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Long-term regulation of hexose transport by insulin in cultured mouse (3T3) adipocytes

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Summary. Observations in vivo suggest that insulin acts as a long-term regulator of hexose uptake in fat cells. In the present study, we examined the long-term effect of insulin on hexose uptake in vitro. Exposure of fully differentiated mouse 3T3-L_1 adipocytes to insulin induced a time-, concentration-, and protein synthesis-dependent increase in basal 2-deoxyglucose uptake (up to 40%) and a decrease in the 'acute' insulin response. The decrease in insulin effect was due to post-receptor alterations, since insulin binding was not substantially altered. The increase in basal 2-deoxyglucose uptake was due to an increase in the apparent V_{max} of the transport system rather than to the observed increase (30%) in hexokinase activity, since the concentration of non-phosphorylated 2-deoxyglucose inside the cell was far below the extracellular concentration. The increase in apparent V_{max} was most likely due to a protein synthesis-dependent increase in de novo synthesis of hexose transporters. Glucose was not essential for the effect. The mechanism responsible for the loss in insulin response remains to be solved. It can be concluded that insulin has the ability to act as a long-term regulator of hexose uptake in fat cells in vitro.

Key words: Hexose uptake, insulin response, 3T3-L_1 adipocytes, cycloheximide, hexose transport kinetics, 2-deoxyglucose.

Several observations in vivo suggest a role of insulin in the long-term regulation of insulin-sensitive hexose uptake in fat cells. In insulin-dependent diabetes mellitus and in streptozotocin-induced diabetes in animals, the hexose uptake by fat cells is decreased [1–6]. Hypoinsulinaemia, induced by fasting, is also accompanied by a low rate of basal hexose uptake [7]. In rats on a high carbohydrate diet, which is accompanied by high plasma insulin values, hexose uptake is increased [8–10], and in experiments in which rats were made hypo- and hyperinsulinaemic, the rate of glucose uptake by the fat cells correlates well with the plasma insulin level [11]. In vitro, a correlation between insulin concentration and hexose uptake has been difficult to establish. In isolated fat cells and in adipose tissue explants, no long-term insulin effects on hexose uptake have been found [12–14], but this could be due to limitations of the methods employed. Isolated fat cells are viable only for several hours, while incubation of explants in culture medium for 17 h induces a 2.5-fold increase in hexose uptake, even in the absence of insulin [13]. Furthermore, mechanical agitation and the use of digesting enzymes in the fat cell isolation procedure are known to affect hexose uptake [15–17]. The present availability of pre-adipocyte cell lines, like the mouse 3T3-L_1 pre-adipocyte cell line [18, 19], offers the opportunity to study the role of insulin in the regulation of hexose uptake in cultured fat cells.

Materials and methods

Cell culture

3T3-L_1 pre-adipocytes (Flow Laboratories, Irvine, Ayrshire, UK) were inoculated at a density of 3×10^3 cells/cm^2 and grown to confluence in Dulbecco's Modified Eagle's medium supplemented with glutamine (2 mmol/l), fetal calf serum (10%) and antibiotics (standard medium). Cells were kept at 37 °C in a humidified atmosphere of 7.5% CO_2 in air, and were fed every other day. Two days post-confluence, adipose conversion was enhanced according to the method of Rubin et al. [20] as modified by Reed and Lane [21]. The cells were cultured in standard medium in the presence of dexamethasone (0.25 μmol/l), 3-isobutyl-1-methylxanthine (0.5 mmol/l) and insulin (1660 nmol/l) for 2 days, and in the presence of insulin alone for 6 days. The cells were then maintained in the absence of insulin for at least 3 additional days. By this procedure, more than 80% of the cells expressed the adipocyte phenotype. In the experiments, fully differentiated cells were cultured in the presence and absence of insulin. After various periods of time hexose uptake and insulin effectiveness were determined.

