Sphingomyelinase stimulates 2-deoxyglucose uptake by skeletal muscle

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The effects of sphingomyelinase, phosphorylcholine, *N*-acetylsphingosine (C2-ceramide), *N*-hexanoylsphingosine (C6-ceramide) and sphingosine on basal and insulin-stimulated cellular accumulation of 2-deoxy-D-glucose in rat soleus muscles were investigated. Preincubation of muscles with sphingomyelinase (100 or 200 m-units/ml) for 1 or 2 h augmented basal 2-deoxyglucose uptake by 29–91 %, and that at 0.1 and 1.0 m-unit of insulin/ml by 32–82 % and 19–25 % respectively compared with control muscles studied at the same insulin concentrations. The sphingomyelinase-induced increase in basal and insulin-stimulated 2-deoxyglucose uptake was inhibited by 91 % by 70 μM cytochalasin B, suggesting that it involves glucose transporters. Sphingomyelinase had no effect on the cellular accumulation of L-glucose, which is not transported by glucose transporters. The sphingomyelinase-induced increase in 2-deoxyglucose uptake could not be reproduced by preincubating the muscles with 50 μM phosphorylcholine, 50 μM C2-ceramide or 50 μM C6-ceramide. Preincubation of muscles with 50 μM sphingosine augmented basal 2-deoxyglucose transport by 32 %, but reduced the response to 0.1 and 1.0 m-unit of insulin/ml by 17 and 27 % respectively. The stimulatory effect of sphingomyelinase on basal and insulin-induced 2-deoxyglucose uptake was not influenced by either removal of Ca^{2+} from the incubation medium or dantrolene, an inhibitor of Ca^{2+} release from the sarcoplasmic reticulum. This demonstrates that Ca^{2+} does not mediate the action of sphingomyelinase on 2-deoxyglucose uptake. Sphingomyelinase also had no effect on basal and insulin-stimulated activities of insulin receptor tyrosine kinase and phosphatidylinositol 3-kinase. In addition, 1 and 5 μM wortmannin, an inhibitor of phosphatidylinositol 3-kinase, failed to inhibit the sphingomyelinase-induced increase in 2-deoxyglucose uptake. These results suggest that sphingomyelinase does not increase 2-deoxyglucose uptake by stimulating the insulin receptor or the initial steps of the insulin-transduction pathway. The data suggest the possibility that sphingomyelinase increases basal and insulin-stimulated 2-deoxyglucose uptake in skeletal muscle as the result of an unknown post-receptor effect.

INTRODUCTION

Studies over the last few years have led to the discovery of a 'sphingomyelin cycle' [1] or a sphingomyelin signalling pathway in which activation of a neutral sphingomyelinase results in hydrolysis of sphingomyelin to ceramide and phosphorylcholine. Observations on various cell lines indicate that the sphingomyelin pathway is stimulated by 1,25-dihydroxyvitamin D_3 [1,2], tumour necrosis factor α [3,4], γ-interferon [3], interleukin 1β [5] and 1,2-diacylglycerols [6]. The potential signalling role of ceramide is suggested by findings that it activates mitogen-activated protein kinase [4], ceramide-activated protein kinase [5] and ceramide-activated phosphatase [7]. In addition, ceramide was reported to stimulate phosphorylation of epidermal growth factor receptor [8], to stimulate cellular proliferation [9] and differentiation [3], and to modulate secretory protein traffic in cells [10]. Treatment of cells with exogenous sphingomyelinase provides a useful tool for the study of the sphingomyelin pathway because sphingomyelin is present in both the outer and inner layer of the plasma membrane. Available evidence indicates that sphingomyelin is not evenly distributed between different subcellular membranes and its highest concentration is found in the plasma membrane [11–13].

Our previous studies have shown that the ceramide concentration in skeletal muscle *in vivo* remains unchanged during insulin-stimulated glucose uptake by the muscle [14], but that ceramide levels are elevated in tissues such as liver and skeletal muscles when they exhibit insulin resistance [15]. These observations suggested the possibility that ceramides are involved in the development of tissue insulin resistance. To investigate the effects of the increased ceramide concentration on basal and insulin-stimulated glucose transport in skeletal muscle, rat soleus muscles were incubated with sphingomyelinase. Contrary to expectations, this treatment increased both the basal and insulin-stimulated transport of 2-deoxyglucose, a non-metabolizable glucose analogue, by soleus muscles. We set out to investigate this novel observation in order to answer the following questions. (a) Does the sphingomyelinase-induced increase in 2-deoxyglucose uptake involve glucose transporters or does it merely reflect damage to cell membrane integrity? (b) Is the stimulation of 2-deoxyglucose uptake due to sphingomyelinase *per se* or contamination of the commercial enzyme preparation with phospholipase C or proteinases that are known to stimulate sugar transport [16–19]? (c) Can the degradation products of sphingomyelin mimic the stimulatory action of sphingomyelinase on basal and insulin-stimulated 2-deoxyglucose uptake by muscle? (d) As hypoxia and contractions appear to stimulate glucose transport in muscle by increasing cytoplasmic Ca^{2+} [20–23], is the increased 2-deoxyglucose uptake by sphingomyelinase-treated muscles mediated by Ca^{2+}? (e) Finally, does sphingomyelinase act by stimulating the insulin receptor or some component of the signal-transduction pathway by which insulin increases muscle glucose uptake? This last question was approached by measuring the activities of muscle insulin receptor tyrosine kinase and phosphatidylinositol 3-kinase. The latter enzyme is part of the signal-transduction pathway that effects the stimulation of glucose uptake by insulin [24–26]. Activation of this enzyme involves the following steps. First, binding of insulin to its receptor and activation of receptor tyrosine kinase causes phosphorylation of insulin receptor substrate 1 (IRS-1). Then

Abbreviations used: C2-ceramide, *N*-acetylsphingosine; C6-ceramide, *N*-hexanoyl-D-sphingosine; IRS-1, insulin receptor substrate 1.

the regulatory subunit of phosphatidylinositol 3-kinase (p85) binds to a phosphorylated src homology-binding domain on IRS-1, and this, in turn, increases the activity of the catalytic subunit (p110) of phosphatidylinositol 3-kinase [27]. Thus measuring phosphatidylinositol 3-kinase activity can be used to assess the potential effect of sphingomyelinase on any of the above steps of the insulin signal-transduction pathway. We also investigated the involvement of the insulin pathway in sphingomyelinase action using wortmannin, an inhibitor of phosphatidylinositol 3-kinase. Wortmannin has been shown to inhibit augmentation of muscle glucose uptake caused by insulin [25,26] but not by hypoxia or contractions [26]. The subsequent report provides a detailed account of the studies.

