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PALMITATE STIMULATES GLUCOSE TRANSPORT IN RAT ADIPOCYTES BY A MECHANISM INVOLVING TRANSLOCATION OF THE INSULIN SENSITIVE GLUCOSE TRANSPORTER (GLUT4)

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SUMMARY. In rat adipocytes, palmitate: a) increases basal 2-deoxyglucose transport 129±27% ($p<0.02$), b) decreases the insulin sensitive glucose transporter (GLUT4) in low density microsomes and increases GLUT4 in plasma membranes and c) increases the activity of the insulin receptor tyrosine kinase. Palmitate-stimulated glucose transport is not additive with the effect of insulin and is not inhibited by the protein kinase C inhibitors staurosporine and sphingosine. In rat muscle, palmitate: a) does not affect basal glucose transport in either the soleus or epitrochlearis and b) inhibits insulin-stimulated glucose transport by 28% ($p<0.005$) in soleus but not in epitrochlearis muscle. These studies demonstrate a potentially important differential role for fatty acids in the regulation of glucose transport in different insulin target tissues.

Plasma fatty acid concentrations have been shown to be elevated in both obesity and non-insulin dependent diabetes mellitus (1-3). Plasma fatty acid concentrations in patients with diabetic ketoacidosis and in some patients with hyperosmolar non-ketotic coma may exceed 2 mM (4-5). The effect of fatty acids on glucose transport in insulin target tissues remains controversial. Fatty acids have been shown to inhibit glucose transport in the well-oxygenated heart (6). However, in skeletal muscle this effect has been supported by some (7,8) but not by others (9,10). Also the rat hindlimb, which is composed of ~50% fast-twitch white muscle (11), has a very low capacity for fatty acid oxidation (12), was used in these investigations. The effect of palmitate on glucose transport in rat adipocytes remains controversial with one report of an increase (13) and another of no effect (14). In the present study we investigated the effects of palmitate on glucose transport in fat and skeletal muscle tissues that represent both fast twitch glycolytic fibers and slow twitch oxidative fibers muscle. In addition we characterize the mechanism by which palmitate stimulates glucose transport in rat adipocytes.

EXPERIMENTAL PROCEDURES

Fatty acid free BSA was prepared by acid-charcoal treatment according to the method of Chen (15). Fatty acids were added back to the fatty acid free albumin via celite by the method of Spector and Hoak (16). Fatty acid concentrations were measured using an enzymatic colorimetric method (NEFAC Wako Pure Chemical Industries Ltd. Osaka, Japan.).

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Adipocytes were isolated from the epididymal fat pads of 120 - 140 g male Sprague-Dawley rats by the collagenase digestion method of Rodbell (17) using Krebs Ringer Phosphate buffer.

Adipocyte 2-Deoxyglucose transport was performed according to the method of Garvey *et al.* (18). Briefly, adipocytes (2 x 10^5 cells/ml) were resuspended in buffer and incubated in plastic tubes with or without insulin (1 nM final concentration) or other agents at 37°C for 15 min. After the 15 min incubation 2-deoxyglucose [6.7 μCi of 2-deoxy-D-[1-^{3}H]glucose (17 Ci/mmole)/ml of reaction mix and a final 2-deoxyglucose concentration of 34 μM] was added to the mixture and transport was measured for 3 min. Nonspecific carryover and diffusion was corrected for by the addition of cytochalasin B (50 μM final concentration cytochalasin B). 3-O-methylglucose transport in adipocytes was assayed for 3 sec according to the method of Whitesell and Gliemann (19).

In order to do the **immunoblot of GLUT4**, adipocytes were fractionated by a modification (21) of the method of McKeel and Jarett (22) and plasma membranes were isolated. The homogenate was spun at 20,000 x g for 15 min and low density microsomes (LDM) were isolated by centrifuging the supernatant at 38,000 x g for 20 min. This supernatant was centrifuged at 190,000 x g for 90 min, and the pellet (LDM) was vortexed, resuspended and frozen at -70°C. Samples were electrophoresed on 10% SDS-PAGE and blotted according to the method of Harris *et al.* (23). After blocking the sheets were probed with the 1F8 monoclonal antibody (supplied by D. James, Washington University, St. Louis, MO.) followed by detection using a second antibody coupled to alkaline phosphatase. Where indicated, immunoblots were quantitated using a laser densitometer (LKB 2202, Ultroscan, Bromma, Sweden).

