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# EFFECT OF SPHINGOID BASES ON BASAL AND INSULIN-STIMULATED 2-DEOXYGLUCOSE TRANSPORT IN SKELETAL MUSCLE

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**Summary:** Incubation of rat soleus muscles with 50 μM sphingosine or 50 μM sphinganine augmented basal 2-deoxy-D-glucose (2DG) transport 32%, but reduced the response to 0.1 and 1.0 mU insulin/ml by 17 and 27%, respectively. When the muscles were incubated with 50 μM phytosphingosine, a 63-93% increase in basal 2DG transport was observed. However, this treatment had no effect on insulin-stimulated 2DG transport. The phytosphingosine-induced increase in basal 2-DG transport was inhibited 93 and 98% with 35 and 70 μM cytochalasin B, respectively, suggesting that it is mediated by glucose transporters. Cellular accumulation of L-glucose, which is not mediated by glucose transporters, was not affected by phytosphingosine. It is concluded that (a) both sphingosine and sphinganine increase basal 2DG transport in muscle but diminish insulin-stimulated transport, and (b) phytosphingosine stimulates basal 2DG transport in muscle by a mechanism involving glucose transporters. 

Previous studies from this laboratory have shown that the ceramide concentration in skeletal muscle *in vivo* remains unchanged during insulin-stimulated glucose uptake by the muscle (1), but that ceramide levels are elevated in tissues such as liver and skeletal muscles when they exhibit insulin resistance (2). These observations suggested the possibility that ceramides are involved in the development of tissue insulin resistance either directly or by increasing the tissue concentration of sphingosine to which ceramides are degraded (3). Sphingosine has been shown to decrease insulin-stimulated glucose transport in 3T3-L1 fibroblasts (4) and to suppress the effect of insulin in rat adipocytes without decreasing the binding of the hormone to its receptor (5,6). Since skeletal muscle represents the major tissue contributing to clinical insulin resistance, the present study investigated the effect of sphingosine on basal and insulin-stimulated glucose transport in skeletal muscle. The effects of related sphingoid bases, sphinganine and phytosphingosine, were also evaluated. The results show that sphingoid bases have a dual effect on glucose transport in skeletal muscle: on one hand, they stimulate basal glucose transport, and on the other hand they inhibit the insulin-stimulated glucose transport.

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## Materials and Methods

Materials: D-Sphingosine from bovine brain sphingomyelin, DL-erythro-dihydrosphingosine (DL-sphinganine), phytosphingosine hydrochloride, 2-deoxy-D-glucose, L-glucose (mixed anomers), insulin from bovine pancreas, and cytochalasin B were obtained from Sigma. [1,2-$^3$H(N)]2-deoxy-D-glucose, [1-$^3$H(N)]-L-glucose, and [1-$^3$H(N)] sucrose were purchased from New England Nuclear.

Studies on soleus muscles *in vitro*: Young male Sprague-Dawley rats weighing 60-70g were fasted overnight (17 hrs) before the experiment. The rats were killed by stunning and rapid exsanguination, and intact soleus muscles with short pieces of tendons were excised from both hindlimbs. The muscles were mounted on clips to maintain them at 100% of their rest length and were preincubated in Krebs-Ringer bicarbonate buffer (110 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, and 1.2 mM $CaCl_2$, pH 7.4) containing 2 mM sodium pyruvate, 1% defatted albumin, 0, 0.1 and 1.0 mU insulin/ml, and other additions as indicated. The concentrations of additions in the preincubation medium were 50 $\mu$M D-sphingosine, 50 $\mu$M DL-sphinganine, 0 to 50 $\mu$M phytosphingosine hydrochloride, and 35 or 70 $\mu$M cytochalasin B. Sphingoid bases and cytochalasin B were dissolved in ethanol and were added in such a way that the medium contained 0.1% ethanol. The corresponding control medium always contained an equivalent concentration of ethanol. The preincubation period lasted 1 or 2 hrs as indicated. At the end of this period, the muscles were transferred into fresh identical medium enriched with 1 mM 2-deoxy-D-glucose (0.5 $\mu$Ci/ml) and were incubated for an additional 15 min. Where indicated, 2-deoxy-D-glucose was replaced by 1 mM L-glucose (0.5 $\mu$Ci/ml). The media were equilibrated with 95% $O_2$-5% $CO_2$ before use, and all incubations were carried out at 37°C under an atmosphere of 95% $O_2$-5% $CO_2$.