Deactivation procedure

Before determination of insulin binding and insulin-sensitive hexose uptake, the cells were subjected to a protocol to remove all the cell-bound insulin and to deactivate the hexose transport system ('deactivation procedure'). The protocol was as follows: the cells were washed rapidly four times with 2 ml Dulbecco's phosphate buffered saline (PBS), pH 7.4. Standard medium (2 ml, adjusted to pH 7.0) was added and the cells were incubated at 37 °C for 30 min. The medium was poured off and the cells were washed three times with 2 ml PBS. This procedure was repeated three times. The deactivation procedure was effective in removing > 85% of the cell-bound insulin. After 1 h of incubation (37 °C) of the cells with 20 pmol/l ^{125}I-insulin, 12.8 ± 1.0% of the insulin was bound, whereas after the deactivation procedure insulin binding was 1.8 ± 0.4% (mean ± SD, $n = 3$). Deactivation of the insulin-stimulated hexose transport system was almost complete also. Exposure of the cells to 1 µmol/l insulin for 30 min stimulated the basal 2-deoxyglucose uptake from 2.96 ± 0.26 to 8.73 ± 0.06 nmol/10 min per well (mean ± SD, $n = 3$). After the deactivation procedure, the 2-deoxyglucose uptake was reduced to 3.33 ± 0.23 nmol/10 min per well, and the deactivated cells showed the same insulin sensitivity and responsiveness as untreated cells. To avoid possible non-specific effects of the deactivation procedure, cells exposed to insulin for 30 min and subjected to the deactivation procedure, were taken as control cells in all the experiments.

Insulin binding

Fat cells (60 mm wells) were incubated with 20 pmol/l mono-A^{14}-^{125}I-insulin (360 Ci/mol) in 1.5 ml Krebs-Ringer Tris-HCl buffer containing 2% serum albumin (Tris-buffer), pH 7.4, at 22 °C. Bacitracin (1.5 mmol/l) was added to inhibited extracellular insulin degradation. The wells were shaken (75 oscillations/min) at 22 °C. After 3 h, the cells were rinsed rapidly four times with 2 ml ice-cold PBS, scraped off the dishes and treated with 1 ml of 0.1 mol/l NaOH. Aliquots (0.1 ml) were taken for protein determination [22] and ^{125}I was counted. Non-specific binding was defined as the amount of ^{125}I bound in the presence of an excess of unlabelled insulin (1000 nmol/l). Cell number was counted in a cell-counting chamber (Tamson, Zoetermeer, The Netherlands) and was constant throughout the experiments (5×10^5 cells/30 mm well; 2×10^6 cells/60 mm well).

2-Deoxyglucose uptake

Hexose uptake was measured using 2-deoxyglucose. Deactivated fat cells (30 mm wells) were incubated in 0.75 ml Tris-buffer (pH 7.4) at 37 °C in the presence and absence of insulin. After 30 min, 2-deoxy-D-(1-^{14}C)glucose (0.1 mmol/l, 710 cpm/nmol) was added, and uptake was followed for 2–10 min. Uptake was stopped by rinsing the cells four times with 1 ml ice-cold PBS. Cells were prepared as for insulin binding, except that ^{14}C was counted in a liquid scintillation counter (Packard Instruments, Grovers Down, Illinois, USA). The nature of the labelled substances present in the cell was determined by column chromatography, as described previously for rat adipocytes [23]. The intracellular concentration of 2-deoxyglucose was calculated after determination of the intracellular waterspace, using the double-labelling technique described by Gliemann et al. [24]. Hexokinase activity was estimated by determination of the rate of phosphorylation of 0.1 mmol/l 2-deoxyglucose by cellular homogenates in Tris-buffer, containing ATP (10 mmol/l), and $MgCl_2$ (10 mmol/l) at 37 °C.

Materials

All culture requisites were purchased from Gibco, Grand Island, New York, except for the culture wells (Greiner, Nürtingen, FRG). Demineralized bovine serum albumin was from Organon, Oss, The Netherlands. 2-deoxy-D-(1-^{14}C)-glucose was obtained from New England Nuclear, Boston, Massachusetts, USA. 3-O-methyl-(^{3}H)-glucose and (U-^{14}C)-sucrose were from Amersham International, Buckinghamshire, UK. Mono-A^{14}-^{125}I-insulin (human and porcine) were generously provided by Eli Lilly Nederland, The Netherlands. Native insulin was from Novo, Copenhagen, Denmark. Dexamethasone and bacitracin were obtained from Sigma, St. Louis, Missouri, USA. Cycloheximide from Serva, Heidelberg, FRG and methylxanthine from Aldrich Chemicals, Milwaukee, Wisconsin, USA.

