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STIMULATION OF GLUCOSE TRANSPORT AND OXIDATION IN ADIPOCYTES BY FATTY ACIDS: EVIDENCE FOR A REGULATORY ROLE IN THE CELLULAR RESPONSE TO INSULIN

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SUMMARY: Exposure of adipocytes of rats to free fatty acids causes accelerated 3-O-[3H]methyl D-glucose transport maximally at 35-70 μM depending on the degree of unsaturation and chain length, like insulin. Glucose oxidation is also stimulated with a relatively greater activity of the hexose monophosphate shunt. The latter is not suppressed in the presence of cytochalasin B which completely inhibits the stimulatory effect of insulin or of the fatty acids on methyl glucose transport. This supports the original evidence of S.P. Mukherjee et al. that a transport-independent, NAD(P)H-oxidase linked glucose uptake in response to insulin or mimicking agents can differentially enhance the shunt pathway (Biophys. J., 15,314, 1975; Fed. Proc. 34,660, 1975). The potency of the major fatty acids of membrane phospholipids were linoleic acid>oleic acid≥ cis-vaccenic acid>palmitic acid.

INTRODUCTION

Glucose transport in adipocytes is known to be via carrier-mediated diffusion and rate-limiting for intracellular metabolism of the sugar (1-4). Intense efforts have been made in the past to identify the components or to elucidate the mechanism for its regulation. The evidence that insulin as well as several cell surface-reactive agents with various chemical specificities, e.g., concanavalin A (5), polyamines (6) or prostaglandins (7) cause accelerated glucose transport, suggests that a physico-chemical alteration in the plasma membrane may trigger both transport and metabolism of glucose. A new insight into these mechanisms has developed with the discovery of a pyridine nucleotide oxidase in the plasma membrane of adipocytes which is activated by insulin and a number of other agents known to mimic some metabolic actions of insulin (8-11). On the basis of the evidence that this reaction and its product, hydrogen peroxide, have a profound role in several acute cellular responses to insulin, viz., glucose transport and oxidation via the hexose monophosphate shunt (8-11) and regulation of the activities of adenylate cyclase (11-15), depot fat lipase (16-18), pyruvate dehydrogenase (16,20-22) and lipogenesis (13,16,18), S.P. Mukherjee first demonstrated that H_2O_2 fulfills the

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Abbreviations:

Nicotinamide adenine dinucleotide phosphate, reduced form = NADPH
Methyl D-glucose = MeGlc
Dimethyl sulfoxide = DMSO
Polyunsaturated fatty acid = PUFA; Maximal effective dose = ED_{max} .

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role of a "second messenger" of insulin (8,18). Our evidence for intracellular H_2O_2 accumulation and induction of peroxidative pathways in insulin-treated fat cells (8-10,13) has also been confirmed more recently by other authors (19). At the same time these investigators observed that insulin-stimulated adipocytes have an enhanced rate of turnover of fatty acid moieties of plasma membrane phospholipids, which is relatively higher in the phosphatidylinositol fraction (23,24). Furthermore, exposure of these cells to small concentrations of exogenous phosphatidylinositol causes stimulation of NADPH oxidase in their plasma membrane and glucose transport and oxidation (23-25). Thus, it is likely that the phospholipid environment of the plasma membrane, and turnover of its free fatty acids may play a critical role in the coupling of insulin receptors with the hormone's effector system, while the NADPH oxidase activity with its product, H_2O_2, appears to be the final pathway of the hormone's transmembrane signal. We present here the evidence in support of our above hypothesis that the polyunsaturated fatty acids and to some extent, palmitic acid, do indeed exert an insulin-like effect on glucose transport and metabolism in intact fat cells.

METHODS AND MATERIALS

Fat cells were prepared from male CD strain rats (150 to 200 g) from Charles River Co., Boston, Mass., by digestion of epididymal fat pads with collagenase (1 mg/ml) in Krebs Ringer phosphate buffer (9), pH 7.4 containing 3% bovine serum albumin. Glucose oxidation via the hexose monophosphate shunt and the Embden-Meyerhof glycolytic pathways was measured by the rate of $^{14}CO_2$ evolution from [1-^{14}C]D-glucose and [6-^{14}C]D-glucose respectively, as described before (9,14). The fatty acids added to the 17 x 100 mm polypropylene (Falcon) tubes were mixed with the medium buffer on a Vortex mixer. The solvents, in which the stock fatty acid solutions were prepared, were dried under N_2 gas. To examine whether the added agents have a direct effect on intracellular glucose metabolic enzyme activities, glucose oxidation was also assayed in a fortified cell-free homogenate containing 0.5 mM NAD, 0.5 mM $NADP^+$, 5 mM ATP and 25 mM nicotinamide and 2.5 mM GSSG and incubated at 37°C for 1 hour.

