Modulation of glucose transport in hamster adipocytes by insulin and by β - and α_2 -adrenoceptor agonists

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Summary. Glucose transport in hamster adipocytes and its modulation by insulin and isoprenaline was characterized with the aid of the non-metabolizable hexose 3-O-methylglucose. Insulin stimulated the initial uptake rates by an increase in $V_{\max}$ of the transport without any detectable change in K_m . The hormone concentration producing half maximal stimulation was identical to that required in rat adipocytes. However, hamster adipocytes were much less responsive to insulin (3-fold stimulation as compared to a 12-fold stimulation in rat fat cells), and maximal transport rates were 10-fold lower than that observed in rat adipocytes. Accordingly, the number of glucose transporters, as assessed by glucose-inhibitable cytochalasin-B binding, was considerably lower in plasma membranes of hamster adipocytes. Moreover, no transporters were detected in the low-density microsomes which in insulin-sensitive cell types represent the intracellular pool of recruitable glucose transporters. The relative insulin resistance of the hamster fat cells may therefore be due to a depleted pool of intracellular glucose transporters. In the presence of adenosine, the β -adrenoceptor agonist isoprenaline produced a

moderate stimulation of the basal transport rate which was antagonized by the α_2 -agonist clonidine. If adenosine deaminase was added in order to remove endogenous adenosine, isoprenaline inhibited the insulin-stimulated transport by 50%. In contrast to the stimulatory effects of insulin and isoproterenol, the inhibitory effect of the catecholamine was reversed by cooling the cells to 22°. Glucagon produced a comparable inhibition, suggesting that the inhibitory effect was mediated by adenylate cyclase or its regulatory subunits. The α_2 -adrenoceptor agonist clonidine fully antagonized the inhibitory effect of isoproterenol. The effect of glucagon was reversed by adrenaline, if propranolol was added in order to unmask the α_2 -agonist action of the catecholamine. The data demonstrate that α_2 -adrenoceptors participate in the regulation of glucose transport by antagonizing both the β -receptor mediated stimulation and inhibition produced by catecholamines.

Key words. Adipose tissue, hamsters, glucose, insulin, adrenoceptor agonists.

Glucose metabolism in the adipocyte is mainly regulated by its transport rate, which is under the hormonal control of insulin [1]. Catecholamines contribute to the regulation of glucose transport [2–4]. Recent evidence suggests that β -adrenoceptors mediate a stimulatory as well as an inhibitory effect on the glucose transport rate [5, 6, 7], the quality of which depends on the inhibitory regulation of adenylate cyclase [8]. In a variety of cells, including the human fat cell [9], the inhibition of adenylate cyclase is controlled by catecholamines via α_2 -adrenoceptors. The aim of the present study was to investigate the regulation of glucose transport as mediated by α_2 -adrenoceptors via the adenylate cyclase complex. The rat fat cell, which is the almost exclusively

used experimental model, apparently lacks α_2 -receptors [10]. Therefore, hamster adipocytes [11] were used in the present study, and their insulin-sensitive glucose transport as well as its modulation by the adrenoceptor agonists isoprenaline and clonidine were characterized.

Materials and methods

Chemicals

Cristalline porcine insulin was a gift from the Hoechst AG (Frankfurt, FRG). Purified human albumin was purchased from Behringwerke (Marburg, FRG). Crude bacterial collagenase (type IV) was supplied by Worthington Biochemicals (Freehold, NJ, USA). Clonidine was a gift from Boehringer (Ingelheim, FRG). Hepes was purchased from Sigma (St. Louis, Mo., USA), silicone oil (Cat. No. 6428 R-15) was obtained from A. H. Thomas (Philadelphia, Pa, USA). All radiochemicals were from Amersham-Buchler (Braunschweig, FRG).

