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OKADAIC ACID INCREASES GLUCOSE UPTAKE IN 3T3-L1 ADIPOCYTES BY STIMULATING GLUCOSE TRANSPORTER 1 EXPRESSION

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We present evidence that chronic 24 hour treatment of 3T3-L1 adipocytes with the phosphatase inhibitor okadaic acid increases deoxyglucose uptake 25 fold with a maximal effect at a concentration of 35nM. This pharmacological response is associated with a 21 fold increase in expression of glucose transporter 1 (glut 1) mRNA. These findings are discussed with respect to glucose transporter gene regulation and insulin signalling and are compared to previous observations describing the acute effects of okadaic acid on glucose transporter translocation.

Okadaic acid, a polyether derivative of a 38-carbon fatty acid, is a potent inhibitor of protein phosphatases 1 and 2A (1,2) and has been used to study the role of protein phosphorylation in a variety of cellular responses (3). Recent reports of its metabolic effects in rat adipocytes indicate that the compound elicits anti-insulin effects by increasing the rate of lipolysis and decreasing the rate of lipogenesis as well as insulin-like effects by acutely stimulating glucose uptake (4). This alteration in lipid metabolism has been attributed to the modulation of two key metabolic enzymes, stimulation of hormone sensitive lipase and inhibition of acetyl-CoA carboxylase upon enzyme phosphorylation (4). The mechanistic basis for the modulation of the glucose transport system is less clear. Lawrence and coworkers demonstrated (5) that in rat adipocytes the elevated glucose transport rate following an acute 20 min exposure to okadaic acid at a concentration of 1μM was accompanied by the plasma membrane translocation of vesicles containing insulin-regulatable glucose transporter molecules (glut 4). It has been further proposed that the recycling of these vesicles and intrinsic activity of glut 4 are controlled by phosphorylation (6). The molecular targets of these phosphorylation reactions have yet to be identified.

In this manuscript the effects of chronic, low dose okadaic acid treatment on the glucose transport system in 3T3-L1 adipocytes are described. We show that this exposure leads to a maximal 25 fold enhancement of deoxyglucose uptake at 35 nM okadaic acid. This response is paralleled by the elevated expression of the HepG2-type glucose transporter (glut 1) in okadaic acid treated 3T3-L1 adipocytes.

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MATERIALS AND METHODS

Materials

Dulbecco's modified Eagles medium (DMEM, high glucose) and fetal calf serum (FCS) were obtained from JRH Biosciences and Hazleton Biologicals respectively. Porcine crystalline insulin was a gift of Eli Lilly . Okadaic acid was purchased from Boehringer Mannheim, 2-[1,2-^{3} H]-deoxyglucose from New England Nuclear (969.4GBq/mmol) and cytochalasin B was obtained from Aldrich.

Cell culture

3T3-L1 cells (ATCC CL 173) were cultured and differentiated into adipocytes in 6 or 12 well plates as described (7) Briefly, a confluent culture containing 4x10^{6} cells/well was differentiated by incubation for 2 days in DMEM containing 5µg/ml insulin, 0.25 µM dexamethasone , 0.5 mM isobutylmethylxanthine and 15% fetal calf serum. The cells were maintained for another 5-6 days in DMEM with 15% FCS. Okadaic acid was diluted in dimethylformamide as indicated in the figure legends and was directly added to the culture not exceeding 1% solvent (vol/vol). Prior to assaying for deoxyglucose uptake, adipocytes were washed 3 times and preincubated for 1 hour in 0.9 ml of serum and glucose free DMEM. Following this period, insulin was added where indicated at 50 nM for 20 min before assaying deoxyglucose uptake. All values were determined in triplicate. The samples for RNA analysis were washed 3 times with PBS and stored at -70^{0} C.

Deoxyglucose uptake assay

[^{3}H]-deoxyglucose (50 µM, 1µCi/ml, 1Ci=37GBq) was added to each well for 6 min at room temperature. Uptake was terminated by rapid removal of the assay medium followed by four washes in ice-cold Dulbecco's phosphate buffered saline. The cells were dissolved in 0.5ml of 1% sodium dodecyl sulfate and the lysate was transferred into 2ml of scintillation fluid for beta counting (Biodegradable counting scintillant, Amersham).