The initial rate of D-glucose transport into adipocytes was assayed using the tracer non-metabolizable glucose analogue, 3-O-[^{3}H]methyl D-glucose by rapid cell separation by oil flotation procedure (4) with some modifications. Cells (approximately 10^7/ml) were incubated at 37°C in the presence or absence of insulin or either of the fatty acids and/or cytochalasin B, for 5 min. 3-O-[^{3}H]methyl D-glucose solution (specific activity: 0.1 μCi/μmol) was added at the final concentration of 50 μM, and at intervals of a few seconds as indicated in the figures, the uptake was stopped by addition of 100 μl of a solution (albumin-free buffer) of 0.3 mM phloretin, a competitive inhibitor of glucose transport. The phloretin was first dissolved in dimethyl sulfoxide (final concentration of DMSO in the cell medium was 0.5%) keeping the DMSO-controls and the contents of the tubes mixed by continuous swirling at 27°C. Aliquots of 100 μl cell suspension were withdrawn in duplicates and transffered to 0.4 ml polypropylene tubes containing 0.15 ml silicon oil (Dow Corning Co.) of specific gravity 0.97, and centrifuged on a Brinkman microfuge for 5 sec, followed by a washing with 0.1 ml of warm buffer and recentrifugation for 5-8 seconds. The tubes were transferred to ice and the congealed fat cakes were cut with a sharp razor and dissolved in Aquasol-2 (New England Nuclear) and counted for radioactivity. Assuming that the hexose carrier(s) is stereospecific, correction for non-carrier diffusion was made by measuring L-[1-^{14}C]glucose uptake using the same procedure as for 3-O-[^{3}H]methyl D-glucose.

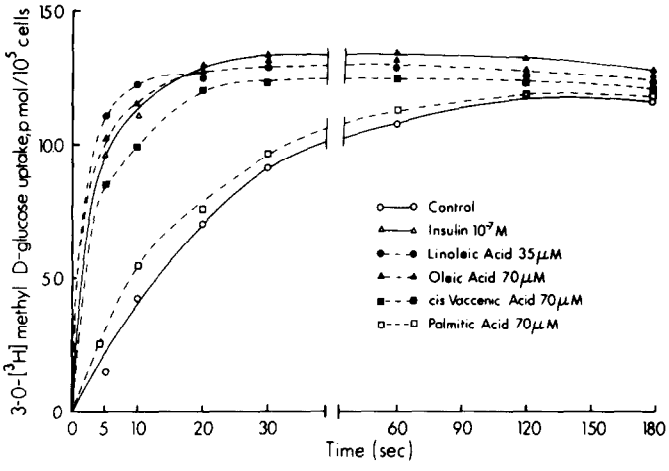

Figure 1: The rapid kinetics of 3-O-[^{3}H]methyl D-glucose transport into adipocytes. The cells were preincubated for 10 min at 37°C in Krebs Ringer phosphate buffer, pH 7.4 containing 1% bovine serum albumin and insulin or the fatty acids (employed at their ED_{max}) as indicated. The tubes were taken to room temperature (27°C) and a solution of 3-O-[^{3}H]methyl D-glucose (Final conc. 50 μM) was added and the uptake was measured by addition of a stopping solution containing phoretin, (final 0.3 mM) followed by centrifugation as described in "methods".