Data analysis

Statistical analysis of the data was performed by Student's t-test for paired comparison.

Results

The long-term effect of insulin on the hexose uptake was examined by culturing the 3T3 fat cells in the presence of insulin (1000 nmol/l) for 24 h and for 30 min (control cells). The cells were then subjected to the deactivation procedure and 2-deoxyglucose uptake was determined (Fig. 1). Prolonged exposure of the cells to insulin increased the basal 2-deoxyglucose uptake by up to 50% compared with control cells ($n = 6$, $p < 0.001$). By contrast, long-term insulin treatment decreased the acute stimulatory effect of insulin on the uptake. Insulin sensitivity decreased approximately fivefold (ED_{50}: 3.10 ± 0.26 versus 0.56 ± 0.15 nmol/l; mean ± SD, $n = 5$, $p < 0.005$), whereas maximal insulin-stimulated 2-deoxyglucose uptake (insulin responsiveness) was decreased by approximately 15% ($n = 6$, $p < 0.05$). ^{125}I-insulin binding (20 pmol/l) was not affected by long-term insulin treatment (9.8 ± 0.5% and 11.0 ± 0.8% bound per well in insulin-treated (24 h) and control cells, respectively, mean ± SD, $n = 6$, NS). At higher insulin concentrations, down-regulation of insulin receptor binding was not a consistent finding either (data not shown).

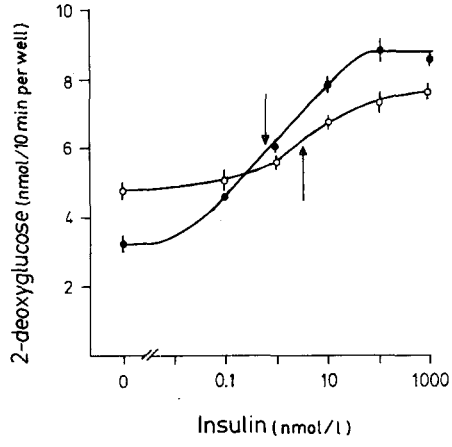

Fig. 1. Dose-response relationship of the insulin-sensitive 2-deoxyglucose uptake. Control cells (○) and cells cultured in the presence of insulin (1000 nmol/l) for 24 h (●) were subjected to the deactivation procedure and uptake of 2-deoxyglucose (0.1 mmol/l) was determined in the presence of increasing amounts of insulin. Arrows indicate the ED_{50} insulin concentration. Data are the mean ± SD of triplicate determinations of one representative experiment

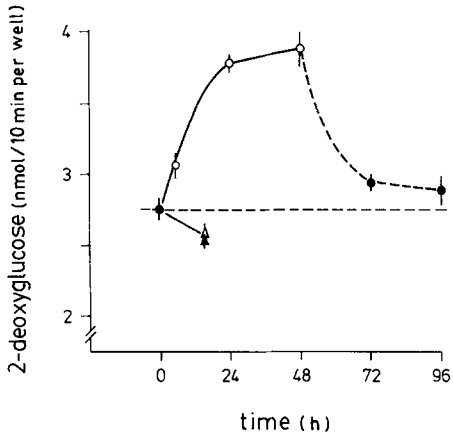

Fig. 2. Time course, reversibility and protein synthesis dependence of the long-term insulin effect. Basal 2-deoxyglucose uptake was determined after culturing fat cells in the presence of insulin (1000 nmol/l) (○), in the absence of insulin following 48-h exposure to insulin (●) and in the absence (▲) and presence (△) of insulin in medium containing cycloheximide (1 μg/ml). The dotted line represents the uptake value of control cells. Data are the mean ± SD of triplicate determinations of one representative experiment

