Enhancement of glucose transport by insulin at 37 °C in rat adipocytes is accounted for by increased $V_{\max}$

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Summary. The kinetics of 3-O-methyl-D-glucose and D-glucose transport was reinvestigated at 37 °C in rat adipocytes exhibiting very slow transport rates in the absence of insulin and approximately 30-fold insulin stimulation. The apparent maximal velocity for 3-O-methylglucose was increased at least 15-fold from 0.04 ± 0.01 to $0.73 \pm 0.17 \text{ mmol} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$ (SD, $n=7$). The apparent half saturation constant was decreased by insulin from $8.1 \pm 1.6 \text{ mmol/l}$ to $3.3 \pm 0.8 \text{ mmol/l}$. However, approximately 10% of the 3-O-methylglucose at a very low concentration was transferred by routes other than the glucose transporter, e.g. via nonmediated diffusion or via the fructose transporter, and the estimated value for the half saturation constant was therefore too high in the nonstimulated cells. After appropriate correction this value was esti-

mated as $5.0 \pm 1.8 \text{ mmol/l}$. The inhibition constant of 3-O-methylglucose on the conversion of $12 \mu\text{mol/l}$ ^{14}C -labelled glucose to cell-associated products was about 4 mmol/l and was not changed significantly by insulin. Transport experiments with labelled glucose gave results similar to those obtained for transport of 3-O-methylglucose. It is concluded that insulin can cause a 15- to 30-fold increase in the maximal velocity for transport of 3-O-methylglucose and glucose, and that a concomitant decrease in the half saturation constant, if present, is insignificant.

Key words: Glucose transport, insulin, 3-O-methylglucose, fructose, adipocytes.

Several authors have reported that insulin increases the maximal velocity ($V_{\max}$) for the nonmetabolisable glucose analogue 3-O-methyl-D-glucose (3OMG) in rat adipocytes with little, if any, concomitant change in the half saturation constant (K_m), [1–4]. On the contrary, a recent report [5] has shown a large insulin-induced reduction in K_m (from 35 mmol/l to 3 mmol/l) and only a moderate increase in $V_{\max}$, i.e. 4-fold for glucose and 2-fold for 3OMG [5]. This discrepancy has quite severe implications with regard to the mechanism of insulin action. Thus, the hypothesis that the cell has both a pool of nonoperative (perhaps intracellular) and operative (plasma membrane) transporters with closely similar kinetic properties [6, 7], and that the insulin effect is largely accounted for by conversion (probably translocation) of the former to the latter pool [8, 9], cannot be valid if insulin mainly acts by reducing K_m . We have therefore reinvestigated the kinetics of hexose transport. In such studies it is crucial to obtain adipocytes with a very low permeability in the nonstimulated ("basal") state to allow a marked stimulation by insulin. In our cell preparation, the transport of labelled 3OMG was enhanced approximately 30-fold by insulin at 37 °C.

Materials and methods

3-O- ^{14}C]methyl-D-glucose (50 mCi/mmol), [U- ^{14}C]glucose and [U- ^{14}C]fructose (both 300 mCi/mmol) were from Amersham International (Amersham, UK) and [1- ^3H]-L-glucose was from New England Nuclear (Dreieich, FRG). Collagenase (type 1, 267 U/mg , batch 43 D 289) was from Worthington, (Freehold, NJ, USA) insulin from Nordisk Gentofte, (Gentofte, Denmark) and phloretin from K & K laboratories (Plainview, NY, USA). Unlabelled 3OMG and bovine serum albumin (fraction V) were from Sigma (St. Louis, Mo, USA). Other chemicals were analytical grade.