Adipocyte insulin receptors, were prepared by wheat germ lectin-Sepharose chromatography as described previously (24) except that the homogenization and fractionation buffer consisted of 0.15 M sucrose, 5 mM ATP, 100 mM NaF, 40 mM sodium pyrophosphate, 40 mM Na_2HPO_4, 5 mM EDTA, 1 mM PMSF, 1 mg/ml bacitracin, 5 mM sodium vanadate, and 50 mM Tris pH 7.4. Equal aliquots of receptor were precipitated with 10% (w/v) trichloroacetic acid. These samples were solubilized, run on 10% SDS-PAGE and immunoblotted by the method of Tashiro-Hashimoto *et al.* (25) using an antiphosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, New York 12946)

Epitrochlearis and soleus muscles were removed from male Sprague-Dawley rats weighing 117 ± 3 g following an overnight fast. These rats were anesthetized using sodium pentobarbital (5 mg/100 g body weight). The epitrochlearis muscles (~20 mg) were used intact, while ~20 mg strips were obtained from the soleus muscles. All muscles were incubated at 35°C with shaking for 60 min in 2 ml of Krebs-Henseleit buffer containing 3% fatty acid-free BSA, 2 mM pyruvate, 38 mM mannitol, with and without palmitate and/or insulin, with a gas phase of 95% O_2-5% CO_2. Glucose transport activity was measured using [^{3}H]-3-O-methylglucose by a modification (26) of the procedure used previously in frog sartorius muscle (27,28). After the initial treatment period of 60 min, the muscles were incubated for 10 min at 29°C in 1.5 ml of Krebs-Henseleit buffer containing 3% fatty acid-free BSA, 2 mM pyruvate, 8 mM 3-O-methyl-D-[1-^{3}H]-glucose (437 μCi/mmol), 30 mM [^{14}C] mannitol (8 μCi/mmol), and the same additions present during the preceding incubation. The muscles were then processed, and the extracellular space and intracellular 3-O-methylglucose concentration were determined as described previously (26). Glucose transport is expressed as μmol 3-O-methylglucose · ml intracellular water^{-1} · 10 min^{-1}.

RESULTS

Effects of Palmitate on Glucose Transport

Palmitate increases basal glucose transport in rat adipocytes. Figure 1 shows a representative experiment in which palmitate stimulates glucose transport 223% over basal, in contrast to insulin which stimulates glucose transport by 609%. In four similar experiments palmitate concentrations of 2-3 mM, stimulates basal glucose transport by 129±27% ($p<0.02$, $n=4$), compared to insulin which stimulates basal glucose transport 619±92% ($n=4$). The effect of palmitate on glucose transport is not additive with insulin ($p>0.5$, $n=4$). The stimulation of basal glucose transport by palmitate is concentration dependent (data not shown). At palmitate

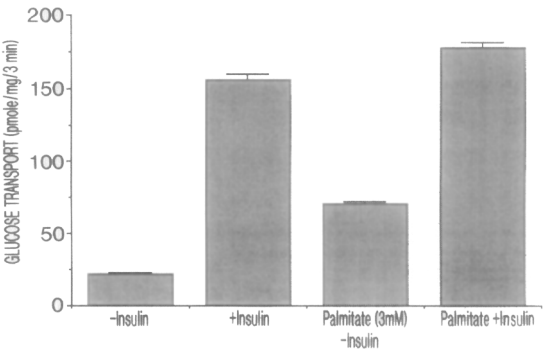


Fig. 1. **Effects of palmitate on glucose transport in rat adipocytes.** 2-Deoxyglucose transport was measured in rat adipocytes as descibed in the Experimental Procedures. Protein (mg) was estimated from cell numbers (45). The insulin concentration used was 1 nM. Each condition in this single representative experiment was done in triplicate and the error bars represent standard error of the mean. The " -/+ Insulin " conditions used KRP buffer containing 3% fatty acid free BSA while the "Palmitate -/+ Insulin" conditions utilized 3% fatty acid free BSA with 3 mM palmitate, added as described in the Experimental Procedures.

