reagents were obtained from Sigma (St. Louis, MO, U.S.A.), Merck (Darmstadt, West Germany) or Boehringer (Mannheim, West Germany).

RESULTS

Validation of the modifications in the methodology

In plasma and microsomal membranes of brown adipose tissue the concentration of glucose that maximally displaced cytochalasin B binding was found to be 400 mM or over (results not shown). At this concentration of glucose or sucrose, the high viscosity of the solution makes the pelleting of the membranes incomplete. When plasma membrane was centrifuged for 1 h at 360 000 g in 400 mM-glucose or -sucrose, 31 $\pm$ 2 % ($n = 4$) of the total protein remained in the supernatant. The amount of protein remaining in the supernatant under these conditions was directly correlated with the concentration of D-glucose, L-glucose or sucrose present in the medium (results not shown). All the data obtained by this approach had therefore to be corrected for the protein remaining in the supernatant, with the assumption that they were not different from those that had been pelleted. For this reason, another technique for separating the membrane from the supernatant had to be found. This was achieved by precipitation with Ba(OH)₂ and ZnSO₄, as described in the Materials and methods section. A comparison of the two techniques is presented in Fig. 1. No difference is found in the shape of the binding curve, the total binding, the Hill coefficients or the K_d values obtained by the two techniques.

Glucose utilization by brown adipose tissue

Table 1 illustrates the glucose utilization by brown adipose tissue of rats after saline infusion, or after an euglycaemic/hyperinsulinaemic clamp. As shown previously by Ferré *et al.* (1986), insulin increases glucose utilization 40-fold. The same experimental conditions

Table 2. Enzyme markers of plasma and microsomal membranes obtained from brown adipose tissue of control rats after saline infusion or hyperinsulinaemic/euglycaemic clamp

Plasma and microsomal membranes were obtained as described in the Materials and methods section. Enrichment of each membrane fraction is assessed by the changes in specific activities of respective marker enzymes compared with the activities of the initial homogenate. Recovery is expressed as percentages of values found in the initial homogenate. Results are means $\pm$ S.E.M. for four individual preparations for each group of rats.

	5'-Nucleotidase		NADPH-cytochrome <i>c</i> reductase		Protein	
	Sp. activity (μ units/mg of protein)	Recovery (%)	Sp. activity (munits/mg of protein)	Recovery (%)	(mg/g of brown adipose tissue)	Recovery (%)
Control						
Homogenate	2.2 $\pm$ 0.1		60.2 $\pm$ 3.6		109.8 $\pm$ 5.7	
Plasma membranes	42.9 $\pm$ 1.0	34.6 $\pm$ 2.4	221.1 $\pm$ 7.4	6.6 $\pm$ 0.4	1.9 $\pm$ 0.1	1.8 $\pm$ 0.1
Microsomes	6.1 $\pm$ 0.9	5.9 $\pm$ 1.3	465.3 $\pm$ 14.3	17.0 $\pm$ 2.6	2.2 $\pm$ 0.3	2.1 $\pm$ 0.3
Hyperinsulinaemic						
Homogenate	2.9 $\pm$ 0.2		62.8 $\pm$ 2.5		111.6 $\pm$ 7.1	
Plasma membranes	48.3 $\pm$ 3.6	33.5 $\pm$ 2.4	236.7 $\pm$ 13.6	8.5 $\pm$ 0.7	2.5 $\pm$ 0.1	2.3 $\pm$ 0.1
Microsomes	6.5 $\pm$ 0.9	4.0 $\pm$ 0.8	500.0 $\pm$ 30.7	13.5 $\pm$ 1.8	1.9 $\pm$ 0.1	1.7 $\pm$ 0.2

Fig. 2. [^3H]Cytochalasin B binding sites in brown-adipose-tissue plasma membranes from rats after saline infusion or euglycaemic/hyperinsulinaemic clamp