Adipocytes were prepared by collagenase digestion [10] of epididymal fat pads from ad lib fed Wistar rats weighing 150–170 g as described [11]. The tissue was minced in 3-ml polyethylene vials containing 3 ml 10 mmol/l Hepes buffer, pH 7.4, with Krebs' salts (in mmol/l): 131 NaCl , 4.7 KCl , 2.5 CaCl_2 , 1.25 MgSO_4 , $2.5 \text{ NaH}_2\text{PO}_4$, 3.5% albumin, 1.0 mmol/l D-glucose and 0.5 mg/ml collagenase. Following gentle stirring for about 60 min with a teflon coated bar the suspension was passed through a $300\text{-}\mu\text{m}$ nylon filter, washed twice in buffer with 1% albumin and once in 37°C buffer with 5% albumin and 1 mmol/l D-glucose. It should be noted that glucose has a K_m for transport of about 7 mmol/l [2] or higher [5], and the presence of 1 mmol/l should therefore have little impact on the measured K_m for 3OMG. Finally, the suspension was adjusted to 40% cytochrome. When needed, insulin was added to a final concentration of $1 \mu\text{mol/l}$ about 20 min before the experiment.

Hexose transport was measured in two ways following previously published methods: I. $12 \mu\text{l}$ albumin free buffer containing $0.1 \mu\text{Ci}$ labelled 3OMG and unlabelled 3OMG, when needed, was

added to a 11 × 50 cm roundbottom tube. The tube was warmed to 37°C and 40 µl cell suspension was squirted on to the isotope [2]. This method was used for measurement of transport both in "basal" and insulin treated cells. II. 30 µl of cell suspension was added to the assay tube and incubated for 10 min. Six µl of buffer containing tracer was then added and the cells were swirled once by hand [5]. This method was only used for measurement of transport in cells not stimulated by insulin since we found that mixing was not fast enough for measurement in stimulated cells. All experiments were carried out using the zero trans protocol, i.e. unlabelled (and labelled) 3OMG was at time zero only present in the extracellular compartment.

The incubations were terminated by the addition of 3 ml albumin-free buffer containing 0.3 mmol/l phloretin. 0.5 ml silicone oil with a density of 0.99 was layered on top of the aqueous phase and the tubes were centrifuged for 40 s at about 2000 g [2]. The cell pellet was collected from the top of the oil phase and the radioactivity measured by liquid scintillation counting.

Uptake of [¹⁴C]glucose (12 µmol/l) was measured by incubation of adipocytes (2% cytochrome) in a volume of 500 µl for 30 min with shaking to keep the cells in a homogeneous suspension. Incubations were terminated by transfer of 400 µl suspension to oil filled microfuge tubes followed by centrifugation and counting of radioactivity in the cell pellet. At this condition, the rate of accumulation of radioactivity is proportional to the glucose transport rate [12].

Statistical analysis

The method of least squares for regression analysis was used.

Results

Figure 1A shows the equilibration of extracellular 3OMG at various concentrations with the intracellular water. Half equilibration was obtained by 3 min when using 0.1 mmol/l 3OMG at 37°C and at about 6 min when using 8 mmol/l, whereas much longer time was required for half equilibration of 40 mmol/l 3OMG. The results suggest an apparent K_m of the order of magnitude 8 mmol/l, since this 3OMG concentration reduced the initial influx of labelled sugar analogue by about 50% and doubled the half time of equilibration. The inset shows that equilibration of 0.1 mmol/l and 8 mmol/l 3OMG approximated exponential curves with rate constants of 0.22 min⁻¹ and 0.11 min⁻¹, respectively. Equilibration of 40 mmol/l was nonexponential in agreement with previous results on more permeable adipocytes [2, 13]. The infinite cis entry K_m was calculated from the 40 mmol/l 3OMG progress curve according to previously published procedures [13] as approximately 10 mmol/l.

The rate of equilibration was markedly increased when the cell suspension was cooled to 22°C and transport measurement was carried out at that temperature. This previously noted phenomenon [13] stresses the importance of the physiological temperature for maintaining the cells at a state with low "basal" glucose permeability.

Additional findings (not shown) were that 0.1 mmol/l 3OMG equilibrated at closely similar rates when using methods I and II. We also found that 1 mmol/l D-glucose had no measurable effect on