concentrations as low as 0.2 to 0.6 mM, palmitate significantly ($p < 0.05$, $n = 4$) stimulates glucose transport. Palmitate also stimulates 3-O-methylglucose transport similarly to 2-deoxyglucose transport (data not shown). In contrast to its effects on adipocytes, palmitate had no stimulatory effect on basal glucose transport in skeletal muscle (Fig. 2). Moreover, in the predominantly (84%) slow-twitch, oxidative soleus muscle (11), palmitate has a significant inhibitory effect on insulin-stimulated glucose transport (-28%, $p<0.005$). However, no effect of palmitate is observed on insulin-stimulated glucose transport in the epitrochlearis, which consists of predominantly fast-twitch white fibers (29).

Mechanism of Action of Palmitate-Stimulated Glucose Transport in Rat Adipocytes

One of the mechanisms by which insulin stimulates glucose transport is by translocation of insulin-sensitive glucose transporters from microsomes to plasma membranes. The effects of

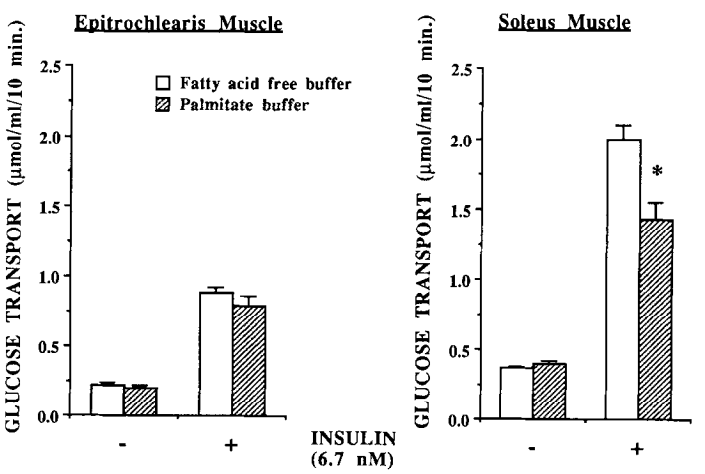
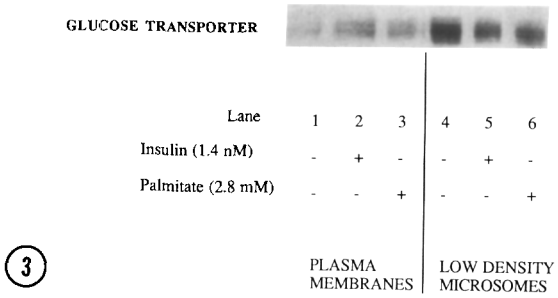


Fig. 2. **Effect of palmitate on glucose transport in rat skeletal muscle.** 3-O-methylglucose transport was measured in rat soleus and epitochlearis muscle. Palmitate (1-2 mM) and insulin (6.7 nM) were added as indicated. For soleus muscle each column represents 12 different muscles assayed on two separate days. For epitochlearis muscle each column is the result of 17 different muscles assayed on three separate days. The value obtained in the presence of palmitate plus insulin * is significantly different ($p<0.005$) from the value obtained in the presence of fatty acid free buffer plus insulin.