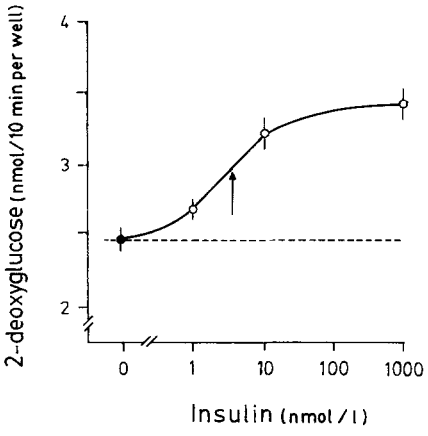

Fig. 3. Concentration dependence of the long-term insulin effect. Fat cells were cultured in the presence of various concentrations of insulin for 24 h. After the deactivation procedure, the uptake of 2-deoxyglucose in the absence of insulin was determined. The arrow indicates the ED_{50} insulin concentration. The dotted line represents the uptake value of control cells. Data are the mean ± SD of triplicate determinations of one representative experiment

Time-course experiments showed that the long-term effect of insulin was maximal within 24 h (Fig. 2). When cells were cultured first in the presence of insulin for 24 h and subsequently in the absence of the hormone, the long-term effect of insulin disappeared within 24 h (Fig. 2). The mediation of the long-term effect of insulin was prevented when cycloheximide (1 μg/ml), an inhibitor of protein synthesis, was present in the culture medium (Fig. 2). In contrast, the acute stimulatory effect of insulin was not affected by cycloheximide (data not shown), suggesting that the acute and long-term effects of insulin were mediated by different mechanisms.

Variations of the insulin concentration in the culture medium revealed that the long-term insulin effect was

Table 1. Characteristics of 2-deoxyglucose uptake into insulin-treated (24 h) and control cells

	Control cells	Insulin-treated cells	p
Hexokinase activity (nmol/min per well)	3.5 ± 0.2	4.9 ± 0.4	< 0.001
Amount of intracellular 2-deoxyglucose (pmol/10 min per well)	98.0 ± 9.0	90.0 ± 11.0	NS
Intracellular water space (μl/well)	3.58 ± 0.12	3.49 ± 0.15	NS
Intracellular 2-deoxyglucose concentration (μmol/l)	27.5 ± 1.4	26.0 ± 1.8	NS
K_m of 2-deoxyglucose uptake (mmol/l)	2.1 ± 0.2	2.3 ± 0.2	NS
V_{max} of 2-deoxyglucose uptake (nmol/min per well)	4.0 ± 0.1	6.1 ± 0.3	< 0.005

Data are expressed as the mean ± SD of four experiments

maximal after exposing cells to 1000 nmol/l insulin (initial concentration) (Fig. 3). The ED_{50} was 4.5 ± 0.36 nmol/l (mean ± SD, $n = 3$), slightly in excess of the ED_{50} of the acute stimulatory effect of insulin in the insulin-pre-treated (24 h) cells (3.10 ± 0.26 nmol/l), and approximately eight times the ED_{50} of the acute insulin effect in the control cells (0.56 ± 0.15 nmol/l). These data, however, should be interpreted with caution, since estimation of the insulin degradation by the trichlorocetic acid precipitation method [25] indicated that the cells degraded 40% of 1 nmol/l ^{125}I-insulin and 4% of 1000 nmol/l ^{125}I-insulin during the incubation period (24 h at 37 °C).

To delineate further the long-term effect of insulin, the 2-deoxyglucose uptake process, which involves transport of hexose across the membrane and formation of 2-deoxyglucose 6-phosphate, was studied in more detail. Long-term exposure (24 h) of the cells to insulin (1000 nmol/l) increased the hexokinase activity of the cells up to 30% (Table 1). This increase was a specific long-term effect, since short-term exposure of the cells (30 min) to insulin did not affect the enzyme activity (control cells) (Table 1). The increase in hexokinase activity might explain the observed increase in 2-deoxyglucose uptake, if phosphorylation and not transport is the rate-limiting step in the 2-deoxyglucose uptake process. However, chromatography of the labelled compounds inside the cells (during the uptake) revealed that the intracellular concentration of non-phosphorylated 2-deoxyglucose, as calculated from data on the amounts of non-phosphorylated 2-deoxyglucose inside the cells and the intracellular water space, was never more than 20–30% of the extracellular concentration (100 μmol/l; $p < 0.001$; Table 1). The same results were obtained when 100 μmol/l cytochalasin B was used to stop the 2-deoxyglucose uptake (data not shown), indi-