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Isolation of fat cells

Male Syrian hamsters (body weight 65–95 g) bred in our institute were used throughout. The animals had free access to food and water. Isolated fat cells were prepared by a modification of the method of Rodbell [12]. The epididymal adipose tissue of 1–2 hamsters was added to 5 ml of Krebs-Hepes buffer containing (mmol/l) Na 131, K 5.8, Ca 2.5, Mg 1.2, Cl 141, PO₄ 1.2, HCO₃ 1.2, Hepes 10, and glucose 1. Human serum albumin (4%), which gave the best insulin response of all lots of albumin we tested, and collagenase (1.5 mg/5 ml) were added. The tissue was cut with scissors for 15 s and incubated for 25 min at 37°C in a shaking water bath (125 cycles/min with an amplitude of 5.5 cm). The cells were filtered through nylon mesh, washed five times with fresh buffer, and distributed to the incubation vials to yield a concentration of approximately 10⁵ cells/ml. Aliquots of the cell suspension were fixed with osmium tetroxide [13], and were counted in a Fuchs-Rosenthal counting chamber. Total lipid volume was determined by centrifugation of aliquots in hematocrit tubes. Adipocytes from rats weighing 160–190 g were prepared by the same procedure, except that the tissue was digested for 60 min in buffer containing 3.5 mg collagenase/5 ml.

Glucose transport assay

The uptake rate of 3-O-methylglucose was determined with the method of Whitesell and Gliemann [15] with minor modifications. After preincubation with the agents under investigation the fat cell suspension was concentrated to yield samples of 200 µl containing approximately 400,000 cells. If indicated, the samples were allowed to equilibrate to the desired temperature, and were added to 5 µl of buffer containing 0.6 µCi of [3H]methylglucose (final concentration 0.15 mmol/l). The uptake of the glucose analogue was stopped after 10 s by addition of 9 ml of ice-cold phloretin solution (1 mmol/l, buffered with 1.3% NaHCO₃, pH 7.4). Silicone oil was added, and the samples were centrifuged (3 min, 300 mg) within 2 min after the termination of the transport. The cell layer was removed, and cell-associated radioactivity was determined. Blanks which had been added to the stopping solution before addition of radioactivity were included in each series, and all data were corrected accordingly. Equilibrium uptake was determined with samples incubating 60 min with methylglucose, and initial velocities were, if necessary, corrected as described [14].

In rat adipocytes the transport rate was determined with the same procedure except that the uptake of the hexose was stopped after 3 s. The timing was performed with a metronome set at half-second intervals. In the experiments designed to compare the glucose transport in hamster with that in rat fat cells the data were calculated as pmol/s/10⁵ cells. For better reproducibility, in all other experiments the data were normalized per lipid volume and expressed as pmol/s/µl lipid.

Preparation of plasma membranes and assay of glucose transporter number

After a 30-min preincubation in the presence or absence of insulin (4.8 ng/ml) the adipocytes were homogenized, and plasma membranes were isolated as previously described [16] with minor modifications. Basically, cells were washed 2 times with homogenization buffer containing (mmol/l) sucrose 250 and Hepes 10 (pH 7.6), and were pressed 5 times through a plastic cannula (0.7 mm width). The homogenate was defatted by centrifugation for 5 min at 600 g (20°). The infranant was centrifuged for 60 min at 48 000 g, and the resuspended pellet was layered on a discontinuous sucrose gradient (15 and 33%). After centrifugation at 100 000 g for

30 min the plasma membranes formed a band at the interface between the two sucrose concentrations. A low density microsomal fraction was isolated as described [17] by centrifugation of the supernatants at 200 000 g for 60 min. Similar to previously reported data [24] the specific activity of a Golgi marker enzyme was 3-fold higher in the low-density microsomes than in the plasma membranes (90 vs. 29 nmol/mg/120 min), indicating that the contamination of plasma membranes with intracellular membranes was not higher than 5% [24]. The membranes were collected, washed with buffer consisting of (mmol/l) NaCl 100, Hepes (pH 7.6) 10, Phenylmethylsulfonylfluorid 0.1, and MgSO₄ 0.2, resuspended to yield a protein concentration of approximately 1 mg/ml, and frozen in liquid nitrogen. The membranes were stored at –50° for up to 3 months.