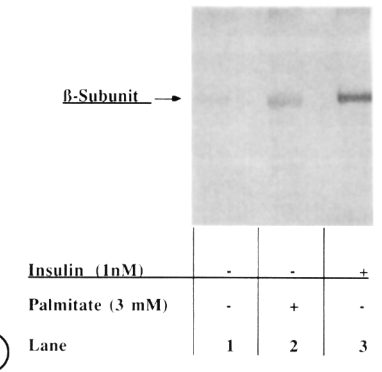


Fig. 3. **Western immunoblot of the insulin sensitive glucose transporter (GLUT4).** Rat adipocytes were prepared and incubated in the presence or absence of insulin and palmitate as indicated, then fractionated as described in the methods into plasma membranes (lanes 1,2,3) and low density microsomes (lanes 4,5,6). Immunoblots were performed using a monoclonal antibody (1F8) to the insulin sensitive glucose transporter (provided by D. James). The position of the insulin sensitive glucose transporter (GLUT4) is indicated.

Fig. 4. **Western immunoblot of the tyrosine phosphorylated β–subunit of the insulin receptor.** Rat adipocytes were treated with insulin or palmitate as indicated, then fractionated into crude plasma membranes which were solubilized and insulin receptors were partially purified using a wheat germ lectin-Sepharose column as described in the experimental procedures. Immunoblots were performed using a monoclonal antiphosphotyrosine antibody. The position of the β subunit of the insulin receptor is indicated.

palmitate are not additive with those of insulin, suggesting that they share a common mechanism. Therefore we determined whether palmitate, like insulin, stimulates translocation of GLUT4 (Fig. 3). Insulin causes an increase in plasma membrane GLUT4 (compare lanes 1 and 2), and a decrease in GLUT4 in the low density microsomes (compare lanes 4 and 5). Palmitate also increases GLUT4 in plasma membranes (compare lanes 1 and 3), while decreasing GLUT4 in low density microsomes (compare lanes 4 and 6). In a series of three experiments the concentration of GLUT4 in low density microsomes is decreased by 32 ± 8% and 63 ± 18% by palmitate and insulin respectively.

Tyrosine phosphorylation of the ß-subunit of the insulin receptor has been proposed as a mediator of at least some of the actions of insulin, including glucose transport (30). To determine if palmitate, like insulin, causes autophosphorylation of the insulin receptor, we investigated the effect of palmitate on the phosphorylation of the ß-subunit of the insulin receptor. Fig. 4 demonstrates an increased number of phosphotyrosine residues in the ß-subunit of the insulin receptor after insulin treatment (compare lanes 1 and 3), and although to a lesser extent than insulin, palmitate treatment (compare lanes 1 and 2).

Phorbol esters activate protein kinase C and stimulate glucose transport in a variety of cells, including rat adipocytes (31). To examine the possibility that activation of protein kinase C mediates palmitate-stimulated glucose transport in rat adipocytes, we utilized the protein kinase C inhibitors staurosporine and sphingosine. Rat adipocytes were incubated with 12-O-tetradecanoylphorbol-13-acetate (1.6 nM), in the presence and absence of sphingosine (25 μM) and staurosporine (100 nM) in fatty acid free or palmitate buffers. Both sphingosine and staurosporine decreased phorbol ester-stimulated glucose transport by 32±7% and 45±3% respectively (data not

shown). In contrast, palmitate(1.8 mM)-stimulated glucose transport was not changed by either sphingosine (+17±7%) or staurosporine (-1±13%).

DISCUSSION

These studies demonstrate that palmitate stimulates glucose transport in rat adipocytes. Although these results confirm the work of Joost and Steinfelder (13), they are in disagreement with the investigations of Mukherjee *et al.* (14) who did not see any significant increase in glucose transport. Differences between our data and that of Mukherjee *et al.* (14) are difficult to explain but may be related to different methods of addition of fatty acids to buffers. To determine if fatty acids have the same stimulatory effect on glucose transport in other insulin-sensitive tissues we evaluated the effect of palmitate on glucose transport in rat skeletal muscle (Fig. 2). Palmitate had no effect on basal glucose transport in either the predominantly glycolytic epitrochlearis or predominantly oxidative soleus muscles. Palmitate also had no effect on insulin-stimulated glucose transport in the epitrochlearis. However, palmitate inhibited insulin-stimulated glucose transport in the rat soleus. Nearly 30 years ago, Randle *et al.* (32) proposed the "glucose fatty acid cycle" whereby products of fatty acid oxidation inhibit glucose transport and utilization. The above results are consistent with the existence of the "glucose fatty acid cycle" in skeletal muscle, but only in fibers with a high respiratory capacity. These fibers have a large capacity to utilize fatty acids as an energy source (7,8). An alternative explanation for our results is that in skeletal muscle palmitate has a direct effect on the plasma membrane and exerts its inhibitory effects via alteration of the activity of GLUT4. Since expression of GLUT4 in the soleus is ~ 3-fold greater than in the epitrochlearis (33), the effect of palmitate would be much greater in the soleus than in the epitrochlearis.