At the end of the 15 min incubation period, the muscles were removed and the cellular uptake of radioactive 2-deoxyglucose (or L-glucose, where present) was determined as follows: muscles and aliquots of the incubation medium were digested separately in Solvable tissue solubilizer (New England Nuclear) and the radioactivity determined after addition of Formula-989 (New England Nuclear) in a liquid scintillation counter. Cellular radioactivity was calculated as the difference between the total tissue radioactivity (disintegrations/min) and the amount of radioactivity present in the tissue extracellular space. The extracellular spaces in muscles were determined separately by incubations with 0.1 mM sucrose (0.5 $\mu$Ci/ml).

Data evaluation: The results are expressed as means ± SE and were analyzed by the paired Student's t-test and by the Mann-Whitney U-test as appropriate.

## Results

Effects of sphingosine and sphinganine. Table 1 shows the results of studies in which muscles were preincubated in the presence of sphingoid bases for 2 hrs before 2-deoxy-D-glucose transport was measured. Sphingosine (50 $\mu$M)

**Table 1. Effects of 50 $\mu$M sphingosine or 50 $\mu$M sphinganine on basal and insulin-induced 2-deoxy-D-glucose transport in soleus muscle**

| | 2-deoxy-D-glucose uptake | | |
|---|---|---|---|
| | Basal | 0.1 mU insulin/ml | 1.0 mU insulin/ml |
| Exp. 1 | | | |
| Control (dpm/mg) | 478 ± 21 | 949 ± 19 | 1818 ± 109 |
| Sphingosine (dpm/mg) | 633 ± 22 | 766 ± 35 | 1321 ± 64 |
| Difference | +32% (p<0.001) | -19% (p<0.005) | -27% (p<0.004) |
| Exp. 2 | | | |
| Control (dpm/mg) | 485 ± 36 | 1081 ± 40 | 2052 ± 172 |
| Sphinganine (dpm/mg) | 642 ± 43 | 910 ± 43 | 1485 ± 99 |
| Difference | +32% (p<0.03) | -16% (p<0.02) | -28% (p<0.004) |

Each value is a mean ± SE from muscles of 6 rats. The values represent cellular accumulation of 2-deoxy-D-glucose in dpm/mg muscle.