Glucose-inhibitable cytochalasin-B binding was determined as described previously [17].

Lipolysis

Lipolytic activity of adipocytes was assessed with glycerol release determined enzymatically as described [18].

Statistical analysis

Statistical analysis was performed with Student's t-test for paired data, and calculated as mean ± SEM. Significance was established at $p < 0.05$.

Results

In preliminary experiments the conditions for the isolation of hamster adipocytes were optimized according to the criteria of cell yield, cell lysis as judged from lipid formation, and insulin responsiveness. The results led to a procedure slightly different from that used for rat adipocytes. A lower collagenase concentration (0.3 mg/

Fig. 1. Time course of 3-O-methylglucose uptake in basal (filled circles) and insulin-stimulated (open circles) hamster adipocytes. Isolated adipocytes were incubated for 20 min with (4.8 ng/ml) or without insulin at 37°C, and transport was initiated (37°C) and stopped at the indicated time points as described in "Materials and methods". Data are means of duplicates from a representative experiment

Fig. 2. Effect of insulin on 3-0-methylglucose transport in hamster (filled circles) and rat adipocytes (open circles). Initial transport rates were determined at 22°C after a pulse time of 10 (hamster) and 3 (rat) s. The arrows symbolize half-maximal effects. Data show means $\pm$ SEM of 7 separate experiments

ml vs. 0.7 mg/ml in rats) and a shorter incubation time (25 vs. 60 min) were sufficient to prepare hamster adipocytes in good yield (usually 5×10^6 cells/hamster vs. 6.5×10^6 cells/rat). The adipocytes prepared with this procedure were larger than those we usually obtain from rats ($160 \text{ pl/cell} \pm 53$ vs. 80 ± 7 , $n = 6$). Attempts to prepare smaller adipocytes were unsatisfactory because of poor yield of cells from adipose tissue of younger hamsters. Since α_2 -receptors are not fully developed in young hamsters [19], we decided to use animals weighing not less than 65 g.

The initial transport rate of methylglucose in hamster adipocytes was considerably smaller than that observed in rat adipocytes. Studies of the time course of uptake (Fig. 1) revealed that the uptake was linear for several seconds, and that a termination of the transport after not less than 10 s was necessary for acceptable reproducibility. Figure 2 shows the effect of insulin on the initial uptake rate of 3-0-methylglucose in rat adipocytes as compared to that in hamster adipocytes. Basal as well as insulin-stimulated transport rates were significantly lower in the hamster cells ($p < 0.01$). We usually observed a 2- to 3-fold effect of insulin in hamsters as compared to a 8- to 12-fold effect in the rat, the maximum rate in hamster adipocytes being only 10% of that of rat adipocytes. Insulin concentrations giving rise to half maximal response were comparable in both cell types (150 pmol/l). As can be seen from Figure 3, which shows the substrate dependent transport rate in hamster adipocytes, insulin produced a significant increase in $V_{\max}$ with no detectable change in K_m ($p < 0.01$).

Fig. 3. Substrate dependent 3-0-methylglucose transport in isolated hamster adipocytes in the absence (open circles) or presence of insulin (24 ng/ml). Data obtained with 0.15 mmol/l methylglucose are not shown in the double reciprocal plot (lower panel) but were included in the calculation. $V_{\max}$ was 0.59 ± 0.09 vs. 1.8 ± 0.4 pmol/s/ μ l lipid ($p < 0.01$ was tested with Student's paired t-test), K_m was 7.2 ± 1.4 vs. 8.2 ± 2.6 mmol/l (not significant), as calculated from 5 experiments analyzed separately by linear regression analysis. Data represent means $\pm$ SEM

The plasma membranes of hamster adipocytes contained a considerably lower amount of glucose transporters than plasma membranes obtained from rat adipocytes as assessed by measurement of glucose-inhibitable cytochalasin-B binding sites (Fig. 4). In contrast to the large effect observed in rat plasma membranes, insulin failed to produce a significant increase of the transporter number in hamster adipocyte plasma membranes. Moreover, in low-density microsomes from hamster adipocytes no glucose transporters were detectable with the cytochalasin-B binding assay.