EXPERIMENTAL

Materials

2-[1,2-^{3}H(n)]Deoxy-D-glucose and L-[1-^{3}H(n)]glucose were purchased from New England Nuclear. [U-^{14}C]Sucrose and [γ-^{32}P]ATP were from ICN Biomedicals. *N*-Acetylsphingosine (C2-ceramide) was kindly provided by Dr. Y. Hannun and Dr. A. Bielawska (Duke University Medical Center, Durham, NC, U.S.A.). *N*-Hexonoyl-D-sphingosine (C6-ceramide) was from Biomol. Sphingomyelinase from *Streptomyces*, sphingomyelinase from *Staphylococcus aureus*, phosphorylcholine chloride (calcium salt), D-sphingosine from bovine brain sphingomyelin, 2-deoxy-D-glucose, L-glucose (mixed anomers) and all other chemicals were obtained from Sigma. Unless indicated otherwise, sphingomyelinase from *Streptomyces* was used throughout the study. According to Sigma, 1 unit of sphingomyelinase will hydrolyse 1 μmol of trinitrophenylaminolaurylsphingomyelin/min at pH 7.4 and 37 °C.

Studies on soleus muscle *in vitro*

The experiments were performed on young male Sprague–Dawley rats weighing 60–70 g. The animals were transferred to clean cages without food at 16:00 h and killed by rapid exsanguination at 09:00–10:00 h the following day. Intact soleus muscles with short pieces of tendons were excised from both hind limbs. The muscles were mounted on clips to maintain them at 100 % of their rest length and were preincubated in Krebs–Ringer bicarbonate buffer (119 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 % (w/v) defatted albumin, 0, 0.1 and 1.0 m-unit of insulin/ml, and other additions as indicated. The concentrations of additions to the preincubation medium were 100 or 200 m-units of sphingomyelinase/ml, 50 μM phosphorylcholine chloride, 50 μM C2-ceramide, 50 μM C6-ceramide, 50 μM D-sphingosine, 50 μg of leupeptin/ml, 1 mM PMSF, 1 mM EGTA, 15 and 25 μM dantrolene, 70 μM cytochalasin B and 1 or 5 μM wortmannin. C2-ceramide, C6-ceramide, sphingosine, PMSF and cytochalasin B were dissolved in ethanol and added in such a way that the medium contained 0.1 % ethanol. The corresponding control medium always contained an equivalent concentration of ethanol. Dantrolene was dissolved in 4.44 % (w/v) D-mannitol with 0.08 % (w/v) NaOH [17]. Addition of dantrolene resulted in 2.5–4.0 mM mannitol concentration in the medium and therefore the corresponding control medium always contained an equivalent concentration of mannitol. Wortmannin was dissolved in DMSO and its addition resulted in 0.1 % DMSO in the medium; the corresponding control medium contained the same concentration of DMSO. The preincubation period lasted 1 or 2 h as indicated. At the end of this period, the muscles were transferred to fresh medium that was identical except that it contained 1 mM 2-[^{3}H]deoxy-D-glucose (0.5 μCi/ml) and were incubated for an additional 15 min as previously described [17]. Where indicated, 2-deoxy-D-glucose was replaced by 1 mM L-[^{3}H]glucose (0.5 μCi/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 was 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 (d.p.m.) and the amount of radioactivity present in the tissue extracellular (sucrose) space.

Determination of 1,2-diacylglycerol and ceramides

Six soleus muscles were pooled for each determination. The muscles were placed in liquid nitrogen immediately after their removal from the incubation medium. They were kept in liquid nitrogen and powdered under liquid nitrogen within 2 h. Powder corresponding to 180–190 mg of tissue was extracted as described by Bligh and Dyer [28] in 6 ml of chloroform/methanol (1:2, v/v) with a sufficient amount of 1 M NaCl to bring the total water (including tissue water) to 1.6 ml. After a 10 min extraction, 2 ml of chloroform and 2 ml of 1 M NaCl were added, and the phases were separated by centrifugation (1100 ***g***; 4 °C; 10 min). The aqueous layer was discarded, and the organic layer containing the lipids was transferred to a clean tube. The extract was kept at −32 °C and analysed within 24 h.

Analysis was performed as described in detail previously [14,15,29]. An aliquot of lipid extract corresponding to 1–2 mg of tissue was used to determine the *sn*-1,2-diacylglycerol content by the diacylglycerol kinase assay described by Preiss et al. [30]. Ceramides were determined in the same assay, as diacylglycerol kinase also catalyses phosphorylation of ceramides and phosphorylated derivatives of diacylglycerol and ceramides can easily be separated by TLC. Known amounts of *sn*-1,2-dioleoylglycerol and ceramides were included in every assay to produce a standard curve.