RESULTS

Glucose transport by a saturable, carrier-mediated diffusion was assayed by measuring the rapid kinetics of equilibration of the non-metabolizable analogue 3-O-[^{3}H]methyl D-glucose. Insulin stimulated the velocity of this rate as expected, about 6 fold over the basal as indicated by the $t_{1/2}$ of saturation at 3 sec (Figure 1). Similar stimulation was also achieved in the presence of exogenous polyunsaturated fatty acids, e.g., linoleic (C18:2, cis 9,12), oleic (C18:1), or cis-vaccenic (C18:1, cis-11) acid. The saturated fatty acid, palmitic acid (C16:0) had only a small effect on MeGlc transport although it enhanced glucose oxidation. From a dose-response study (not illustrated) the maximal stimulatory effect of each of these fatty acids was observed at 35 μM (linoleic acid) to 70 μM. Insulin and the potent unsaturated fatty acids did not have an additive effect, suggesting thereby a common locus of action in the membrane (data not shown).

Cytochalasin B (50 μM) inhibited the basal rate as well as the stimulation of MeGlc transport by either insulin or the fatty acids (Figure 2). These effects of cytochalasin B at MeGlc concentration (50 μM) which is considerably lower than our measured K_m of the sugar (about 2.6 mM; not illustrated) was progressively overcome by increasing the concentration of MeGlc (Figure 3) which permits non-carrier diffusion. Thus, the observed changes in the rate of this transport due to insulin or the fatty acids reflect those in a saturable process. The transport of L-[1-^{14}C] glucose, assayed to determine

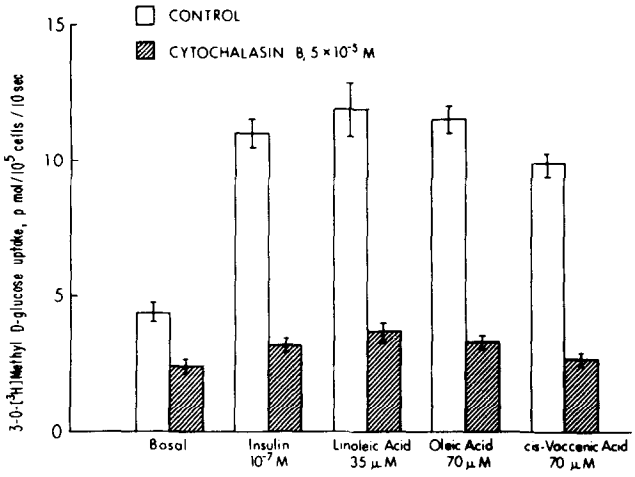

Figure 2: Inhibitory effect of cytochalasin B on the basal rate and on the stimulatory effect of insulin or the free fatty acids on 3-O-[^{3}H]methyl D-glucose uptake. The values represent the chosen equilibrium at 10 sec as average ± S.E. of 4 experiments in duplicate. The other details are the same as in Figure 1.

the non-saturable diffusion parameter, was minimal, about 3% of methyl D-glucose, but was enhanced to some extent in cells treated with insulin or oleic acid or cis-vaccenic acid (not shown).

Glucose oxidation was also enhanced severalfold in the presence of the fatty acids, as well as of insulin (Table 1). The hexose monophosphate shunt activity, reflected by [1-^{14}C]D-glucose oxidation was increased to a greater extent than the glycolytic pathway

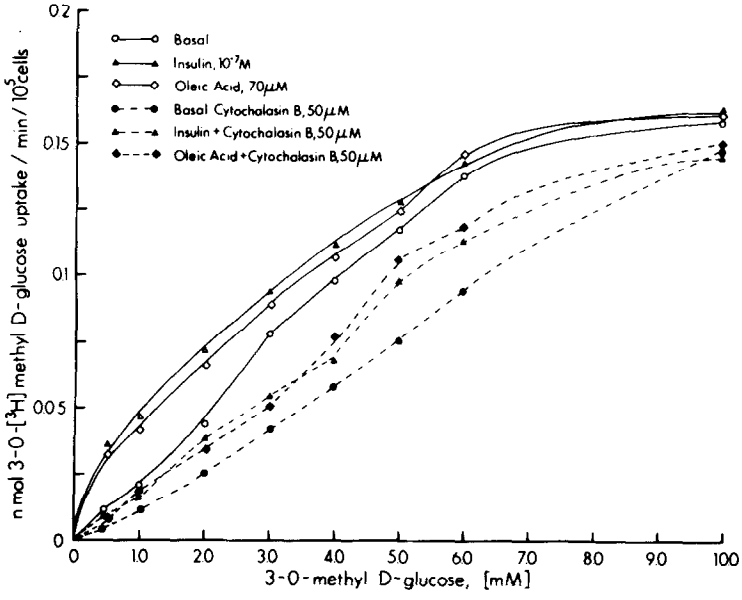

Figure 3: Effect of insulin or the free fatty acid (shown with oleic acid) upon 3-O-[^{3}H]methyl D-glucose uptake by adipocytes at 1 min at increasing concentrations of the sugar, in the presence or absence of cytochalasin B. The other details are the same as in Figures 1 and 2.

