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# SPHINGOLIPIDS INHIBIT INSULIN AND PHORBOL ESTER STIMULATED UPTAKE OF 2-DEOXYGLUCOSE

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SUMMARY: Studies are presented demonstrating inhibition of both insulin and phorbol myristate acetate stimulated uptake of 2-deoxyglucose uptake by 3T3-L1 fibroblasts. Greatest inhibition of uptake was seen with sphinganine while sphingosine was also potent in this regard. Ceramide inhibited phorbol myristate acetate but not insulin stimulation of uptake. It is suggested that sphingolipid inhibition of glucose transport relates to the previously demonstrated effect of corticosteroids to increase membrane sphingomyelin and inhibit glucose transport.


A role for the calcium and phospholipid dependent protein kinase in glucose transport has been suggested by studies demonstrating a stimulatory effect of phorbol esters on glucose transport (1,2). Recent studies have demonstrated an action of sphingolipids, including sphinganine, sphingosine, and gangliosides, to inhibit phorbol ester stimulated functions (3,4,5,6). The present study was undertaken to determine whether these and other sphingolipids would inhibit 2-deoxyglucose uptake in 3T3-L1 fibroblasts stimulated by insulin or 4-phorbol 12-myristate 13-acetate. An inhibitory effect of sphingolipids on glucose uptake may relate to the corticosteroid induced increase in membrane sphingomyelin and inhibition of glucose transport (8,9,10).

## ABBREVIATIONS

PMA (4-phorbol 12-myristate 13-acetate; Sa (sphinganine or dihyrosphingosine); So (sphingosine or trans-4 sphinganine); Cer (ceramide or N-acyl sphingosine); Sm (sphingomyelin); BSA (bovine serum albumin); protein kinase C (calcium and phospholipid dependent protein kinase).

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## METHODS

3T3-L1 fibroblasts were kindly supplied by Harold Green (Harvard). Cells were maintained in Dulbecco's Modified Eagle's medium with 10% defined calf serum (HyClone Laboratories) and were grown at 37° C in an atmosphere of 5% $CO_2$ on 100 X 10 mm plastic tissue culture plates. Media was changed three times weekly. The cells were subcultured before reaching confluency and experiments were carried out two or three days after confluence and prior to conversion to adipocytes at a stable but not highly responsive time (7). The culture media was changed at least 24 hours prior to starting an experiment.

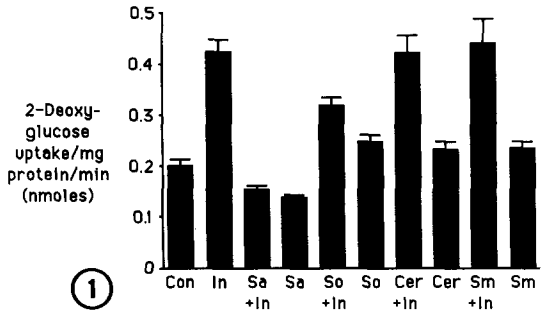
Glucose transport was determined by measuring [$^{14}$C] 2-deoxyglucose incorporation by the fibroblasts (2). The media was replaced with Krebs-Ringer phosphate buffer and after one wash with the buffer, sphingolipids were added to appropriate groups. The cells were incubated for 30 minutes with the sphingolipid. Following this incubation, insulin (65 mU/ml), or 4-phorbol 12-myristate 13-acetate (16.2 nM), was added to appropriate groups and incubation was continued for another 30 minutes. During the last ten minutes of this incubation [$^{14}$C] 2-deoxyglucose (0.1 μCi/μmol) was added to the cells. The incubation was stopped by quickly aspirating off the buffer and washing four times with cold phosphate buffered saline (pH 7.4). The cells were then solubilized with a solution containing 3% $Na_2CO_3$, 0.1 M NaOH, and 1% deoxycholate. The solubilized cells and a single wash of the plates that contained the cells were combined and aliquots removed for scintillation counting and protein determination (11).

Sphinganine, sphingosine, ceramide and sphingomyelin were dissolved in warm ethanol and added to 37° C Krebs-Ringer phosphate buffer containing 40 μM bovine serum albumin while it was being vortexed (ethanol/buffer ratio was 1/100, v/v). The lipid solutions were made fresh daily (all 40 μM). Control groups were incubated with the same ratios of ethanol and BSA containing buffer.

PMA was dissolved in acetone and added to the buffer to make 16.2 μM and an acetone to buffer ratio of 1/1000 (v/v). Acetone was added to control groups to produce a similar ratio.