Measurement of insulin receptor tyrosine kinase activity

Insulin receptor tyrosine kinase activity was assayed in partially purified insulin receptors as described by Heydrick et al. [31] with minor modifications. Soleus muscles from *in vitro* incubations were homogenized in 0.5 ml of ice-cold homogenization buffer [50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 2 mM Na_3VO_4, 30 mM *bis*-(*p*-nitrophenyl) phosphate, antipain (10 μg/ml), leupeptin (5 μg/ml), aprotinin (2 μg/ml), benzamidine (1.5 mg/ml), PMSF (34 μg/ml) and 1 % Triton X-100, pH 7.4]. After homogenization, the samples were centrifuged for 15 min at 7000 ***g***. Aliquots of the resulting supernatants, corresponding to approx. 2.5 mg of protein, were added to 150 μl of wheat-germ agglutinin–agarose (pre-equilibrated with homogenization buffer), and the resulting suspensions were gently agitated for 2 h at 4 °C. The suspensions were then centrifuged for 5 min at 10000 ***g*** at 4 °C, washed once in the homogenization buffer + 0.1 % Triton X-100 and twice in an assay buffer [30 mM Hepes, 30 mM NaCl, 30 mM *bis*-(*p*-nitrophenyl) phosphate and 0.1 % Triton X-100, pH 7.4]. After the washes, the bound glycoproteins were eluted from the beads by the addition of 250 μl of 0.45 M *N*-acetyl-

Table 1 Effect of sphingomyelinase on ceramide and 1,2-diacylglycerol levels in soleus muscles

The muscles were incubated for either 1 or 2 h in Krebs–Ringer bicarbonate buffer with 2 mM sodium pyruvate, 1% (w/v) defatted albumin, and 0, 100 or 200 m-units of sphingomyelinase/ml. Each value is a mean ± S.E.M. from three determinations. Muscles of six rats were pooled for each determination. NS, Not statistically significant.

Incubation time (h)	Sphingomyelinase (m-units/ml)	Ceramide		1,2-Diacylglycerol	
		(nmol/g)	Change (%)	(nmol/g)	Change (%)
1	0	72 ± 3		143 ± 6	
	100	173 ± 13	+140 ($P < 0.01$)	111 ± 9	−22 ($P < 0.008$)
2	0	68 ± 2		143 ± 17	
	100	238 ± 19	+250 ($P < 0.004$)	138 ± 6	NS
1	0	84 ± 6		273 ± 27	
	200	263 ± 10	+213 ($P < 0.001$)	276 ± 23	NS
2	0	67 ± 3		161 ± 14	
	200	221 ± 10	+230 ($P < 0.001$)	125 ± 8	NS

glucosamine in assay buffer. After 30 min at 4 °C, the eluate/bead mixtures were centrifuged at 10000 ***g*** for 5 min and the resulting supernatants containing partially purified insulin receptors (eluate) were stored at −80 °C until use. The activity assay was performed by adding a 40 μl aliquot of the eluate to 10 μl of 1.5 mg/ml poly(glutamate/tyrosine, 4:1), and the kinase reaction was initiated by the addition of 10 μl of an ATP mixture (1 μM [γ-^{32}P]ATP (0.125 μCi/60 μl)/48 mM $MgCl_2$; final concentrations). Tyrosine phosphorylation was allowed to proceed for 30 min at room temperature. The reaction was stopped by spotting 50 μl of the reaction mixture on to Whatman ET 31 filter paper squares (2 cm × 2 cm) and the papers were then placed in a 10% trichloroacetic acid bath containing 10 mM sodium pyrophosphate for 15 min. The papers were then washed extensively with 5% trichloroacetic acid/10 mM sodium pyrophosphate and dried; radioactivity incorporated into the substrate was quantified by liquid-scintillation counting.

Measurement of phosphatidylinositol 3-kinase activity

Soleus muscles were assayed immediately after removal from the incubation medium as described by Serunian et al. [32] and Ruderman et al. [33] with modifications. Muscles were homogenized in ice-cold solubilization buffer [20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_3VO_4, 10 mM pyrophosphate (tetrasodium salt anhydrous), 10 mM NaF, 2 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Nonidet P40, aprotinin (2 μg/ml), antipain (10 μg/ml), leupeptin (5 μg/ml), benzamidine (1.5 mg/ml) and PMSF (34 μg/ml)]. After homogenization, all samples were rocked at 4 °C for 45 min and then insoluble material was removed by centrifugation at 7000 ***g*** and 4 °C for 30 min. Tyrosine-phosphorylated proteins were immunoprecipitated from the supernatant containing 5 mg of protein with a monoclonal anti-phosphotyrosine antibody coupled directly to agarose (PT-66; Sigma). Immunoprecipitation was carried out at 4 °C for 2.5 h with constant rocking. The immunoprecipitates were washed successively in PBS containing 1% (v/v) Nonidet P40 and 100 μM Na_3VO_4 (three times), 100 mM Tris/HCl, pH 7.5, containing 500 mM $LiCl_2$ and 100 μM Na_3VO_4 (three times), and 10 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA and 100 μM Na_3VO_4 (twice) [34]. Immunoprecipitates were then assayed for phosphatidylinositol 3-kinase phosphorylation activity directly on the agarose beads. The beads were incubated in a 100 μl reaction mixture containing (final concentrations): 0.2 mg of phosphatidylinositol/ml sonicated in 10 mM Tris/HCl, pH 7.5, 0.4 mM EGTA, 0.4 mM sodium phosphate, 9.2 mM $MgCl_2$ and [γ-^{32}P]ATP (43.6 μM at 0.36 μCi/μl). The reaction was allowed to proceed at room temperature with constant rocking for 15 min and was stopped by the addition of 400 μl of 1 M HCl. Lipids were then extracted from the reaction mixture with 260 μl of chloroform/methanol (1:1, v.v). The samples were centrifuged and the lower organic phase was removed and applied to a silica-gel TLC plate (Analtech) coated with 1% potassium oxalate. TLC plates were developed in chloroform/methanol/NH_4OH (90:70:20, by vol) and dried. The radioactive spots were visualized by autoradiography, scraped and quantified by liquid-scintillation counting.

Data evaluation

The results are expressed as means ± S.E.M. and were analysed by analysis of variance with the Newman–Keuls test.