Fig. 1. Time course of sugar uptake. Panel A shows the uptake (37°C) at the following 3-O-methylglucose (3OMG) concentrations in mmol/l: 0.1 (●—●); 8.0 (○—○); and 40.0 (▲—▲). The points are the mean values of 4 replicates with 1 SD. The broken line shows uptake of 0.1 mmol/l 3OMG at 20°C. The inset shows a logarithmic transformation with f_i as the fraction of the intracellular 3OMG distribution space filled at a given time. The uptake of 0.1 mmol/l 3OMG at 30 min (37°C) was taken to indicate the intracellular distribution space. The following exponentials provided the best fit: 0.1 mmol/l 3OMG: $y = 0.7 (1 - e^{-0.22x})$; 8.0 mmol/l: $y = 0.7 (1 - e^{-0.11x})$. Thus, the rate constant for uptake of 0.1 mmol/l 3OMG (v/S) was 0.22 min⁻¹. The uptake curve was, as expected, nonexponential with 40 mmol/l 3OMG. Panel B shows the following uptakes at 37°C: 0.1 mmol/l 3OMG (●—●) or D-glucose (○—○) in the presence of 20 µmol/l cytochalasin B. These data are described by the equation: $y = 0.7 (1 - e^{-0.019x})$ i.e., the rate constant of entry is 0.019 min⁻¹. ³H-labelled L-glucose in the presence of 40 mmol/l 3OMG (x—x). The broken lines show uptake of 0.1 mmol/l fructose in the absence (▲) or presence (△) of 20 µmol/l cytochalasin B.

3OMG transport in agreement with the measured K_m for glucose of 7 mmol/l or higher [2].

It is a necessary premise for the calculation of a meaningful K_m that one considers 3OMG transferred only via the D-glucose transporter. However, sugar may also cross the cell membrane via other routes. Figure 1B shows that both 0.1 mmol/l 3OMG and 0.1 mmol/l D-glucose enter the intracellular distribu-

Fig. 2. Concentration dependence of uptake. The reciprocal rate constants for uptake (cf. legend to Fig. 1) at different 30MG concentrations are plotted against 30MG concentration (●—●, regression line: $y = 20.0x + 180$) and half saturation constant (K_m) is calculated as 9.0 mmol/l from Hanes' form of the Michaelis-Menten equation: $S/v = K_m/V_{max} + S/V_{max}$ (V_{max} = maximal velocity). When data are corrected for cytochalasin B-insensitive 30MG uptake by subtracting 0.020 from all rate constants (▲—▲, regression line: $y = 28.0 + 173$), then K_m for the glucose transporter is calculated as 6.2 mmol/l. For comparison the analogous data is shown for insulin-treated cells (○—○, regression line: $y = 1.1x + 5.9$) with K_m calculated as 5.4 mmol/l. Note that the rate constant of 0.1 mmol/l 30MG uptake is about 30-fold higher in the insulin-stimulated cells and correction for cytochalasin B-insensitive uptake has therefore practically no influence

Fig. 3. Inhibition by 30MG of the uptake and metabolism of D-glucose. Fat cells (2% v/v) were incubated for 30 min with 12 $\mu\text{mol/l}$ [$U\text{-}^{14}\text{C}$]-D-glucose, and uptake of radioactivity in the cell pellet was measured in the absence and presence of 30MG at various concentrations. The ordinate shows the fraction of radioactivity taken up into the cell pellet by 30 min, i.e. equivalent to a rate constant of uptake. The plot is therefore analogous to that of Figure 2. The data in the absence of insulin (●—●, regression line: $y = 26x + 88$) gave a 30MG inhibition constant (K_i) of 3.4 mmol/l. In the presence of insulin (○—○, regression line: $y = 1.2x + 5.0$) the K_i value was 4.2 mmol/l

tion space with a rate constant of about 0.019 min^{-1} in the presence of 20 $\mu\text{mol/l}$ cytochalasin B. The rate constant in 5 analogous experiments with 0.1 mmol/l 30MG was $0.020 \pm 0.002 \text{ min}^{-1}$ (1 SD) and this value

remained unchanged when the 30MG concentration was increased to 40 mmol/l (not demonstrated). In separate experiments (not shown) half maximal inhibition was obtained with 0.1 $\mu\text{mol/l}$ cytochalasin B and 20 $\mu\text{mol/l}$ should therefore be a saturating concentration. Figure 1B also shows that the fairly rapid uptake of fructose, which in cells not stimulated by insulin occurs almost exclusively via a specific fructose transporter [14], is not inhibited by cytochalasin B. It is therefore possible that 30MG and D-glucose are taken up by the fructose transporter, although at slow rates. Finally, Figure 1B shows a very slow uptake of L-glucose in the presence of 40 mmol/l 30MG. L-glucose is taken up by the D-glucose transporter, although with a very low affinity [2], and the residual uptake in the presence of 40 mmol/l 30MG may therefore represent non-mediated diffusion. We conclude from these experiments that 30MG is in part transported through a pathway which is not the glucose transporter, since it is not inhibited by cytochalasin B. This transport, which may occur via the fructose transporter, is a significant fraction of the total 30MG transport in the "slow" cells not treated with insulin.