Table 1

EFFECTS OF INSULIN AND FREE FATTY ACIDS ON GLUCOSE OXIDATION PATHWAYS IN ADIPOCYTES

Additions	nmol glucose oxidized/hr/2 x 10^6 cells	
	from [1-^{14}C]D-glucose	from [6-^{14}C]D-glucose
Control	8.4 ± 1.4	5.2 ± 0.44
Control + cytochalasin B	8.0 ± 1.6	3.4 ± 0.61
Insulin	23.6 ± 4.5	9.6 ± 0.82
Insulin + cytochalasin B	17.5 ± 1.4	5.5 ± 0.35
Linoleic acid	25.6 ± 3.2	8.3 ± 1.2
Linoleic acid + cytochalasin B	19.2 ± 2.4	5.6 ± 0.72
Oleic acid	24.3 ± 2.8	7.2 ± 0.6
Oleic acid + cytochalasin B	18.6 ± 2.0	4.8 ± 0.54
Cis-vaccenic acid	26.4 ± 4.0	7.8 ± 0.4
Cis-vaccenic acid + cytochalasin B	18.0 ± 1.6	4.9 ± 1.1
Palmitic acid	14.2 ± 2.7	5.7 ± 1.4
Palmitic acid + cytochalasin B	7.9 ± 1.6	4.2 ± 0.4

Additions: cytochalasin B: 50 μM; insulin: 10^{-7}M; linoleic acid: 35 μM and other fatty acids: 70 μM. The fatty acids were employed at their respective maximal effective concentrations. The details are described in the "methods". The values are the averages ± S.E of 3 experiments in duplictae.

(oxidation of [6-^{14}C]-D-glucose), as seen with insulin. The relative potencies of the fatty acids and their maximal effective concentration in eliciting the response of the shunt activity (Table 1) were found to be similar to those for MeGlc transport (Figure 1). The dose-responses to cis-monoenoic acids, e.g. oleic acid or cis-vaccenic acid, were closely related. With palmitic acid, however, only oxidation via the shunt was increased. The concentration-dependent stimulatory effect of the saturated and unsaturated fatty acids on [1-^{14}C]D-glucose oxidation is illustrated in Figure 4. Moreover, insulin added together with the most potent fatty acid, linoleic acid, did not produce any additive effect on glucose oxidation (not illustrated). The inhibitor of transport, cytochalasin B at its maximal effective concentration (50 μM) also inhibited the effect of insulin or of the fatty acids on the glycolytic pathway, but failed to completely inhibit the stimulation or the basal rate of the hexose monophosphate shunt. We did not detect any lipid peroxidation in these cells under the present experimental conditions (10). Also, neither of the fatty acids had any appreciable effect on glucose oxidation in the cell-free system (not shown). Thus, we may consider their observed effects on MeGlc transport and glucose oxidation in these cells as due to a physico-chemical effect on the plasma membrane reactive sites.

DISCUSSION

Our evidence that glucose transport and intracellular utilization in adipocytes are stimulated by small concentrations of externally added fatty acids, suggests that they may have a critical role in the physiological functions associated with the plasma mem-