RESULTS AND DISCUSSION

Effects of sphingomyelinase

Incubation of soleus muscles with 100 or 200 m-units of sphingomyelinase/ml for either 1 or 2 h resulted in 140–250% increases in muscle ceramide level (Table 1). No increases in muscle 1,2-diacylglycerol concentration were observed, indicating that the enzyme preparation was not contaminated by phospholipase C activity (Table 1).

Treatment with sphingomyelinase stimulated both basal and insulin-induced glucose transport by muscles (Figure 1). After a 1 h preincubation of muscles with 100 m-units of sphingomyelinase/ml, basal 2-deoxyglucose uptake was elevated by 49% ($P < 0.02$), and uptakes at physiological (0.1 m-unit/ml) and supraphysiological (1 m-unit/ml) insulin concentrations were increased by 82 and 19% ($P < 0.001$) respectively compared with corresponding control muscles studied at the same insulin concentrations. A 2 h preincubation under the same conditions produced a similar pattern of changes. Compared with corresponding control muscles, sphingomyelinase augmented 2-deoxyglucose uptake at 0, 0.1 and 1.0 m-unit of insulin/ml by 29% ($P < 0.02$), 54% ($P < 0.001$) and 25% ($P < 0.001$) respectively. To test whether increasing the concentration of the enzyme in the incubation medium would magnify these effects, muscles were also studied in the presence of 200 m-units of sphingomyelinase/ml (Figures 1b and 1d). Preincubation of muscles with 200 m-units of sphingomyelinase/ml for 1 h resulted in an 80% ($P < 0.02$) and 69% ($P < 0.001$) higher 2-deoxyglucose uptake at 0 and 0.1 m-unit of insulin/ml re-

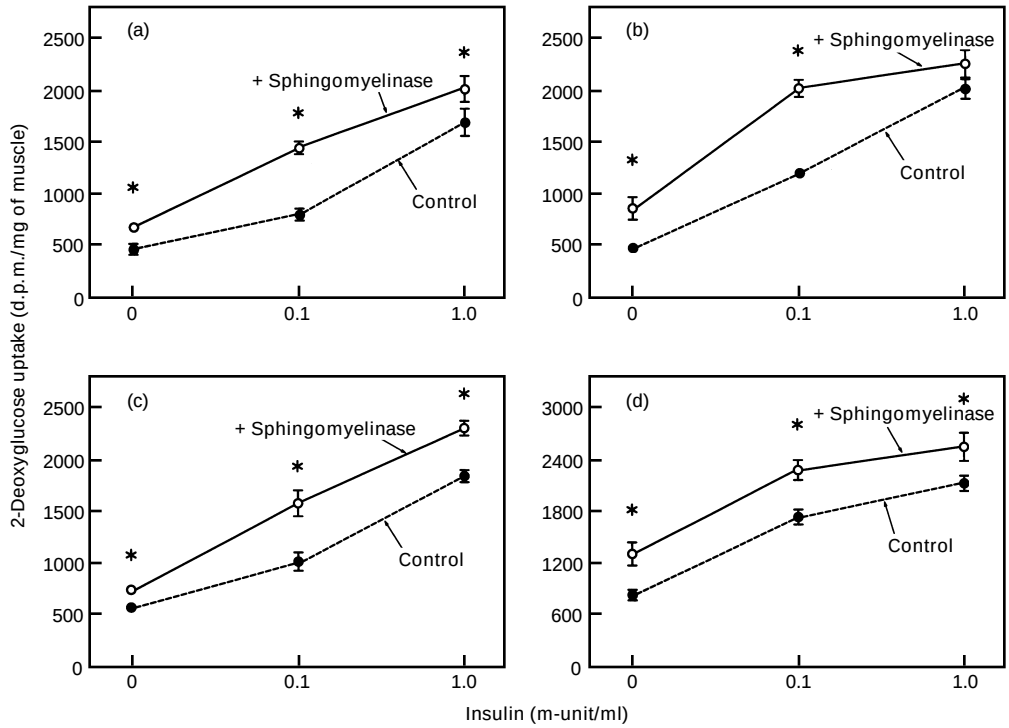

Figure 1 Effect of sphingomyelinase on basal and insulin-induced uptake of 2-deoxyglucose by soleus muscles.

The muscles were first preincubated for either 1 h (**a**, **b**) or 2 h (**c**, **d**) in Krebs–Ringer bicarbonate buffer with 2 mM sodium pyruvate, 1% (w/v) defatted albumin, 0, 0.1 or 1.0 m-unit of insulin/ml, and 0 or 100 m-units (**a**, **c**) or 200 m-units (**b**, **d**) of sphingomyelinase/ml. The muscles were then transferred to fresh identical medium supplemented with 1 mM [^{3}H]2-deoxy-D-glucose (0.5 μCi/ml), and incubated for an additional 15 min to measure 2-deoxyglucose uptake. In each rat, the soleus muscle from one hind limb was always incubated with sphingomyelinase (○), and the soleus muscle from the contralateral hind limb served as the control (●). Each value is a mean ± S.E.M. from muscles of six rats. S.E.M. bars are not indicated if they were smaller than the size of the symbols. *Statistically significant difference ($P < 0.05$) from control muscles studied at the same insulin concentration.

Table 2 Basal and sphingomyelinase-stimulated 2-deoxyglucose uptake by soleus muscles in the presence of proteinase inhibitors

The muscles were first preincubated with 0 or 200 m-units of sphingomyelinase/ml and with or without individual inhibitors for 2 h, and then incubated for 15 min in identical fresh medium supplemented with 1 mM 2-[^{3}H]deoxy-D-glucose (0.5 μCi/ml) to measure 2-deoxyglucose uptake. Ca^{2+} was omitted from media containing EGTA. Each value is a mean ± S.E.M. from muscles of six to twelve rats.