[^3H]Cytochalasin B binding to plasma membranes from brown adipose tissue of control ($\circ$, $\square$; *a* and *c*) or insulin-treated rats ($\bullet$, $\blacksquare$; *b* and *d*), was measured at ten concentrations of the ligand, in the presence ($\square$, $\blacksquare$) or in the absence ($\circ$, $\bullet$) of D-glucose (*a* and *b*). Glucose-displaceable cytochalasin B binding (*c* and *d*) was obtained by subtracting the binding in the absence from that in the presence of 500 mM-D-glucose extrapolated by means of linear regression to the same free cytochalasin B concentration. The curves were drawn as described in the Materials and methods section: (*c*), R_0 8.1 pmol/mg of protein, h 1.0, K_d 178 nM; (*d*), R_0 10.5 pmol/mg of protein, h 2.3, K_d 106 nM.

Fig. 3. Glucose-displaceable [^3H]cytochalasin B binding to brown-adipose-tissue plasma and microsomal membranes from rats after saline infusion or euglycaemic/hyperinsulinaemic clamp

Specific cytochalasin B binding sites (i.e. displaceable by 500 mM-unlabelled D-glucose) were measured in brown-adipose-tissue plasma and microsomal membranes at six standard concentrations, as described in the Materials and methods section: (*a*) plasma-membrane fraction after saline infusion ($\circ$) or euglycaemic/hyperinsulinaemic clamp ($\bullet$); (*b*) microsomal membrane fraction after saline infusion ($\circ$) or euglycaemic/hyperinsulinaemic clamp ($\bullet$). Results are the means $\pm$ S.E.M. for four independent experiments in duplicate. When error bars are not visible, they are contained in the symbol.

were then used to study the properties of the brown-adipose-tissue glucose transporter, as described below.

Characteristics of brown-adipose-tissue plasma and microsomal membranes

For preparation of plasma and microsomal membranes, brown adipose tissue was collected from rats 60 min after a saline infusion, or a hyperinsulinaemic/euglycaemic clamp as described in Table 1, except that no 2-deoxy[1- ^3H]glucose was injected. The enrichment of the plasma and microsomal membrane was comparable

in the two groups: a 19.4 ± 0.4 - and 17.0 ± 0.6 -fold increase in 5'-nucleotidase specific activity was achieved in brown-adipose-tissue plasma membrane of control and insulin-treated rats respectively. Similarly, a 7.8 ± 0.6 - and 8.0 ± 0.4 -fold increase in NADPH-cytochrome *c* reductase specific activity was measured in microsomal membranes of control and insulin-treated rats. The recovery of the two types of membranes was also similar, as shown by Table 2.

Glucose transporters were measured in plasma membranes by studying the D-glucose-displaceable [^3H]-

Table 3. Hill coefficient, K_d and number of D-glucose-inhibitable cytochalasin B binding sites in plasma and microsomal membranes of rat brown adipose tissue after saline infusion (control) or hyperinsulinaemic/euglycaemic clamp

Plasma and microsomal membranes were obtained as described in the Materials and methods section. Results are means $\pm$ S.E.M. of four independent preparations for each group: * $2P < 0.01$; ** $2P < 0.002$; *** $2P < 0.001$. Brown adipose tissue was collected after 60 min of saline infusion or euglycaemic/hyperinsulinaemic clamp. †These results take into account the recovery of protein and the specific activity of marker enzymes.