Northern blots

RNA from frozen 3T3-L1 adipocytes was extracted by the hot phenol procedure (8). For Northern blots, 20 µg of total RNA was denatured in 70% formamide/6% formaldehyde in MOPS buffer (20 mM morpholinepropanesulfonic acid, MOPS, 5 mM sodium acetate, 1mM EDTA) at 55 ^{0}C for 15 min and electrophoresed in a 6% formaldehyde/1% agarose gel in MOPS buffer. Conditions for transfer, hybridization and washing were as described previously (8). The specific probes for mouse glut 1 and glut 4 span approximately 400 bases each in the 5'-coding region and were generated by polymerase chain reaction. Specificity of the glut 1 and glut 4 probes was verified in an RNA analysis using RNA preparations from various tissues (data not shown). Probes for glut 1, glut 4 and a mouse ribosomal gene (gene 32, 9) were gel purified and labelled with ^{32}P-dCTP using an oligonucleotide labelling kit (Pharmacia). To normalize the amount of RNA in each lane, the blots were hybridized with glut 1 and glut 4 probes first, and then rehybridized with the gene 32 probe. Autoradiograms were scanned with a LKB Ultrascan XL densitometer. The densitometric value obtained by glut 1 hybridization to RNA isolated from vehicle treated 3T3-L1 cells was arbitrarily set as 1 unit.

RESULTS

Dose dependence of deoxyglucose uptake in chronic okadaic acid treatment

The experiments described were carried out to study the effect of chronically elevated intracellular phosphoprotein concentrations on glucose transporter expression and signal transduction by insulin in adipocytes.

For that purpose 3T3-L1 adipocytes were treated with various concentrations of okadaic acid or vehicle solvent for 24 hours. The adipocytes were then washed and preincubated for 60 minutes in serum- and glucose-free medium and the uptake of 2-deoxyglucose was determined as described under Experimental Procedures. Where indicated, cells were additionally exposed to insulin at a concentration of 50 nM before assaying deoxyglucose uptake.

As shown in Figure 1, okadaic acid stimulated deoxyglucose uptake over the vehicle control in a dose dependent manner with a maximum response at 35 nM. Above this concentration, deoxyglucose uptake declined to basal level and no stimulation was observed at 1μM okadaic acid. This decline is not due to cytotoxicity since the RNA and protein content per 10^6 cells is unaltered up to a concentration of 330 nM okadaic acid (data not shown). When okadaic acid was tested in conjunction with insulin at 50nM, deoxyglucose uptake was enhanced additively below 35 nM of okadaic acid, however addition of insulin did not alter the maximum response at 35 nM. Furthermore, above 35 nM okadaic acid, there was no additive effect of okadaic acid and insulin.

The response elicited by either okadaic acid or okadaic acid plus insulin was completely abolished when the adipocytes were exposed to cytochalasin B (10) at 50nM for 20 minutes prior to measuring uptake (data not shown).

Time course of okadaic acid stimulated deoxyglucose uptake

Based on our results in Figure 1, we chose the optimum concentration of 35 nM okadaic acid for various time points as indicated in Figure 2. As shown, deoxyglucose

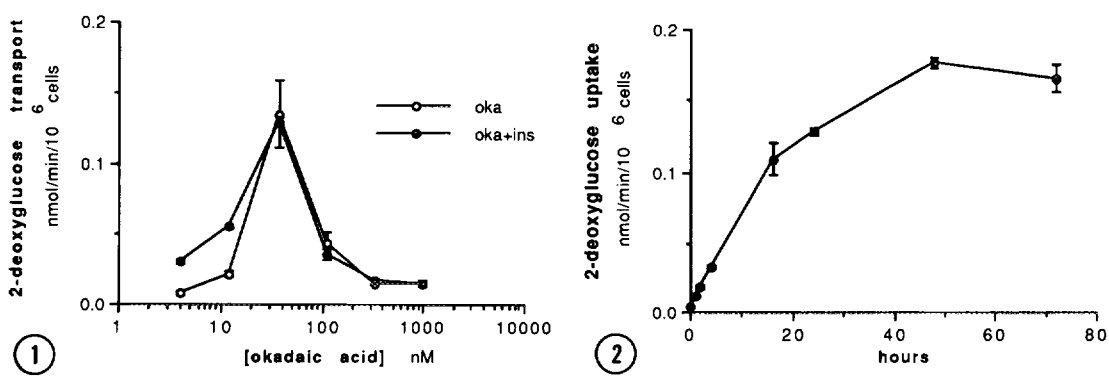

Fig.1. Dose dependence of okadaic acid stimulated glucose transport. 3T3-L1 adipocytes were exposed to various concentrations of okadaic acid for 24 hrs (oka) or were additionally incubated for 20 min with 50 nM insulin (oka+Ins). Control adipocytes received only the solvent dimethylformamide for 24 hrs and yielded values of 0.004 and 0.020 nmol/min /10^6 cells for the basal and insulin stimulated uptake, respectively.