In the following experiments the entry velocities were calculated from the early parts of the curves which did not deviate significantly from exponential uptakes as also shown previously [5]. Figure 2 shows the concentration dependence of transport. When no correction was applied in the calculation, insulin caused a decrease in the apparent K_m from 9.0 mmol/l to 5.4 mmol/l and an increase in V_{max} from $0.04 \text{ mmol} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$ to $0.73 \text{ mmol} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$. However, when the data were corrected for transfer through a cytochalasin B insensitive pathway, K_m in the nonstimulated cells was reduced to 6.2 mmol/l.

Figure 3 shows the inhibition by 30MG of the uptake of 12 $\mu\text{mol/l}$ [$U\text{-}^{14}\text{C}$]glucose into the cell pellet. At this condition, glucose uptake is proportional to glucose transport [14]. It appears that the inhibition constant, K_i , for 30MG remains about 4 mmol/l whether or not the cells are treated with insulin.

Table 1 summarizes the effect of insulin on the kinetic parameters for 30MG, and it appears that insulin causes a large increase in V_{max} . It also decreases the apparent K_m for 30MG transport, but this effect disappears when data are corrected for the contribution of cytochalasin B insensitive transport of 30MG.

Finally, transport experiments were carried out with labelled D-glucose. Table 2 shows that insulin caused a large increase in the apparent V_{max} and only a small decrease in the apparent K_m . Due to the uptake of glucose by a cytochalasin B insensitive pathway, the latter value is likely to be overestimated in the non-stimulated cells in the same way as when labelled 30MG was used for transport. It can therefore be concluded that insulin increased transport of both 30MG and glucose by increasing V_{max} with little, if any, concomitant increase in K_m .

Table 1. Transport parameters for 3-O-methylglucose

	K_m (mmol/l) ($n=7$)		V_{max} (mmol·l ⁻¹ ·s ⁻¹) ($n=7$)		K_i (mmol/l) ($n=4$)
	app	corr	app	corr	
No insulin	8.1 ± 1.6	5.0 ± 1.5	0.04 ± 0.01	0.03 ± 0.01	4.2 ± 1.1
Plus insulin	3.3 ± 0.8	3.3 ± 0.8	0.73 ± 0.17	0.73 ± 0.17	4.4 ± 0.9

K_m and V_{max} values, apparent or corrected, were calculated as described in the legend to Figure 2. K_i values were calculated as described in the legend to Figure 3. The results are mean values ± 1 SD

Table 2. Transport parameters for D-glucose

	App K_m (mmol/l)	App V_{max} (mmol·l ⁻¹ ·s ⁻¹)
No insulin	7.0 ± 2.8	0.04 ± 0.01
Plus insulin	4.2 ± 1.7	0.75 ± 0.28

Cells were incubated with [U-¹⁴C]glucose and unlabelled glucose to give the same concentration range as that shown for 3OMG in Figure 2. The incubations were timed to give an uptake of labelled glucose equivalent to no more than 25% of the distribution space for 3OMG, i.e. initial velocities were approximated. The apparent K_m and V_{max} values were calculated as shown for 3OMG in Figure 2; no correction was applied. The results are the mean values of 5 experiments ± 1 SD

Discussion

It is a valid argument [5] that the effect of insulin on the glucose transport system should be studied in cells responding very markedly to insulin. Adipocytes can exhibit an extremely low permeability to glucose; when this is the case, insulin can increase transport 30- to 50-fold. However, this state is vulnerable, and the permeability can be increased by a variety of factors; for instance, when the temperature is lowered [13], the pH increased [15], and when the collagenase, the albumin or the mechanical treatment are unsuitable for cell preparation [16]. Therefore, when the cells only respond moderately to insulin (say, 3- to 5-fold) because the preparation is not quite optimal, then a possible effect on K_m may have been lost.