		2-Deoxyglucose uptake (d.p.m./mg of muscle)		
Study	Treatment	Control	+ Sphingomyelinase	Change (%)
1	–	458 ± 38	834 ± 47	+ 82 ($P < 0.001$)
	Leupeptin (50 μg/ml)	452 ± 16	899 ± 59	+ 99 ($P < 0.004$)
2	–	559 ± 28	858 ± 56	+ 53 ($P < 0.004$)
	1 mM PMSF	519 ± 54	730 ± 56	+ 41 ($P < 0.04$)
3	–	436 ± 50	704 ± 78	+ 61 ($P < 0.007$)
	1 mM EGTA	857 ± 76	1418 ± 100	+ 65 ($P < 0.007$)

spectively compared with corresponding control muscles studied at the same insulin concentrations. The apparent 13% increase at 1 m-unit of insulin/ml was not statistically significant. After a 2 h preincubation of muscles with 200 m-units of sphingomyelinase/ml, 2-deoxyglucose uptake at 0, 0.1 and 1.0 m-unit of insulin/ml was increased by 59% ($P < 0.003$), 32% ($P < 0.001$) and 20% ($P < 0.02$) respectively compared with corresponding control muscles.

To test for contamination of sphingomyelinase with proteinases, the sphingomyelinase preparation was incubated with a synthetic proteinase substrate, azocasein, and the proteolytic activity of sphingomyelinase was compared with that of trypsin with an activity of 12000 units/mg [35]. To magnify the possible proteinase action, 2, 10 and 20 units of sphingomyelinase/ml were tested. These concentrations were 10-fold, 50-fold and 100-fold higher than those used in the muscle incubations. The resulting trypsin-like activity of these concentrations was minimal, corresponding to 3, 8 and 14 units of trypsin/ml respectively. The possible contamination of sphingomyelinase preparation with proteinases was also assessed by

Table 3 Comparison of the effect of sphingomyelinase from *Streptomyces* and sphingomyelinase from *Staphylococcus aureus* on 2-deoxyglucose uptake by soleus muscles

The muscles were first preincubated with or without the enzyme for 2 h, and then incubated for an additional 15 min in fresh medium that was identical except that it also contained 1 mM 2-[^{3}H]deoxy-D-glucose (0.5 μCi/ml) to measure 2-deoxyglucose uptake. The concentration of both enzymes was 200 m-units/ml of medium. Each value is a mean ± S.E.M. from muscles of six rats.

	2-Deoxyglucose uptake (d.p.m./mg of muscle)		
Control	+ Sphingomyelinase from *Streptomyces*	+ Sphingomyelinase from *Staphylococcus aureus*	Change (%)
483 ± 55	920 ± 99	–	+ 91 ($P < 0.004$)
352 ± 50	–	658 ± 79	+ 87 ($P < 0.004$)

Table 4 Effect of cytochalasin B on sphingomyelinase-stimulated 2-deoxyglucose transport in soleus muscles

The muscles were first preincubated with 0 or 200 m-units of sphingomyelinase/ml, 0 or 0.1 m-unit of insulin/ml and 0 or 70 μM cytochalasin B for 2 h, and then incubated for 15 min in fresh identical medium supplemented with 1 mM 2-[^{3}H]deoxy-D-glucose (0.5 μCi/ml) to measure 2-deoxyglucose uptake. Each value is a mean ± S.E.M. from muscles of six to twelve rats.

	2-Deoxyglucose uptake (d.p.m./mg of muscle)		
Treatment	Control	+ Sphingomyelinase	+ Sphingomyelinase + cytochalasin B
–	510 ± 62	845 ± 47	92 ± 26
Insulin	938 ± 110	1646 ± 74	122 ± 27

measuring the effect of proteinase inhibitors on sphingomyelinase-stimulated 2-deoxyglucose uptake by soleus muscles (Table 2). Sphingomyelinase alone increased muscle 2-deoxyglucose uptake by 53–82% ($P < 0.007$). Addition of leupeptin (50 μg/ml), PMSF (1 mM) or EGTA (1 mM, in the absence of exogenous Ca^{2+} in the medium) did not significantly diminish or abolish the effect of sphingomyelinase. Thus the stimulation of 2-deoxyglucose uptake by sphingomyelinase cannot be readily explained by contamination of the commercial sphingomyelinase with proteinases.

Addition of sphingomyelinase had no effect on the pH of the incubation medium. The stimulatory action of sphingomyelinase on muscle 2-deoxyglucose uptake was abolished by boiling the enzyme solution at 100 °C. Furthermore, when the solution of sphingomyelinase from *Streptomyces* (molecular mass 36–42 kDa) was filtered through Criticon filters with 30 and 10 kDa molecular-mass cut-offs, neither of the filtrates stimulated muscle 2-deoxyglucose uptake above the basal value (results not shown). Finally, sphingomyelinase from *Staphylococcus aureus* (molecular mass 32.5–33 kDa) induced an increase in 2-deoxyglucose uptake by muscle that was almost identical with that caused by sphingomyelinase from *Streptomyces* used in the preceding studies (Table 3). Thus it appears that the stimulation of 2-deoxyglucose uptake is caused by sphingomyelinase *per se*.

To test whether the sphingomyelinase-induced increase in basal and insulin-stimulated glucose transport involves glucose transporters, soleus muscles were first preincubated for 2 h with or without 200 m-units of sphingomyelinase/ml, 0.1 m-unit of insulin/ml and 70 μM cytochalasin B, and 2-deoxyglucose uptake during the subsequent 15 min incubation period was then measured. As shown in Table 4, sphingomyelinase alone stimulated 2-deoxyglucose uptake by 66% ($P < 0.003$) compared with controls. Cytochalasin B inhibited the sphingomyelinase-induced 2-deoxyglucose transport by 89% ($P < 0.001$). The same pattern of changes was observed when the media contained insulin (Table 4). Sphingomyelinase increased the insulin-stimulated 2-deoxyglucose transport by an additional 76% ($P < 0.002$), and the uptake stimulated by the combined action of insulin and sphingomyelinase was inhibited by 93% ($P < 0.001$) by cytochalasin B. These results suggest that sphingomyelinase-induced 2-deoxyglucose uptake involves glucose transporters.