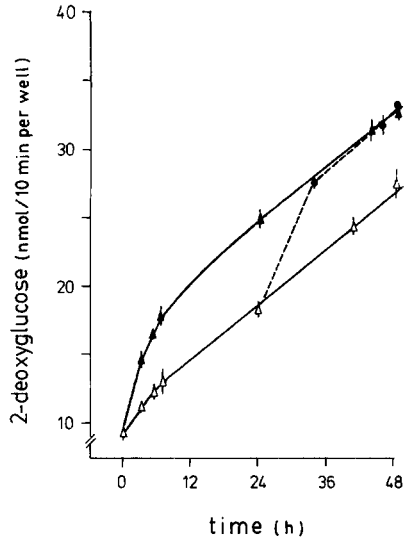

Fig. 4. Long-term insulin effect on glucose-deprived cells. Fat cells were cultured in the absence of glucose for 48 h in the absence (△) and presence of insulin (1000 nmol/l). Insulin was added at the start (▲) and at 24 h (●) after the initiation of the glucose deprivation. At the time points indicated 2-deoxyglucose uptake (0.1 mmol/l) was assayed in the presence of insulin (1000 nmol/l). Data are the mean of triplicate determinations of one representative experiment

cating that efflux of non-phosphorylated 2-deoxyglucose was negligible. The absence of substantial accumulation of non-phosphorylated 2-deoxyglucose inside the cells suggests that transport rather than phosphorylation was rate-limiting in the uptake process and, therefore, that the long-term effect of insulin on the 2-deoxyglucose uptake is mediated by an alteration in activity of the hexose transport system.

Theoretically, an increase in 2-deoxyglucose transport can be attained by an increase in the number and/or activity of the hexose transporters and by an increase in the affinity of the hexose transporters for the hexose. Lineweaver-Burk analysis of a concentration curve of 2-deoxyglucose uptake (0.1–5 mmol/l for 2 min) by insulin-treated (24 h) and control cells revealed that the long-term insulin effect was primarily due to an increase in the apparent V_{max} of the 2-deoxyglucose uptake process (Table 1), i.e. to an increase in the number and/or activity of the hexose transporters. The alteration in hexose transport kinetics was further explored by determining the long-term insulin effect in cells cultured in the absence of glucose. Glucose deprivation has been shown to increase the 2-deoxyglucose uptake by decreasing the rate of inactivation or degradation ('turnover') of hexose transporters (personal observations). As shown in Figure 4, the long-term effect of insulin is still present in cells cultured in the absence of glucose ($n=4$, $p<0.001$). This observation indicates that glucose is not essential for mediation of the long-term insulin effect and, furthermore, that the insulin-induced increase in hexose transport activity is most likely due to an increase in transporter synthesis.

Discussion

In the present study, we tried to delineate the role of insulin in the long-term regulation of hexose uptake in cultured 3T3-L_1 fat cells. The data demonstrate that in cultured fat cells insulin is a long-term regulator of 2-deoxyglucose uptake. Prolonged exposure to insulin increased the basal 2-deoxyglucose uptake and decreased the acute stimulatory effect of insulin on 2-deoxyglucose uptake. The effect was time-, concentration-, and protein-synthesis dependent, and reversible. It was not due to insufficient removal of insulin from the cells, since the long-term effect was inhibited by cycloheximide, in contrast to the acute insulin effect. Furthermore, the effect was not due to non-specific mechanisms, such as a mitogenic effect or an insulin-induced enhancement of the differentiation of the cultured cells, as shown by the reversibility of the effect, the constant cell number and the observation that upon differentiation the basal hexose uptake tends to decrease instead of increase [26]. In the same cell line, Karlsson et al. [27] have noticed similar alterations in the insulin-stimulated hexose uptake after 20 h of exposure to insulin. Our data and Karlsson's data contrast with the results of Rosen et al. [28], who have reported an insulin-induced increase in basal and insulin-stimulated hexose uptake. The reason for this discrepancy is not clear, but could be due to their use of cells at an early stage of differentiation.