Figure 5 shows the effect of isoprenaline on the basal transport rate in the absence of adenosine deaminase. As in all preceding experiments, the transport assay was performed at 22°, after the cells had been incubated with the agents under investigation at 37°. The catecholamine produced a significant stimulation at a concentration of 10^{-7} mmol/l which was reversed at 10^{-6} mmol/l. Clonidine (10^{-5} mmol/l) fully antagonized

Fig. 4a and b. Effect of insulin on glucose transporter distribution in plasma membranes (a) and low-density microsomes (b) from rat (left) and hamster adipocytes (right). Isolated adipocytes were incubated for 20 min with (4.8 ng/ml) or without insulin, membrane fraction were prepared, and glucose-displaceable cytochalasin-B binding was assayed as described under "Materials and methods". The data represent means $\pm$ SEM of 3 separate experiments, each of which was done with cells obtained from 20 rats or hamsters. The dotted bar in the lower panel (low-density-microsomes) symbolizes the limit of sensitivity of the assay. ND = not detectable

Table 1. Effects of isobutylmethyl-xanthine (IBMX) (0.1 mmol/l) and clonidine (10 μ mol/l) on glucose transport and lipolysis in isolated hamster adipocytes

	Glucose transport pmol/10s per μ l lipid		Glycerol release nmol/30 min per 10^5 cells	
	Control	Clonidine	Control	Clonidine
Control	0.11 $\pm$ 0.01	0.11 $\pm$ 0.02	4.1 $\pm$ 0.6	4.9 $\pm$ 0.1
IBMX	0.07 $\pm$ 0.01	0.09 $\pm$ 0.14	73.1 $\pm$ 6.0	35.8 $\pm$ 6.0
	NS		$p < 0.01$	

Isolated cells were incubated at 37°C for 30 min in the presence of the agents as indicated, and the medium was removed for assay of glycerol. Glucose transport was determined at 22°C in aliquots of the concentrated cell suspension, and was normalized per lipid volume as assayed in a separate aliquot. The data represent means $\pm$ SEM of 7 separate experiments. Differences were tested with Student's paired t-test

ized the stimulatory effect of isoprenaline (Fig. 5). The addition of adenosine deaminase, which in rat adipocytes turns the stimulatory effect of catecholamines into a inhibition of the insulin-stimulated transport rate [6, 7], failed to produce any significant effect if cells were incubated according to the conditions applied in the experiment shown in Figure 5 (37.5 ± 2 vs. 32.3 ± 2 fmol/s/ μ l lipid in controls). However, experiments in which the transport assay was performed at 37°C clearly

Fig. 5. Stimulatory effect of isoprenaline and its reversal by clonidine on the initial 3-0-methylglucose transport rate in hamster adipocytes. Isolated cells were incubated for 30 min at 37°C with the indicated concentrations of the agents. The cell suspensions were concentrated and glucose transport was determined at 22°C as described under "Materials and methods". The data represent means $\pm$ SEM of six separate experiments. Significance was tested with Student's paired t-test

Fig. 6. Inhibitory effect of isoprenaline or glucagon and its reversal by α_2 -adrenoceptor agonists. Isolated hamster adipocytes were incubated for 20 min with insulin (4.8 ng/ml). The cells were thereafter incubated for 15 min in the presence of adenosine deaminase (ADA, 2.5 μ g/ml), isoprenaline (1 μ mol/l), clonidine (10 μ mol/l), glucagon (1 μ g/ml), adrenaline (10 μ mol/l), and propranolol (10 μ mol/l) as indicated, and transport was assayed at 37°C. The data are means $\pm$ SEM of 4 separate experiments

revealed a large inhibitory effect of isoproterenol (Fig. 6) which was fully reversed by clonidine. Further, glucagon produced a comparable inhibition which was completely antagonized by adrenaline and propranolol, the combination of which was chosen in order to produce the most specific and efficient stimulation of α_2 -receptors [10].