The differential effect of palmitate on glucose transport in adipocytes and skeletal muscle may affect both insulin resistance and obesity in the intact animal. Plasma fatty acids are increased in non-insulin dependent diabetes mellitus and obesity (1-3). 80% of patients with non-insulin dependent diabetes mellitus are overweight, and obesity is associated with insulin resistance and is a recognized risk factor for type 2 diabetes (34). Skeletal muscle represents the major tissue responsible for insulin-dependent glucose uptake [~ 75% (35)] and palmitate inhibits insulin-stimulated glucose transport in muscle (Fig. 2). Inhibition of glucose transport in muscle was only significant in the soleus muscle which is predominantly (84%) composed of slow-twitch oxidative fibers (11) that have a relatively high capacity for glucose utilization. Most muscles are composed of mixed fibres, consequently slow-twitch oxidative fibers will make a large contribution to the uptake of glucose. Therefore inhibition of glucose transport in oxidative skeletal muscle alone can account for insulin resistance. In adipocytes, glucose would be preferentially taken up due to the specific effect of fatty acids on basal glucose transport. This would enhance the ability of fatty acids to cause increased triglyceride formation in adipocytes (36) and increase fat deposition even in the face of insulin resistance. Therefore our results are consistent with fatty acids contributing simultaneously to both obesity and insulin resistance.

Insulin does not act alone in regulating glucose and fatty acid metabolism. Catecholamines increase plasma fatty acids and have profound effects on insulin target tissues. For example,

epinephrine stimulates lipolysis (37), fatty acid transport (38) and glucose transport (39) in rat adipocytes. It is very likely that these three processes are linked. That is, epinephrine causes a local increase in fatty acids which would increase both fatty acid and glucose transport. Metabolically, it would be advantageous for fatty acids to increase glucose transport in this setting because it would provide the adipocyte with an insulin-independent supply of glycerolphosphate which is required for re-esterification of fatty acids.

The non-additivity of the effects of palmitate and insulin on glucose transport in adipocytes (Fig. 1) suggests that common mechanisms are involved. Consistent with this, both insulin and palmitate stimulate translocation of GLUT4 and tyrosine phosphorylation of the insulin receptor (Figs. 3 & 4). Although controversial, insulin stimulation of glucose transport may involve protein kinase C (31) . Interestingly, our data, using the protein kinase C inhibitors staurosporine and sphingosine, indicate that palmitate-stimulated glucose transport does not involve protein kinase C. Therefore palmitate stimulates glucose transport by a mechanism which is shared at least in part by insulin. Since tyrosine phosphorylation of the ß-subunit of the insulin receptor has been proposed as a mediator of glucose transport (30) it will be important to investigate the mechanism of palmitate stimulated phosphorylation and to determine if palmitate stimulates the phosphorylation of other receptors that demonstrate tyrosine kinase activity. Although the mechanism whereby palmitate stimulates phosphorylation of the insulin receptor and translocation of GLUT4 in rat adipocytes is unknown it is possible that palmitate acts by altering membrane fluidity (40) or by acylating the insulin receptors (41,42), glucose transporters (43), or other regulatory molecules [e.g. palmitate acylation of phospholipids is decreased in erythrocytes from diabetic versus control rats (44)].

In conclusion fatty acids may be important regulators of glucose transport in vivo. Elucidation of the mechanism of action of fatty acids on glucose transport in adipocytes may yield important new information concerning not only the activation of glucose transport, but also its tissue specificity.

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