## RESULTS

The well-known stimulation of glucose transport by insulin is illustrated in Figure 1. Preincubation of the cells with sphinganine totally inhibited the insulin stimulated increase in uptake. There was a small but significant inhibition of control transport by incubation with sphinganine. Preincubation of the cells with sphingosine also significantly decreased insulin stimulated transport. Surprisingly, however, incubation with sphingosine alone increased glucose transport moderately, but significantly, over that found in the control cells. Neither ceramide nor sphingomyelin had an affect on glucose transport in



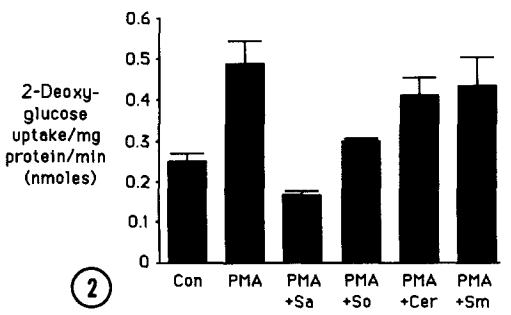




Figure 1. The effect of sphingolipids (40 μM) on control and insulin (65 mU/ml) stimulated 2-deoxyglucose uptake by 3T3-L1 fibroblasts. Mean and SEM is given for each group. Significance: control vs. insulin, n = 18, P <.001; control vs. sphinganine, n = 8, P <.001; control vs. sphingosine, n = 10, P <.01; control vs. ceramide, n = 8, NS; control vs. sphingomyelin, n = 7, NS; insulin vs. sphinganine plus insulin, n = 14, P <.001; insulin vs. sphingosine and insulin, n = 12, P <.001; insulin vs. ceramide plus insulin, n = 8, NS; insulin vs. sphingomyelin plus insulin, n = 8, NS.

Figure 2. Effect of sphingolipids (40 μM) on phorbol myristate acetate (16.2 nM) stimulated 2-deoxyglucose uptake in 3T3-L1 fibroblasts. N = 8 in all groups. Significance: control vs. PMA, P <.001; PMA vs. cells treated with sphinganine, P <.001; and PMA vs. sphingosine, P <0.01; PMA vs. ceramide, P <0.05; PMA vs. sphingomyelin, NS.

control or insulin treated cells under the conditions of this experiment. It is apparent, therefore, that some sphingolipids, but not others, have a significant influence upon glucose transport in the cells studied.

The effect of a number of sphingolipids upon phorbol stimulated glucose transport is presented in Figure 2. The action of phorbol myristate acetate to increase uptake of 2-deoxyglucose is illustrated first. Incubation of the cells with sphinganine significantly decreased the PMA effect upon glucose uptake. As is also seen in Figure 1, sphinganine significantly reduced glucose uptake below control levels. It is not clear that this can be considered inhibition of non-stimulated glucose transport as the cells had recently been exposed to media containing calf serum which had low levels of insulin and perhaps other factors which influenced the uptake of the 2-deoxyglucose. As was the case with insulin stimulated uptake, sphingosine produced a

significant decrease over that observed with PMA alone but not to the same degree as was observed with sphinganine. Ceramide had a small but significant inhibitory effect on the PMA stimulated transport but sphingomyelin failed to have an effect. The decreasing inhibitory effect as the lipid progresses along the pathway of sphingomyelin synthesis is of interest. With PMA, as with insulin, sphinganine was the most significant inhibitor employed.

## DISCUSSION

Phorbol esters have been demonstrated to stimulate glucose transport in a number of cell types including undifferentiated and differentiated preadipose cells (1,2). The mechanism by which this stimulation comes about is not clear but phorbol esters may stimulate phosphorylation of insulin receptors, induce translocation of the hexose carrier to the plasma membrane, and/or phosphorylate glucose transporters (12,13,14,15). Recent studies have demonstrated effects of sphingolipids on phorbol stimulated protein kinase C activity. Protein kinase C activity is inhibited by gangliosides, and the long-chain lipid bases, sphinganine and sphingosine, which also inhibit binding of phorbol dibutyrate to the kinase (3,4,5,6). Previous studies have demonstrated an effect of sphingomyelin to inhibit diacylglycerol stimulated phospholipase $A_2$ activity (16). All of these studies suggest an important influence of sphingolipids upon membrane related events.

An important physiologic role for sphingomyelin has been suggested by studies carried out in this laboratory which demonstrated an effect of corticosteroids to increase the level of sphingomyelin in membranes and particularly the plasma membrane (8,9,10). It was suggested that corticosteroid effects on superoxide anion production and glucose transport might be mediated

by an increase in the sphingomyelin of the plasma membrane. Levels of corticosteroids that inhibit superoxide anion production or insulin stimulated glucose transport also produce significant increases in membrane sphingomyelin in polymorphonuclear leukocytes and isolated fat cells (8,9,16). An increase in sphingomyelinase activity in dexamethasone treated leukocytes may be important in increasing the levels of the sphingolipids which were found to be most effective in inhibiting glucose transport in this study (16). It is not certain that the sphingolipids incubated with fibroblasts in the present studies would have as ready access to important inhibitory sites, as would endogenously produced lipids. The physiologic relevance of specific sphingolipids will require further study, but a likely relation to the corticosteroid stimulated increase in membrane sphingomyelin is suggested.

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