The cells used in this study showed a permeability to 0.1 mmol/l 3OMG in the absence of insulin which was only 2–4% of that in its presence. This may or may not be a measure of the lowest possible permeability. It is conceivable that the membrane, if completely unperturbed, could exhibit a permeability for D-glucose just as low as that for L-glucose. If so, we do not know whether a change from “near zero” to about 3% of the maximal permeability would involve a change in K_m . However, it can be argued that this problem is only of academic interest. What matters is what happens when the cells, due to the action of insulin shift from 3–100% of their maximal permeability.

This shift is obviously brought about by a large increase in V_{max} and the only remaining question is whether there is a small concomitant decrease in K_m . This is where the problem of corrections comes into

the picture. It must be realised that the lower the glucose permeability is due to the glucose transporter, the higher is the relative impact of permeability due to transfer through any other pathway which does not show saturation. This impact is not negligible in the present nonstimulated cells and particularly not at high substrate concentrations.

We have corrected the 3OMG transport data for transfer due to the nonsaturable, cytochalasin B insensitive pathway, and the result is that the small apparent influence of insulin on K_m disappears. It has previously been argued that the nonmediated diffusion can be higher for the slightly more lipophilic 3OMG than for glucose [17] but this was not the case in the present experiments (Fig. 1B). Transport data for D-glucose (Table 2) can therefore be corrected in the same way as those for 3OMG, and the result is again that insulin only increases V_{max} . The same answer was obtained when more indirect measures were used, since the inhibition constant of 3OMG on the rate of conversion of glucose to products was the same whether or not insulin was present.

The present data (Fig. 1) show that in the absence of insulin and at a very low 3OMG concentration, approximately nine-tenths of the sugar analogue is transported into the cell via the glucose transporter and one-tenth via other pathways. The contribution of these pathways, which are not easily saturable, to the total sugar transport, increases with increasing 3OMG concentration as the glucose transporter begins to saturate. Nonmediated diffusion apparently accounts for one-third to one-half of the cytochalasin B-insensitive transport occurring outside the glucose transporter. The remaining part probably occurs via spillover into the fructose transporter – which is the only other known hexose transporter in adipocytes – and which turned out not to be blocked by cytochalasin B.

The remaining question is why insulin in other experiments has been found to cause a large decrease in the apparent K_m . One possibility is that the nonstimulated cells used in that study [5] were even less permeable to glucose due to transfer via the glucose transporter and/or more permeable due to transfer via other pathways with very high K_m values. It should be noted that Whitesell and Abumrad [5] found the presence of glucose (about 1 mmol/l) necessary to maintain a low permeability of the nonstimulated cells. This is in contrast to our results, suggesting a difference between the two cell preparations. Another possibility is that mixing was not sufficient with the method employed, i.e. when a small volume of isotope was added to a larger volume of viscous cell suspension, as was the case in our hands when insulin was present. In any case, the present data allow the conclusion that insulin can cause an approximate 30-fold increase in 3OMG and glucose transport which is very predominantly, and probably exclusively, accounted for by an increase in V_{max} of the glucose transporter.

Two papers on the effect of insulin on glucose transport in rat adipocytes have become available since the submission of this manuscript. Whitesell and Abumrad [18] maintain that large insulin effects are only seen when K_m for 3OMG transport is about 35 mmol/l in nonstimulated cells and that insulin reduces this values to 3.5 mmol/l. Deprivation of glucose and agitation decreases K_m in non-stimulated cells to 2 mmol/l and insulin can under these conditions stimulate transport only about 4-fold. On the other hand, Martz et al. [19] found that insulin caused a 21-fold increase in V_{max} without a significant change in the K_m of around 6 mmol/l. They suggest incomplete mixing as a possible reason for the estimation of a K_m around 35 mmol/l. Thus, the combined evidence seems very strong that in rat adipocytes and under normal laboratory conditions increase in V_{max} can account for at least 95% of the insulin-induced increase in the cells' permeability to glucose, although this mechanism may not be the only one operative under all conditions.

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