Fig.2. Time course of okadaic acid stimulated deoxyglucose uptake 3T3-L1 adipocytes were exposed for various lengths of time to 35 nM okadaic acid before assaying deoxyglucose uptake as described.

uptake was gradually increased over the time span tested reaching a plateau value of approximately 37 fold stimulation after 48 hours.

Effect of okadaic acid on glucose transporter mRNA levels

The time course of the okadaic acid effect on the glucose transport system suggests that its effect might require de novo synthesis of RNA or protein. James and coworkers (11) demonstrated that glucose uptake in 3T3-L1 adipocytes is mediated by the transporter isoforms glut 1 and 4. We determined their steady state mRNA levels in okadaic acid and solvent treated cells by Northern blots.

Figure 3a and c show that the level of glut 1 mRNA as a function of okadaic acid concentration coincides with the deoxyglucose uptake shown in Figure 1, i.e. steady increment reaching a maximum at 35 nM and declining to basal level at 300 nM. Data from glut 1 hybridization are expressed as relative values in Fig. 3c by normalizing them to the vehicle control, set as 1 unit. In contrast to the profile of glut 1 mRNA expression, glut 4 mRNA levels were not significantly changed at 35 and 100 nM okadaic acid when compared to RNA obtained from vehicle treated cells (Fig. 3a).

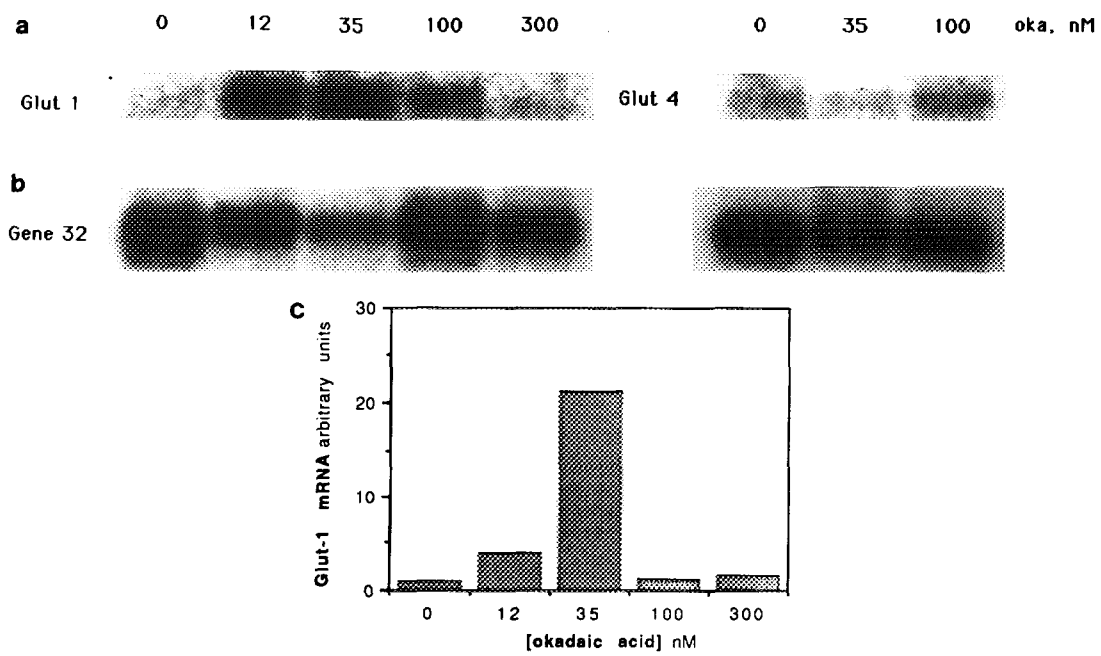

Fig.3. **Titration of okadaic acid stimulated glut 1 mRNA expression.** 3T3-L1 adipocytes were treated with indicated concentrations of okadaic acid for 24 hours. Total RNA was prepared and analyzed by Northern hybridization. The filter was first hybridized with the glut 1 and glut 4 probes (a), it was then stripped and rehybridized to the gene 32 probe to quantify the amount of RNA per lane (b). The filters hybridized to glut 1 and 4 were exposed for 24 and 72 hours, respectively. Autoradiograms were scanned and the glut 1 signal, corrected for total RNA per lane, is given as arbitrary units (c). The value of the solvent control (0 nM oka) has been set to 1 unit.