Table 5 Effect of sphingomyelinase on cellular accumulation of 2-deoxy-D-glucose or L-glucose

The muscles were first preincubated with 0 or 200 m-units of sphingomyelinase/ml for 2 h, and then incubated for 15 min in identical fresh medium supplemented with either 1 mM 2-[^{3}H]deoxy-D-glucose (0.5 μCi/ml) or 1 mM L-[^{3}H]glucose (0.5 μCi/ml) to assess cellular accumulation of the respective sugars. Each value is a mean ± S.E.M. from muscles of six rats. NS, Not statistically significant.

	Cellular uptake (d.p.m./mg of muscle)		
Sugar	Control	+ Sphingomyelinase	Change (%)
2-Deoxy-D-glucose	442 ± 27	707 ± 65	+ 60 ($P < 0.03$)
L-glucose	20 ± 24	20 ± 42	NS

To eliminate the possibility that sphingomyelinase increases the cellular accumulation of 2-deoxyglucose by damaging the integrity of the cell membrane, the effects of sphingomyelinase on the cellular accumulation of 2-deoxy-D-glucose and L-glucose were compared (Table 5). 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 this experiment, soleus muscles were first preincubated for 2 h with or without 200 m-units of sphingomyelinase/ml and then incubated for 15 min in fresh medium that was identical except that it also contained either 2-deoxy-D-glucose or L-glucose. As shown in Table 5, sphingomyelinase increased the cellular accumulation of 2-deoxy-D-glucose but had no effect on the cellular accumulation of L-glucose. Since L-glucose is not transported by glucose transporters, the results (a) demonstrate that sphingomyelinase does not make the cell membrane leaky and (b) provide supporting evidence for the conclusion that sphingomyelinase-stimulated 2-deoxyglucose transport involves glucose transporters.

Effect of degradation products of sphingomyelin

Since sphingomyelinase degrades sphingomyelin to phosphorylcholine and ceramide, the ability of these substances to mimic the action of sphingomyelinase on basal and insulin-stimulated transport of 2-deoxyglucose was investigated. Phosphorylcholine (50 μM) had little, if any, effect on the measured parameters (results not shown). Similarly, 50 μM concentrations of cell-permeable C2-ceramide or C6-ceramide had no statistically significant effect on basal or insulin-stimulated 2-deoxyglucose uptake by muscles (results not shown). Attempts to use ceramide with a long acyl side chain were unsuccessful because addition of ceramide to the albumin-containing incubation medium resulted in precipitation.

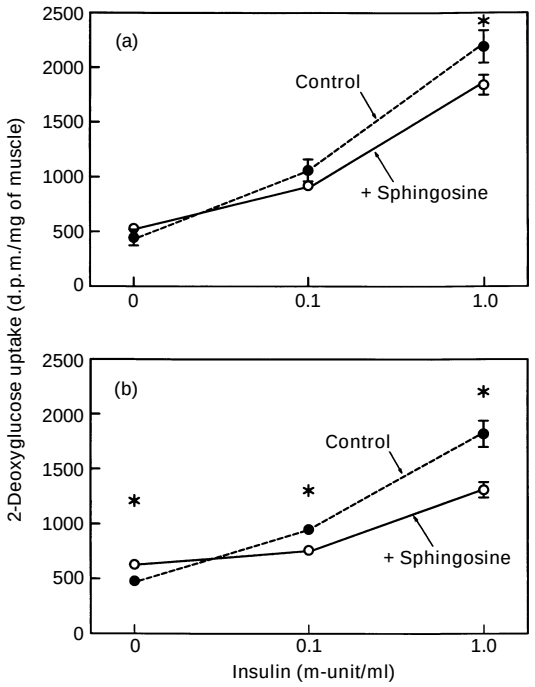

Figure 2 Effect of D-sphingosine on basal and insulin-induced transport of 2-deoxyglucose in soleus muscles

The muscles were first preincubated with 0, 0.1 or 1.0 m-unit of insulin/ml and 0 or 50 μM D-sphingosine for either 1 h (**a**) or 2 h (**b**), and then incubated for 15 min in identical fresh medium supplemented with 1 mM 2-[^{3}H]deoxy-D-glucose (0.5 μCi/ml) to assess 2-deoxyglucose uptake. Each value is a mean $\pm$ S.E.M. from muscles of six rats. S.E.M. bars are not indicated if they were smaller than the size of the symbols. *Statistically significant difference ($P < 0.05$) from control muscles studied at the same insulin concentration.

As an increased level of endogenous ceramides in sphingomyelinase-treated muscles would be expected to result in an elevated tissue sphingosine level [36], the present study evaluated whether sphingosine can mimic the action of sphingomyelinase on glucose transport in skeletal muscle. We have previously demonstrated that 50 μM sphingosine does not increase the release of the cytosolic enzyme lactate dehydrogenase by muscle, indicating that a 50 μM concentration of this lipid does not affect cell viability or impair cell membrane integrity [37]. As shown in Figure 2(a), preincubation of muscles with sphingosine for 1 h had no statistically significant effect on either the basal 2-deoxyglucose uptake or that in the presence of a physiological concentration of insulin (0.1 m-unit/ml). However, it produced a 16 % ($P < 0.05$) decrease in 2-deoxyglucose uptake at a supraphysiological level of insulin (1.0 m-unit/ml). When the muscles were preincubated with sphingosine for 2 h (Figure 2b), a 32 % ($P < 0.002$) increase in basal 2-deoxyglucose uptake was observed, and the response to 0.1 and 1.0 m-unit of insulin/ml decreased 19 and 27 % ($P < 0.004$) respectively. These findings are in agreement with stimulation of basal 2-deoxyglucose uptake and inhibition of insulin-stimulated uptake in sphingosine-treated 3T3 L1 fibroblasts [38] and adipocytes [39]. It should be noted, that this pattern of action differs from that of sphingomyelinase which stimulates both basal and insulin-induced 2-deoxyglucose uptake (Figure 1). Thus, it can be concluded that sphingosine does not mediate the sphingomyelinase-induced augmentation of glucose transport in muscle.