The long-term effect of insulin concerns two different aspects of the insulin-sensitive hexose uptake: the (acute) stimulatory effect on the hexose uptake and the basal hexose uptake. The effect on the 'acute' insulin response cannot be explained by an alteration in insulin binding, since down-regulation of insulin receptor binding was not a consistent finding. Whether 3T3 adipocytes have the ability to down-regulate is still controversial [20, 27, 29–32], but it might depend on the stage of differentiation of the cells. At an early stage of differentiation insulin has been shown to enhance the number of insulin receptors [20, 27, 29, 31]. The absence of substantial alterations in insulin binding implies that the loss in insulin response is due to post-receptor alterations. There is a close resemblance between the long-term insulin effect and the desensitization process observed after incubating fat cells with serum of insulin-resistant patients [33] and anti-insulin receptor antibodies [34]. These observations suggest that the mediation of these processes have steps in common, but further studies are required to elucidate the mechanisms responsible for decreased insulin effectiveness.

The long-term effect of insulin on basal 2-deoxyglucose uptake was found to be due to an increase in the apparent V_{max} of the 2-deoxyglucose uptake, a process which involves transport and phosphorylation of the hexose. In rat adipose cells, it has been argued that 2-deoxyglucose uptake reflects its transport only, when low concentrations are used and uptake is measured

over short time intervals. At higher 2-deoxyglucose concentrations and with prolongation of the uptake period, the phosphorylation reaction would become rate-limiting [35]. In the cultured fat cells, prolonged insulin treatment increased the hexokinase activity of the cells. Since during the uptake period the intracellular concentration of the hexose of non-phosphorylated 2-deoxyglucose was far below the extracellular concentration, even when cytochalasin B was present to prevent efflux of non-phosphorylated 2-deoxyglucose, in the cultured fat cells 2-deoxyglucose transport or perhaps transport-associated phosphorylation, but certainly not intracellular phosphorylation appears to be the rate-limiting step in the uptake process. Consequently, the insulin-induced increase in basal 2-deoxyglucose uptake can be attributed to an increase in the number or activity of hexose transporters rather than to an increase in hexokinase activity. The discrepancy between our results and those of Foley et al. [35] regarding the amount of non-phosphorylated 2-deoxyglucose inside the fat cells is unclear, but it may be related to differences in the extraction procedure, or to their use of phloretin to stop the hexose uptake process. Phloretin has been shown to have drastic effects on fat cell metabolism [36], including rapid dephosphorylation of 2-deoxyglucose 6-phosphate [23].

A protein synthesis-dependent increase in the number or activity of hexose transporters can be attained by an increase in de novo synthesis of hexose transporters and by a decrease in their degradation ('turnover'). A protein-synthesis dependent activation or inactivation of hexose transporters has never been described, but nevertheless it cannot be ruled out completely. The finding that the long-term effect of insulin was present even when glucose was omitted from the culture medium, which has been shown to slacken the 'turnover' of transporters (unpublished observations), suggests that the insulin-induced increase in basal 2-deoxyglucose transport is due to an increase in 'synthesis' of transporters rather than to an inhibition of the 'turnover'. The glucose deprivation experiments further indicate that glucose is not essential for mediation of the long-term insulin effect.

The observation that insulin acts as a long-term regulator of hexose uptake in vitro fits well with observations in vivo: high concentrations of insulin are accompanied by a high basal hexose uptake, whereas low insulin concentrations are parallelled by a low basal hexose uptake [1–11]. Recently, Ciaraldi et al. [37] reported the existence of insulin resistance and a low basal hexose uptake in fat cells of patients with Type 2 (non-insulin-dependent) diabetes. Considering our data, it is possible that in Type 2 diabetic patients the long-term insulin effect of stimulating the 'synthesis' of transporters is decreased, resulting in a low basal hexose uptake. This idea is strengthened by the finding of Scarlett et al. [38] that prolonged intensive insulin therapy in Type 2 diabetes significantly reverses the low basal hexose transport activity. Obviously, more insight into the regulation of hexose transporter kinetics is required to make more definitive statements on the role of the long-term effects of insulin in glucose homeostasis.

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