	Control	Hyperinsulinaemic
Hill coefficient		
Plasma membranes	1.1 $\pm$ 0.1	2.5 $\pm$ 0.2***
Microsomes	1.3 $\pm$ 0.1	1.2 $\pm$ 0.1
K_d (nM)		
Plasma membranes	148 $\pm$ 12	82 $\pm$ 6*
Microsomes	154 $\pm$ 12	129 $\pm$ 10
Cytochalasin B binding sites (pmol/mg of membrane protein)		
Plasma membranes	6.8 $\pm$ 0.6	13.3 $\pm$ 1.3*
Microsomes	15.2 $\pm$ 1.0	6.0 $\pm$ 0.6***
Cytochalasin B binding sites (pmol/g of tissue)†		
Plasma membranes	37.4 $\pm$ 4.5	101.0 $\pm$ 8.2***
Microsomes	196.9 $\pm$ 15.3	92.6 $\pm$ 10.1**
Total	234.3 $\pm$ 17.2	193.6 $\pm$ 16.2

cytochalasin B, in the presence of increasing concentrations of the ligand. Fig. 2 describes this measurement, in the presence or absence of 500 mM-D-glucose (Figs. 2a and 2b), with ten different concentrations of cytochalasin B, in plasma membranes obtained from brown adipose tissue studied under basal conditions (saline) or after euglycaemic/hyperglycaemic clamps. The specific (glucose-displaceable) cytochalasin B binding is shown by Figs. 2(c) and 2(d), and illustrates the stimulatory effect of insulin on the number of glucose transporters present within plasma membranes compared with that measured in brown adipose tissue obtained from saline-infused controls. When the data are analysed as described by Hill (1910), the curve obtained (Fig. 2c) for plasma membranes from control brown adipose tissue shows a Hill coefficient of 1.0, indicating no interaction between the transporters. Similar analyses performed in plasma membrane after exposure to insulin (Fig. 2d) show an increase in the maximal binding and an increase in the Hill coefficient to 2.3. These measurements were further performed in four independent preparations of plasma, and microsomal membrane preparations, now with six concentrations of the ligand, as shown in Fig. 3. Insulin treatment clearly increases the number of glucose-displaceable cytochalasin-B-binding sites on brown-adipose-tissue plasma membranes while decreasing those associated with the microsomal membranes [Figs. 3(a) and 3(b), Table 3]. These results are not corrected for cross-contamination, so that they actually represent an under-estimation of the effect promoted by insulin. The total number of binding sites was unchanged and, in addition, insulin increases the Hill coefficient of those glucose transporters present within the plasma mem-

branes, but it does not modify that of the microsomal membranes (Table 3). Furthermore, insulin also significantly decreases ($P < 0.01$) the apparent K_d of the glucose transporter for cytochalasin B in plasma membranes, but not in microsomal membranes (Table 3).

DISCUSSION

The aim of the present study was to determine the effect of insulin on the state of the glucose transporters of brown adipose tissue, by the labelled cytochalasin-B-binding assay described previously (Cushman & Wardzala, 1980). In order to do so, a modified method to pellet brown-adipose-tissue plasma and microsomal membrane had to be adopted, since, at 400 mM-glucose (the concentration of glucose that optimally displaces cytochalasin B binding), plasma and microsomal membranes of brown adipose tissue do not completely sediment.

A comparison of this method (based on protein precipitation) with the original one (using ultracentrifugation) yield similar results in terms of total plasma glucose transporter number, K_d values and Hill coefficients (Fig. 1). The advantages of the new method lie in a greater rapidity and, more importantly, in the completeness of the recovery of membrane proteins, and hence of the glucose transporter proteins.

Besides the technical improvement of a previously described method, the present series of investigations aimed at providing some explanations for the very large stimulatory effect of insulin *in vivo* on glucose transport and utilization. Indeed, it was observed that increasing the steady-state concentrations of insulin *in vivo* from a basal value of 58 to 632 munits/l during an euglycaemic clamp resulted in a 40-fold increase in glucose uptake and utilization by brown adipose tissue, an observation in keeping with previous data of Ferré *et al.* (1986).

This striking effect of insulin *in vivo* on the transport and utilization of glucose by brown adipose tissue prompted us to define the state of the glucose transporter system in plasma and microsomal membranes obtained, as described in the Materials and methods section, either after 1 h of saline infusion (control group) or after 1 h of hyperinsulinaemic/euglycaemic glucose clamps. All data obtained by the cytochalasin-B-binding assay was analysed by the Hill equation, which provides information not only on the total number of glucose transporters, but also on their properties.