DISCUSSION

The experiments presented were performed to study the impact of protein phosphorylation on glucose transporter expression and insulin signaling in the 3T3-L1 adipocyte cell line. Although the molecules involved in signal transduction from the insulin receptor to the glucose transporter are still unknown there is ample evidence that protein phosphorylation plays a key role in the pleiotropic intracellular signals initiated by insulin (12).

Our key findings are that 24 hour treatment of 3T3-L1 adipocytes with the phosphatase inhibitor okadaic acid leads to a 25 fold increase in the basal, non insulin stimulated deoxyglucose uptake and that this response is maximal at 35 nM okadaic acid, at higher concentrations glucose uptake declines to control value. Finally, the maximal enhancement of basal uptake at 35 nM okadaic acid is paralleled by a 21 fold increase of glut 1 mRNA expression.

The magnitude as well as the steady increase of the okadaic acid induced deoxyglucose uptake over 48 hours suggests that de novo syntheses of RNA or protein might be required, thus highlighting a novel activity of okadaic acid. Its effect on glucose transport has been previously studied in primary rat adipocytes under different experimental conditions. These studies focused on the acute effects of okadaic acid on the glucose transport sytem and were performed at a higher (1μM) drug concentration than used in the current experiments(4,5). While okadaic acid alone stimulated hexose transport 4 fold in adipocytes following a 20 min incubation, it was found that the compound partially antagonizes the stimulating effect of insulin under these experimental conditions (5). These acute effects of okadaic acid have been attributed to the membrane dynamics of glucose transporter carrying vesicles and it has been proposed that phosphorylation regulates membrane fusion and recycling of transporter containing vesicles (5). The data presented in Fig.1 on combined okadaic acid and insulin treatment are in agreement with previous published data since no acute insulin stimulation of deoxyglucose uptake could be elicited in 3T3-L1 cells that had been exposed to okadaic acid at 100 nM or higher.

The fact that okadaic acid exhibits a bell shaped dose response curve for glucose uptake raises an intriguing possibility. Okadaic acid is a specific inhibitor of the phosphatase isoforms 2A(PP2A) and 1(PP1). The purified enzymes are inhibited by okadaic acid with IC 50 values of 0.2 and 10-15 nM respectively while substantially higher inhibitor concentrations are required in intact cells (3). Our observation that okadaic acid exerts opposing effects on deoxyglucose transport at low and high concentrations could be interpreted as a result of inhibiting PP2A and PP1 in 3T3-L1 cells in a concentration dependent manner. According to this hypothesis two distinct phosphatase substrates could remain phosphorylated as compared to untreated cells, the first being involved in stimulating and the second in suppressing the glucose transport system.

Based on the observation of Takai and coworkers (13) that okadaic acid does not inhibit protein kinases, it seems conceivable to conclude from our data that a

phosphatase sensitive to okadaic acid is active in untreated 3T3-L1 cells, thus counteracting the effects of phosphorylation reactions catalyzed by serine/threonine protein kinases. Several studies have suggested that protein kinases regulate glut 1 gene expression: Mountjoy and coworkers (14) demonstrated elevated glut 1 mRNA level in fibroblasts upon stimulation of protein kinase C with phorbol ester. Similar studies established a role for cAMP dependent protein kinase in stimulating glut 1 expression in NIH 3T3 fibroblasts (15) and 3T3-L1 adipocytes (16). The molecular identity of the protein kinase(s) involved in the experiments presented here is not known.

It is striking that the okadaic acid induced glucose uptake is nearly superimposable with the effect on glut 1 mRNA levels. Since our Northern blots could only measure the steady state concentration of messages, the changes could be either due to stabilization of glut 1 mRNA or to enhancement of its transcription rate. The shape of the dose response curve to okadaic acid suggests that distinct mechanisms or molecules are modulated in response to phosphatase inhibition at low and high okadaic acid concentrations.

Recent advances in molecular biology enable us to study such molecules regulating transcription. The gene for rat glut 1 has been isolated and consensus binding sites for the transcription factors have been identified (17). The flanking region of the rat glut 1 gene contains a phorbol ester responsive element and this very domain has been identified as the binding site for transcription factor AP 1 in other genes (18). It remains to be investigated whether the binding of specific factors to the glut 1 gene is altered in 3T3-L1 cells upon phosphatase inhibition by okadaic acid.

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