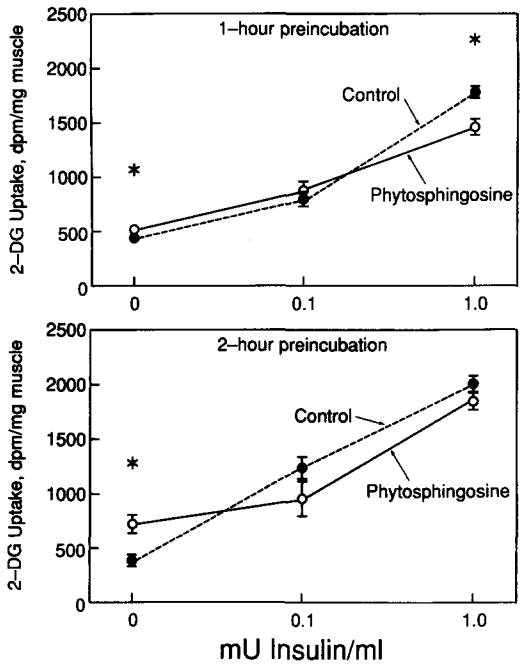



**Fig. 1.** Effect of phytosphingosine on basal and insulin-induced transport of 2-deoxyglucose by soleus muscles. The muscles were first preincubated for either 1 hr (upper panel) or 2 hrs (lower panel) in Krebs-Ringer bicarbonate buffer with 2 mM sodium pyruvate, 1% defatted albumin, 0.1% ethanol, 0, 0.1, or 1.0 mU insulin/ml, and 0 or 50 $\mu$M phytosphingosine. The muscles were then transferred into fresh identical medium enriched with 1 mM 2-deoxy-D-glucose (0.5 $\mu$Ci/ml), and incubated for an additional 15 min to assess 2-deoxyglucose transport. Each value is a mean ± SE from muscles of 6 rats. SE bars are not indicated if they were smaller than the size of the symbols. The asterisk indicates a statistically significant difference ($p<0.05$) from control muscles studied at the same insulin concentration.

produced a 32% increase in basal 2-deoxyglucose transport, but inhibited the response to 0.1 and 1.0 mU insulin/ml by 19 and 27%, respectively. The actions of sphinganine were almost identical to those of sphingosine. Sphinganine (50 $\mu$M) caused a 32% increase in basal 2-deoxyglucose transport, but decreased the response to 0.1 and 1.0 mU insulin/ml by 16 and 28%, respectively. If the preincubation period was shortened to 1 hr, neither sphingosine nor sphinganine had any statistically significant effect on either basal 2-deoxyglucose transport or that at a physiological concentration of insulin (0.1 mU/ml); however, sphingosine and sphinganine produced a 16% ($p<0.05$) and a 25% ($p<0.001$) inhibition of transport at a supraphysiological level of insulin (1.0 mU/ml), respectively (data not shown).

Effects of phytosphingosine. As shown in the upper panel of Fig. 1, a 1-hr preincubation with phytosphingosine increased basal 2-deoxyglucose transport 20% ($p<0.02$), but decreased the response to the supraphysiological concentration of insulin (1.0 mU/ml) by 18% ($p<0.008$). No effect was observed at 0.1 mU insulin/ml. When the muscles were preincubated with phytosphingosine for 2 hrs (lower panel of Fig. 1), a 93% ($p<0.004$) increase in basal 2-deoxyglucose transport was noted. However, this treatment had no effect on 2-deoxyglucose transport at 0.1 or 1.0 mU insulin/ml.

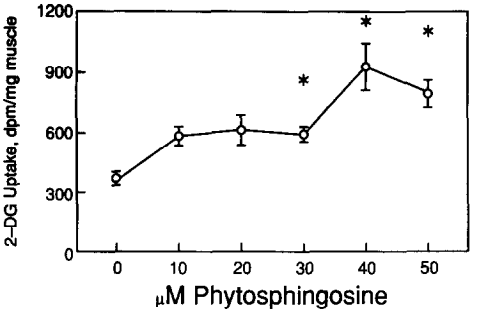
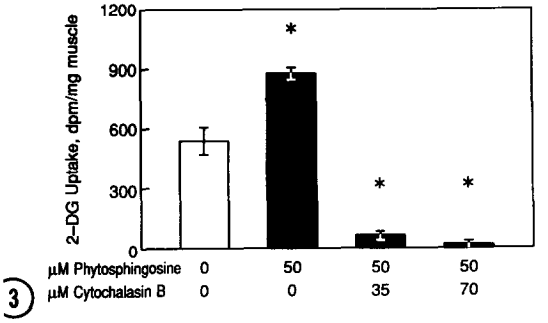



**Fig. 2.** Dose-response relationship of the effect of phytosphingosine on 2-deoxyglucose transport by soleus muscles. Each value is a mean ± SE from muscles of 6 rats. The asterisk indicates a statistically significant difference ($p<0.05$) from muscles studied without phytosphingosine.