Table 6 Effect of lack of extracellular Ca^{2+} (A) and of inhibition of Ca^{2+} release from the sarcoplasmic reticulum with dantrolene (B) on sphingomyelinase-induced increase in 2-deoxyglucose uptake by soleus muscles

The muscles were first preincubated with 0 or 200 m-units of sphingomyelinase/ml and indicated omissions or additions for 2 h, and then incubated for 15 min in identical fresh medium supplemented with 1 mM 2-[^{3}H]deoxy-D-glucose (0.5 μCi/ml) to measure cellular accumulation of 2-deoxyglucose. Each value is a mean $\pm$ S.E.M. from muscles of five to six rats.

		2-Deoxyglucose uptake (d.p.m./mg of muscle)		
	Treatment	Control	+ Sphingomyelinase	Change (%)
(A)	–	436 $\pm$ 50	704 $\pm$ 78	+61 ($P < 0.02$)
	No $CaCl_2$	593 $\pm$ 30	910 $\pm$ 68	+53 ($P < 0.003$)
	No $CaCl_2$ +1 mM EGTA	857 $\pm$ 76	1418 $\pm$ 100	+65 ($P < 0.001$)
(B)	–	576 $\pm$ 58	1068 $\pm$ 41	+85 ($P < 0.001$)
	15 μM dantrolene	478 $\pm$ 69	919 $\pm$ 108	+92 ($P < 0.001$)
	25 μM dantrolene	333 $\pm$ 27	686 $\pm$ 56	+106 ($P < 0.001$)

Role of Ca^{2+}

The skeletal muscle appears to have two distinct pathways for stimulation of glucose transport: one is activated by insulin, and the other by muscle contractions or hypoxia. Available evidence suggests that the stimulatory actions of exercise and hypoxia are mediated by an increase in the cytoplasmic Ca^{2+} level [20–23]. Therefore we tested whether Ca^{2+} plays a role in the sphingomyelinase-induced augmentation of basal and insulin-stimulated 2-deoxyglucose uptake. Table 6(A) shows the results of lack of Ca^{2+} in the incubation medium. Omitting $CaCl_2$ from the medium increased control 2-deoxyglucose uptake by 36 % ($P < 0.007$), and inclusion of the Ca^{2+} chelator EGTA caused an additional 45 % ($P < 0.007$) augmentation of control uptake. These changes did not influence the effect of sphingomyelinase as evidenced by comparable relative increases in muscle 2-deoxyglucose uptake over respective controls.

To examine the role of Ca^{2+} further, the effect of sphingomyelinase was tested in muscles incubated in the presence of dantrolene, an inhibitor of Ca^{2+} release from the sarcoplasmic reticulum [40]. In agreement with the report by Sowell et al. [17], dantrolene decreased control 2-deoxyglucose uptake by muscles (Table 6B). Although this effect was not statistically significant with 15 μM dantrolene, control uptake was decreased by 42 % ($P < 0.004$) when the muscles were incubated with 25μM dantrolene. The latter concentration of dantrolene also decreased sphingomyelinase-induced 2-deoxyglucose uptake by 36 % ($P < 0.004$) but in relation to the respective controls the sphingomyelinase-induced augmentation of 2-deoxyglucose uptake remained unchanged. These results and those obtained in the absence of Ca^{2+} demonstrate that Ca^{2+} is not a mediator of sphingomyelinase-induced augmentation of 2-deoxyglucose uptake in muscle.

Insulin receptor tyrosine kinase

To investigate whether sphingomyelinase increases 2-deoxyglucose uptake by stimulating the insulin receptor, the tyrosine kinase activity of this receptor was measured in soleus muscles exposed to 0 or 200 m-units of sphingomyelinase/ml of medium for 15, 30, 60 and 120 min. As shown in Table 7(A), it did not differ from timed controls at any of the intervals. As

Table 7 Tyrosine kinase activity of the insulin receptor in control and sphingomyelinase-treated soleus muscles

(A) The muscles were incubated with 0 or 200 m-units of sphingomyelinase/ml for 15–120 min. (B) The muscles were first preincubated with 0 or 200 m-units of sphingomyelinase/ml for 90 min, and then incubated in identical fresh medium supplemented with 0 or 1 m-unit of insulin/ml for an additional 30 min. Each value is a mean ± S.E.M. from muscles of three to four rats. In no case was the change statistically significant.

		Tyrosine kinase activity (d.p.m.)	
	Treatment	Control	+ Sphingomyelinase
(A)	15 min	243 ± 6	264 ± 1
	30 min	241 ± 3	236 ± 10
	60 min	236 ± 5	244 ± 18
	120 min	254 ± 22	232 ± 1
(B)	–	322 ± 23	350 ± 8
	Insulin	722 ± 87	766 ± 38

Table 8 Phosphatidylinositol 3-kinase activity in control and sphingomyelinase-treated soleus muscles

(A) The muscles were incubated with 0 or 200 m-units of sphingomyelinase/ml for 5–120 min; 2.5 mg of protein from the supernatant of each muscle homogenate was assayed. Each value is a mean ± S.E.M. from muscles of three rats. (B) Soleus muscles were first preincubated with 0 or 200 m-units of sphingomyelinase/ml for 110 min, and then incubated in identical fresh medium supplemented with 0 or 1 m-unit of insulin/ml for an additional 5 min. A portion (3.4 mg of protein) from the supernatant of each muscle homogenate was assayed. Each value is a mean ± S.E.M. from muscles of four to six rats. In no case was the change statistically significant.