It is noteworthy that, when exposed to saline or to hyperinsulinaemia *in vivo*, subsequently isolated plasma and microsomal membranes of brown adipose tissue have a different glucose transporter number. Thus, in the control group, most of the transporters are intracellularly located and few are at the plasma-membrane level. In contrast, in the insulin-exposed group, most of the glucose transporters are within the plasma membrane, whereas the number of intracellular ones decreases without a change in the total number of transporters (Fig. 3; Table 3). This is in keeping with the concept described by others, either in white fat-cells (see the Introduction for references) or in the diaphragm (Wardzala & Jeanrenaud, 1981, 1983), showing that insulin, by some unknown mechanism favours the translocation of glucose transporters from an intracellular compartment to the plasma membrane.

It should be emphasized that the insulin-induced translocation process *in vivo* resulted only in a 2-fold

increase in the number of plasma-membrane glucose transporters of brown adipose tissue (Table 3), whereas a 40-fold *in vivo* increase in glucose uptake and utilization was observed (Table 1). This could be due to some alterations related to the membrane preparation process, but this possibility is unlikely, as two different methods for isolating membranes yielded identical results (Fig. 1). Although it is conceivable that one glucose transporter could carry more than one glucose molecule, alternative explanations can be provided on the basis of the present work. Thus, when plasma membranes are obtained from brown adipose tissue of saline-infused rats, a Hill coefficient of 1 is found, suggesting the absence of interaction between glucose-transporting molecules. This is in contrast with the observation of an increase in the Hill coefficient, to 2.5, of those transporters once translocated to the plasma membranes under the influence of insulin (Table 3). Furthermore, the affinity of plasma-membrane glucose transporters for cytochalasin B is low in control brown adipose tissue ($K_d = 148$ nM) and becomes greater under the influence of insulin ($K_d = 82$ nM). The affinity of glucose transporters for cytochalasin B cannot be equated to that of glucose itself, but it may be suggested that the observed changes in K_d values could reflect those of the transporters toward glucose. In addition, one should stress that, when glucose transporters are not at the plasma membrane but within the microsome, no change in Hill coefficient or K_d value are brought about by insulin exposure.

To sum up, it is suggested that the marked effect of insulin on glucose uptake (and metabolism) by brown adipose tissue *in vivo* is due to at least three complementary mechanisms: (a) translocation of glucose transporters from an intracellular pool(s) to the plasma membrane, as previously described for other tissues (see the Introduction and above for references); (b) the possible occurrence (based on the K_d values just mentioned) of an increase in the affinity of the transporters for glucose once translocated in the plasma membrane; (c) changes in the properties of the glucose transporters present at the plasma membrane, indicative of an interaction bringing about a positive co-operativity between transporters. This insulin-induced change (reflected by the increase in the Hill coefficient) could be related to as yet unidentified modifications of the transporters, thereby increasing their intrinsic activity. Such changes in the properties of the transporters have been previously hypothesized (Hyslop *et al.*, 1985; Kahn & Cushman, 1985). Analogous (though not identical) changes in glucose transporters have been obtained upon stimulation of brown adipose tissue by cold exposure (Greco-Perotto *et al.*, 1987).

The above conclusions are drawn with the reservation that precise correlations between changes in glucose transport and changes in glucose transporters should ultimately be carried out with the same pool of isolated brown-fat cells.

In summary, the marked stimulation *in vivo* of glucose uptake and utilization by insulin in brown adipose tissue could be related not only to an insulin-mediated translocation of transporters to the plasma membrane but also to genuine insulin-induced changes of the

properties of the plasma-membrane glucose transporters that increase their capacity for transporting glucose.

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