To investigate the dose-response relationship of the pronounced stimulatory effect of phytosphingosine on basal 2-deoxyglucose transport, muscles were first preincubated with 0, 10, 20, 30, 40, and 50 μM phytosphingosine for 2 hrs, and then incubated for 15 min to measure 2-deoxyglucose transport. As shown in Fig. 2, treatment of muscles with 30, 40, and 50 μM phytosphingosine augmented muscle 2-deoxyglucose uptake 33% ($p<0.04$), 112% ($p<0.007$), and 77% ($p<0.007$), respectively. The apparent 28% and 41% increases in muscle 2-deoxyglucose transport at 10 and 20 μM phytosphingosine, respectively, were not statistically significant ($p = 0.0546$ and 0.109, respectively).

To test whether the phytosphingosine-induced increase in basal 2-deoxyglucose transport is mediated by glucose transporters, soleus muscles were first preincubated for 2 hrs with or without 50 μM phytosphingosine, and with or without 35 or 70 μM cytochalasin B, and basal 2-deoxyglucose transport was measured during the subsequent 15-min incubation period (Fig. 3). Compared to

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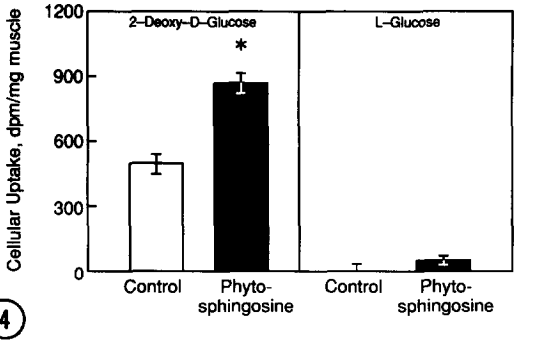


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**Fig. 3.** Effect of cytochalasin B on phytosphingosine-stimulated transport of 2-deoxyglucose by soleus muscles. Each value is a mean ± SE from muscles of 6-10 rats. The asterisk indicates a statistically significant difference ($p<0.05$) from control muscles studied without phytosphingosine or cytochalasin B, and also from muscles studied with phytosphingosine alone.

**Fig. 4.** Effect of phytosphingosine on cellular accumulation of 2-deoxy-D-glucose or L-glucose. Each value is a mean ± SE from muscles of 6 rats. The asterisk indicates a statistically significant difference ($p<0.05$) from corresponding control muscles studied without phytosphingosine.

control incubations, phytosphingosine-treated muscles exhibited a 63% ($p<0.002$) augmentation of 2-deoxyglucose transport. This phytosphingosine-induced 2-deoxyglucose transport was inhibited 93 and 98% ($p<0.004$) with 35 and 70 $\mu$M cytochalasin B, respectively, suggesting that it is mediated by glucose transporters.

To eliminate the possibility that phytosphingosine impairs the integrity of the cell membrane and permits entry of 2-deoxyglucose by a mechanism independent of glucose transporters, the effects of phytosphingosine on the cellular accumulation of 2-deoxy-D-glucose and L-glucose were compared. The latter sugar was used because it has a comparable molecular size to 2-deoxy-D-glucose but, unlike 2-deoxy-D-glucose, it is not transported by glucose transporters. In the experiment depicted in Fig. 4, the muscles were first preincubated with or without 50 $\mu$M phytosphingosine for 2 hrs, and then incubated for an additional 15 min in fresh identical medium, enriched either with 2-deoxy-D-glucose or L-glucose, to measure cellular accumulation of the respective sugars. Phytosphingosine-treated muscles exhibited a 74% ($p<0.001$) increase in cellular accumulation of 2-deoxy-D-glucose. In contrast, phytosphingosine had no statistically significant effect on cellular accumulation of L-glucose, demonstrating that treatment with phytosphingosine did not make muscle cell membranes "leaky".