Table 1 demonstrates the stimulatory effect of the adenosine antagonist isobutylmethyl-xanthine (IBMX)

on lipolyses in hamster adipocytes which was accompanied by an inhibitory effect on glucose transport (35%). As anticipated, the α_2 -agonist clonidine significantly, although not completely, antagonized the stimulatory effect of IBMX on lipolysis ($p < 0.01$), but failed to significantly reverse the inhibitory effect of the methylxanthine on glucose transport.

Discussion

As in rat adipocytes, glucose transport in hamster adipocytes was modulated by catecholamines in both a stimulatory and inhibitory way. This complex regulation is under control of the inhibitory regulatory subunit of adenylate cyclase (N_i) in that its activation, e.g. by adenosine, precludes the inhibitory effect of catecholamines. Removal of adenosine or irreversible inhibition of N_i by *Bordetella pertussis* toxin uncovers their counterregulatory effects [5–8]. The present data confirm and extend this concept by showing that α_2 -adrenoceptors counteract both the stimulatory and the inhibitory effect of the β -agonist isoprenaline on glucose transport.

Since the response of target cells to a catecholamine depends on the type of receptors present rather than on the specificity of the agent, inhibitory effects of adrenaline and noradrenaline are likely to be predominant in rat adipose tissue, provided that adenosine is effectively removed by adenosine deaminase which is present in the connective tissue. In adipocytes equipped with α_2 -adrenoceptors, e.g. from hamster [11, 20, 21] or human [9], the balance between α_2 - and β -receptors will be as crucial for the quality of the response as the catecholamine concentration. Further, differences in the regional distribution of α_2 -receptors might account for different lipolytic or lipogenic activities in the various sites of adipose tissue localization.

The failure of clonidine to significantly reverse the inhibitory effect of IBMX on the transport rate does not exclude the above described conclusions. Methylxanthines inhibit glucose transport probably by a mechanism unrelated to their effects on adenylate cyclase, since their effects on lipolysis can be dissociated from those on glucose transport with respect to the concentration dependencies as well as to the reversibility of the inhibition by antilipolytic agents [22, 23]. In addition, IBMX inhibited glucose transport in plasma membranes under conditions which preclude formation of cAMP [5]. Thus methylxanthines appear to affect the activity of the transporter directly rather than through an elevation of cAMP levels.

A striking difference between the inhibitory effect of catecholamines and the stimulatory effects of both insulin and catecholamines was their temperature dependence: insulin stimulates glucose transport even at 17° [24, 25]; the stimulatory effect, once produced at 37°, is retained after equilibration of cells to 22°. In contrast,

the inhibitory effect of isoprenaline was spontaneously reversed under these conditions. Therefore, the latter effect appears to be dependent on a relatively high activation energy. Thus, the data stress a fundamental difference in the effects of catecholamines and insulin, and it may be speculated that the inhibitory effect of the β -agonist does not involve re-translocation of transporters to the intracellular pool. Indeed, recent work on rat fat cells substantiates the view that isoproterenol produces a change in intrinsic activity of the transporter without any difference in the transporter number in the plasma membrane [26, 32].