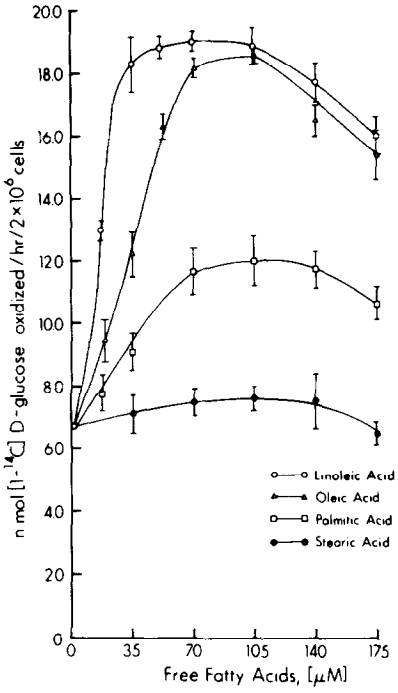

Figure 4: A dose-response curve of the effect of the free fatty acids on the rate of [1-^{14}C]D-glucose oxidation in fat cells. The fetails are given in the "methods". The values are the averages ± S.E. of 4 experiments in duplicate.

brane. The response of the cells to the fatty acids is found to be specific, i.e., linoleic acid>oleic acid≥cis-vaccenic acid>>palmitic acid. Palmitic acid, which is a major constituent acyl fatty acid of these cells (26) also has a relatively smaller stimulatory effect on the hexose monophosphate shunt pathway than the polyunsaturated fatty acids though no significant effect of it could be detected on MeGlc transport. The more readily oxidizable PUFA's, viz., arachidonic acid and linoleic acid (which are precursors of the prostaglandins) were found to be relatively more potent than linoleic or oleic acid in the presence or absence of indomethacin, an inhibitor of prostaglanin synthesis. We have not considered here these PUFA's in assessing the role of fatty acids because agents such as indomethacin may introduce a chemical artefact. However, the effect of the free fatty acids apparently is dependent on the chain length as well as on the degree of unsaturation. An important consideration in relation to the specificity of fatty acids is that the naturally occurring constituents of membrane phospholipids, viz., linoleic acid or oleic acid (mostly occurs in the 2-position) or palmitic acid (1-position) are very effective in eliciting these responses.

The insulin-like effect of the fatty acids on the fat cells' glucose utilization suggests that release of free fatty acids in the plasma membrane, possibly due to activation of a phospholipase A, may in some manner be involved in the transmission of insulin's

signal. Previously some authors noted that treatment of adipose tissue with low concentrations of the enzymes, phospholipase A (27) or phospholipase C (28) elicits insulin-like stimulation of net glucose oxidation, but no evidence has so far existed for the occurrence of a hormone-sensitive inherent phospholipase activity in the membrane. We have indeed found that insulin causes an increased incorporation of labelled oleic acid and palmitic acid into plasma membrane phospholipids, especially in phosphatidylinositol, without any corresponding change in the net contents of these lipid constitutents (23,24) Moreover, exogenous phosphatidylinositol has been found to activate the plasma membrane reactions, e.g., glucose transport and NADPH oxidase, like insulin in normal (23,24) and insulin-refractory fat cells of naturally obese rats (25).

The molecular mechanism of insulin's action in the plasma membrane of target cells such as adipocytes is complex, but is being unfolded rapidly. The discovery that insuli activates a pyridine nucleotide oxidase in the plasma membrane of these cells with the generation of H_2O_2 (8-10) raised the interesting possibility that this may represent a unique electron transport system in the membrane, apparently linked to glucose transport and the regulation of adenylate cyclase activity (11-15). This may also mediate several metabolic effects of insulin which occur independently of glucose transport (18,20). A transport-independent mechanism for glucose uptake and utilization was first revealed clearly by the demonstration by S.P. Mukherjee of a stimulation of the hexose monophosphate shunt by some chemical agents which inhibit MeGlc transport and the glycolytic pathway to the same extent (8-11), even in the presence of glucocorticoids (29).The evidence that unsaturated fatty acids and to a less extent, palmitic acid, elicit both cytochalasin-inhibitable transport and non-inhibitable glucose oxidation via the shunt at a sugar concentration$<<K_m$ reflects on this distinct mechanism for hexose uptake other than a simple diffusion (which may occur at high sugar concentration). The NAD(P)H oxidase activity in the plasma membrane, as discussed above, represents one major effector system. It is possible that localized increase in fluidity or a change in polarity in the micro-environment of the membrane due to the release of unsaturated fatty acids may enhance the activity of enzymes such as NAD(P)H oxidase as well as that of hexose carriers On this logical assumption, we can envision a critical role for a turnover of the fatty acid moieties of the plasma membrane phospholipids in coupling of insulin's receptors with the hormone's effector system.

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