## Discussion

The present study shows that sphingoid bases have a dual effect on 2-deoxyglucose transport by skeletal muscle. All three lipids tested stimulated basal 2-deoxyglucose transport, phytosphingosine being the most potent in this regard. On the other hand, the lipids either did not affect the insulin-stimulated 2-deoxyglucose transport, as in the case of phytosphingosine, or did inhibit the response to physiological and supraphysiological concentration of insulin, as in the case of sphingosine and sphinganine. The size of the changes may be an underestimation because sphingoid bases were kept in solution bound to albumin, and the sphingoid base/albumin complex may not have been able to readily penetrate to muscle fibers located deeper within the incubated muscle. Furthermore, exogenous sphingoid bases may not be able to reach important regulatory sites within the cell as readily as would endogenously produce lipids. This may explain the more pronounced effects of sphingoid bases after a 2-hr preincubation compared with the 1-hr preincubations.

The mechanism of stimulation of basal 2-deoxyglucose transport by sphingoid bases is not fully understood. We have previously noted that 50 $\mu$M sphingosine does not increase the release of the cytosolic enzyme lactate dehydrogenase by the incubated muscle, demonstrating that such a concentration of the lipid does not affect cell viability and cell membrane integrity (7). This conclusion was confirmed and extended in the present study by demonstrating that phytosphingosine did not increase the cellular accumulation of L-glucose. This sugar was used because it has a comparable molecular size to 2-deoxy-D-glucose, but unlike 2-deoxy-D-glucose, it is not transported by glucose transporters. The lack of an effect of phytosphingosine on cellular accumulation of L-glucose indicates that the cell membrane is not freely permeable even to small molecules the size of 2-deoxyglucose, and therefore the increased entry of 2-deoxyglucose into cells of muscles treated with sphingoid bases is not due to leakage through a damaged

cell membrane. This conclusion and the fact that the phytosphingosine-induced increase in basal 2-deoxyglucose transport is inhibitable by cytochalasin B, suggest that sphingoid bases stimulate basal 2-deoxyglucose transport in muscle by a physiological mechanism which includes glucose transporters.

The inhibitory effect of sphingosine and sphinganine on insulin-stimulated 2-deoxyglucose transport by skeletal muscle is consistent with similar observations on 3T3-L1 fibroblasts (4) and rat adipocytes (5,6). It should be noted, however, that unlike in adipocytes, the effect of sphingosine in skeletal muscle was slow to develop, and was not pronounced. Although this may mean that exogenous sphingosine does not readily penetrate to all cells comprising the muscle, the possibility cannot be ruled out that sphingosine is not an important modulator of insulin-stimulated glucose transport in skeletal muscle.

Since sphingosine inhibits protein kinase C (8), inhibition of insulin-induced hexose transport by sphingosine was taken as evidence that protein kinase C plays an essential role in the activation of glucose transport by insulin (5,6). Consistent with this hypothesis are reports that insulin increases the level of 1,2-diacylglycerol and the activity of protein kinase C in skeletal muscle (9,10). However, we were unable to observe any statistically significant changes in skeletal muscle 1,2-diacylglycerol level *in vivo* during the period when exogenous insulin augmented muscle glucose uptake 6-fold (1). It is also uncertain whether an increase in muscle 1,2-diacylglycerol would result in stimulation of glucose transport in this tissue even if it actually occurred. Several groups of investigators reported that interruption of the nerve supply to rat soleus muscle increases this muscle's 1,2-diacylglycerol concentration (2,11,12). Despite this increase, which is comparable to the reported insulin-induced increases in muscle 1,2-diacylglycerol (9,10), glucose transport by the denervated soleus muscle *in vivo* does not increase above the control level (13). These observations, and the fact that the activation of muscle protein kinase C by insulin has been contested (14), lead us to suggest that the diacylglycerol/protein kinase C pathway does not play an important role in stimulation of muscle glucose transport by insulin. This would be consistent with the relatively small effect of sphingosine on this process.

**Acknowledgment**

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