The possibility that the described effects of the catecholamine are secondary to the fatty acids released by stimulation of lipolysis must be considered. Lipolytic agents may produce a decrease of ATP levels in adipocytes which affects insulin action as well as its receptor binding [16, 22]. Further, the rapid fading of adenylate cyclase stimulation usually observed in adipocytes has recently been attributed to accumulation of fatty acids, and under proper incubation conditions a steady-state activation has been obtained [27]. The cell concentration employed in the present study ($1\text{--}2 \times 10^5$ cells/ml) appears to be sufficiently low to preclude intracellular fatty acid accumulation. In addition, the presence of glucose assured that excess fatty acids can rapidly be re-esterified. Further, when the inhibitory effect of isoprenaline was observed, insulin which largely inhibits lipolysis was present. It appears to be unlikely that the inhibitory effect of the catecholamine was secondary to intracellular fatty acid accumulation. However, it cannot be excluded that the stimulatory effect of the catecholamine was mediated by fatty acids, since fatty acids added to the incubation medium produced a moderate stimulation of the transport which is comparable to that observed in the presence of catecholamines and adenosine [28].

The effect of insulin in hamster fat cells resembled that observed in rat fat cells in that the hormone produced an increase in the $V_{\max}$ of the transport with no change in K_m . Further, the concentrations producing half maximal stimulation were essentially identical. However, the results presented above show a marked quantitative difference between the two cell types: hamster adipocytes were considerably less responsive to insulin, which produced a 2- to 3-fold stimulation of the transport rate was compared to the 8- to 12-fold effect observed in rat adipocytes. Further, the maximal transport rate in hamster adipocytes was only about 10% of that of rat adipocytes. Since the K_m of the transport (7–8 mmol/l) was similar to that previously determined in rat adipocytes (3–5 mmol/l, [15]), a difference in affinity to glucose is unlikely to account for the observed difference. Therefore, hamster adipocytes are equipped with either a smaller number or with a less active form of glucose transporters.

Previous studies have demonstrated a good correlation between the number of cytochalasin-B binding

sites and the transport activity in fat cells [29]. Given a proportional relationship, the lower number of cytochalasin-B binding sites as measured in the plasma membranes of the hamster adipocytes can sufficiently explain the 10-fold lower transport activity of these cells. According to the translocation hypothesis, which has been developed on the basis of the cytochalasin-B binding assay, insulin stimulates the insertion of glucose transporters recruited from an intracellular pool (Golgi-enriched low density microsomes) into the plasma membrane [17]. The cytochalasin-B binding data (Fig. 4) of our rat adipocyte plasma membranes and low-density microsomes were in good agreement with the translocation hypothesis, although insulin produced a lower effect in the plasma membranes (2.5-fold) than that observed in intact cells (10-fold stimulation of glucose transport). This discrepancy is routinely observed [24], and has led to the suggestion of an additional effect of insulin on the transporter activity. However, the lower response to insulin can be explained with an elevation of the transporter number in basal plasma membranes: firstly, part of the cytochalasin-B binding sites appears to be inactive (see below), and secondly, the homogenization process may produce a translocation of the transporters to the membrane.

In the plasma membranes of hamster adipocytes, no significant effect of insulin was visible. Moreover, no intracellular transporters could be detected. It might be tempting to speculate, therefore, that insulin increases transport activity in hamster adipocytes by activation of transporters residing in the membrane rather than by recruitment of additional transporters. However, it has to be emphasized that the number of transporters which would account for the observed small transport activation is very small (2–3 pmol/mg) and approximately equals the limit of sensitivity of the assay. Further, a comparative study of cytochalasin-B binding and transport activity in plasma membrane vesicles revealed that a small number of binding sites (3–5 pmol/mg) does not contribute to transport activity (Joost and Cushman, unpublished data). These inactive binding sites form an elevated basal level which renders it difficult to detect small changes of the transporter number. Thus, the translocation mechanism cannot be discounted on the basis of the present data. From the absence of detectable intracellular transporters it can be concluded, however, that the low response of hamster adipocytes to insulin is due to a depleted (or perhaps absent) pool of recruitable transporters. Thus, the mechanism of insulin resistance of hamster adipocytes appears to be very similar to that of the guinea pig adipocyte [30] and to that of several insulin-resistant states [31].

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