		Phosphatidylinositol 3-kinase activity (d.p.m.)	
	Treatment	Control	+ Sphingomyelinase
(A)	5 min	312 ± 41	256 ± 7
	30 min	301 ± 24	283 ± 1
	60 min	298 ± 52	274 ± 6
	120 min	281 ± 46	276 ± 15
(B)	–	121 ± 18	–
	Insulin	253 ± 26	265 ± 16

sphingomyelinase increases not only basal but also insulin-stimulated 2-deoxyglucose uptake, the effect of sphingomyelinase on insulin-stimulated tyrosine kinase activity of the insulin receptor was also evaluated. The muscles were first preincubated with 0 or 200 m-units of sphingomyelinase/ml for 90 min, and then incubated in identical fresh medium supplemented with 0 or 1 m-unit of insulin/ml for an additional 30 min. As shown in Table 7(B), insulin increased the tyrosine kinase activity by 124% ($P < 0.003$), but treatment of muscles with sphingomyelinase had no additional effect on either the basal or insulin-stimulated activity. These results suggest that sphingomyelinase does not augment basal and insulin-induced 2-deoxyglucose uptake by stimulating the insulin receptor.

Phosphatidylinositol 3-kinase

Available evidence suggests that phosphatidylinositol 3-kinase is part of the signal-transduction pathway that effects the stimulation of glucose uptake by insulin [24–26]. Activation of this enzyme involves the following steps. First, binding of insulin to its receptor and activation of receptor tyrosine kinase causes phosphorylation of IRS-1. Then the regulatory subunit of phosphatidylinositol 3-kinase (p85) binds to a phosphorylated src homology-binding domain on IRS-1, and this, in turn, increases the activity of the catalytic subunit (p110) of phosphatidylinositol 3-kinase [27]. To investigate whether sphingomyelinase increases 2-deoxyglucose uptake by stimulating any of the above steps, phosphatidylinositol 3-kinase activity in muscles treated with sphingomyelinase was examined. Table 8(A) shows that incubation of soleus muscles with sphingomyelinase for 5, 30, 60 and 120 min had no effect on phosphatidylinositol 3-kinase activity compared with timed control muscles. This agrees with the results of another experiment in which muscles were incubated for 2 h with either 0.1 m-unit of insulin/ml or 200 m-units of sphingomyelinase/ml or without additions (controls). The activity of phosphatidylinositol 3-kinase in the insulin-treated muscles was 83% ($P < 0.05$) higher than that of controls, but the activity in sphingomyelinase-treated muscles did not differ from that in controls (results not shown). As sphingomyelinase augments not only basal 2-deoxyglucose uptake but also insulin-stimulated uptake, the effect of preincubation of muscles with sphingomyelinase on the subsequent stimulation of phosphatidylinositol 3-kinase activity with insulin was evaluated. Soleus muscles were preincubated with or without 200 m-units of sphingomyelinase/ml for 110 min and then incubated in identical fresh medium supplemented with 1 m-unit of insulin/ml for an additional 5 min [31,41]. As shown in Table 8(B), insulin caused a 109% ($P < 0.02$) increase in phosphatidylinositol 3-kinase activity in muscles preincubated without sphingomyelinase. In muscles preincubated with sphingomyelinase, insulin elicited a 119% ($P < 0.02$) increase in kinase activity. However, the effect of insulin in these two groups of muscles was not statistically different. These data demonstrate that sphingomyelinase stimulates basal and insulin-induced 2-deoxyglucose uptake by a mechanism distal from phosphatidylinostiol 3-kinase. Since IRS-1 is also a substrate for insulin-like growth factor I receptor [42], the data suggest that sphingomyelinase does not act by stimulating this receptor.

Table 9 Effect of wortmannin on sphingomyelinase-induced increase in 2-deoxyglucose uptake by soleus muscle

The muscles were first preincubated with 0 or 200 m-units of sphingomyelinase/ml and with 0, 1 or 5 μM wortmannin for 2 h, and then incubated for 15 min in identical fresh medium supplemented with 1 mM 2-[^{3}H]deoxyglucose (0.5 μCi/ml) to measure 2-deoxyglucose uptake. Each value is a mean ± S.E.M. from muscles of six rats.

	2-Deoxyglucose uptake (d.p.m./mg of muscle)		
Treatment	Control	+ Sphingomyelinase	Change (%)
–	414 ± 25	819 ± 48	+98 ($P < 0.001$)
1 μM wortmannin	376 ± 43	750 ± 66	+99 ($P < 0.001$)
5 μM wortmannin	393 ± 9	865 ± 60	+120 ($P < 0.001$)

To verify that sphingomyelinase acts at a site(s) distal from phosphatidylinositol 3-kinase, we investigated whether the sphingomyelinase-induced augmentation of 2-deoxyglucose uptake can be prevented by wortmannin, an inhibitor of phosphatidylinositol 3-kinase. Wortmannin has been shown to inhibit the increase in muscle glucose uptake caused by insulin [25,26] but not that induced by hypoxia or contractions [26]. This selectivity makes it a useful tool for verifying whether the early steps of the 'insulin pathway' are involved in the sphingomyelinase-induced increase in 2-deoxyglucose uptake by muscle. Table 9 demonstrates that neither 1 nor 5 μM

wortmannin inhibited the sphingomyelinase-induced augmentation of 2-deoxyglucose uptake by soleus muscles. As we are able to inhibit the insulin-stimulated 2-deoxyglucose uptake under identical experimental conditions [25], we conclude that sphingomyelinase does not stimulate the insulin receptor or the initial steps of the insulin signalling pathway and that sphingomyelinase increases muscle 2-deoxyglucose uptake at a step(s) distal from phosphatidylinositol 3-kinase.

In summary, the present study demonstrates that exogenous sphingomyelinase stimulates both basal and insulin-induced 2-deoxyglucose uptake by skeletal muscle. This action does not appear to be mediated by the degradation products of sphingomyelin, the 'Ca^{2+} pathway' or the initial steps in the insulin signal-transduction pathway. The data suggest that sphingomyelinase exerts its action at a step distal from phosphatidylinositol 3-kinase which ultimately results in increased glucose transport by glucose transporters.

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