

N-Acetylsphingosine stimulates phosphatidylglycerolphosphate synthase activity in H9c2 cardiac cells

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Cardiolipin and phosphatidylglycerol biosynthesis were examined in H9c2 cells incubated with short-chain ceramides. Incubation of cells with *N*-acetylsphingosine or *N*-hexanoylsphingosine stimulated [$1,3\text{-}^3\text{H}$]glycerol incorporation into phosphatidylglycerol and cardiolipin, with *N*-acetylsphingosine having the greater effect. The mechanism for the ceramide-mediated stimulation of *de novo* phosphatidylglycerol and cardiolipin biosynthesis appeared to be an increase in the activity of phosphatidylglycerolphosphate synthase, the committed step of phosphatidylglycerol and cardiolipin biosynthesis. The presence of the potent protein phosphatase inhibitors calyculin A or okadaic acid attenuated the *N*-acetylsphingosine-mediated stimulation of phosphatidylglycerolphosphate synthase activity and of phosphatidylglycerol and cardiolipin biosynthesis, indicating the involvement of a ceramide-activated protein phosphatase(s). The presence of 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) stimulated enzyme activity and [$1,3\text{-}^3\text{H}$]glycerol incorporation into phosphatidylglycerol and cardiolipin. The effects of CPT-cAMP and *N*-acetylsphingosine on phosphatidylglycerol

and cardiolipin biosynthesis and on phosphatidylglycerolphosphate synthase activity were additive. Phosphatidylglycerol biosynthesis from *sn*-[^{14}C]glycerol 3-phosphate in permeabilized H9c2 cells was stimulated by preincubation with *N*-acetylsphingosine, and this was attenuated by okadaic acid. *N*-Acetylsphingosine treatment of cells elevated mitochondrial phospholipase A_2 activity. Since the pool sizes of phosphatidylglycerol and cardiolipin were unaltered in these cells, the observed increase in phosphatidylglycerolphosphate synthase activity may be a compensatory mechanism for the *N*-acetylsphingosine-mediated elevation of mitochondrial phospholipase A_2 activity. Finally, addition of tumour necrosis factor α to H9c2 cells resulted in an elevation of both phosphatidylglycerolphosphate synthase and phospholipase A_2 activities. The results suggest that phosphatidylglycerol and cardiolipin metabolism in H9c2 cells may be regulated by intracellular ceramide signalling.

Key words: ceramide-activated protein phosphatase, ceramide signalling, phosphatidylglycerol biosynthesis, phospholipase A_2 .

INTRODUCTION

Cardiolipin (CL) and phosphatidylglycerol (PG) are important structural and functional phospholipids in eukaryotic and prokaryotic cells (for reviews, see [1–5]). PG is synthesized via the Kennedy [or CDP-1,2-diacyl-*sn*-glycerol (CDP-DG)] pathway in mammalian tissues [6]. In this pathway, phosphatidic acid (PA) is charged to CDP-DG in the presence of CTP in a reaction catalysed by PA:CTP cytidylyltransferase (EC 2.7.7.41). In the second or committed step of the pathway, CDP-DG in the presence of *sn*-glycerol 3-phosphate is converted into phosphatidylglycerolphosphate (PGP) in a reaction catalysed by PGP synthase (EC 2.7.8.5). In the third step of the pathway, the PGP formed is rapidly converted into PG in a reaction catalysed by PGP phosphatase (EC 3.1.3.27). In most tissues examined, this enzymic activity is high compared with that of PGP synthase, and this is why PGP does not accumulate in mammalian tissues. Finally, PG condenses with another molecule of CDP-DG to form CL in a reaction catalysed by CL synthase [7].

In the past few years, ceramides have been identified as important metabolites that are produced from the hydrolysis of sphingomyelin (SM) in many different cells (for reviews, see [8,9]). For example, tumour necrosis factor α (TNF α), vitamin D $_3$ and γ -interferon all promote the hydrolysis of SM to ceramide

in HL-60 human leukaemia cells [10–12]. More recently, ceramides generated from this sphingomyelin cycle of signal transduction have been shown to be involved in the regulation of cellular growth, differentiation and apoptosis (for reviews, see [8,13,14]). Short-chain cell-permeant ceramides [*N*-acetylsphingosine (C $_2$ -Cer) and *N*-hexanoylsphingosine (C $_6$ -Cer)] have been shown to stimulate both protein phosphatase and protein kinase activities [15,16]. CL is important for proper functioning of the mitochondrial respiratory chain in Chinese hamster ovary cells [17], and alterations in CL during dexamethasone-induced apoptosis in thymocytes is well documented [18–20].

Since the mitochondrial respiratory chain and apoptosis are both modulated by ceramides [21,22], it is possible that CL metabolism may be regulated by ceramide signalling. In the present study, we took advantage of the use of short-chain cell-permeant ceramides to examine whether PG and CL biosynthesis is regulated by short-chain ceramides and ceramide signalling. Our surprising results demonstrate that addition of C $_2$ -Cer to H9c2 cells dramatically stimulates PG and CL biosynthesis and that the mechanism appears to be an increase in mitochondrial PGP synthase activity. We show that the signalling action of C $_2$ -Cer may be mediated through a ceramide-activated protein phosphatase(s) which is inhibited by okadaic acid and calyculin A. We further show that the pool sizes of PG and CL are

Abbreviations used: C $_2$ -Cer, *N*-acetylsphingosine; C $_6$ -Cer, *N*-hexanoylsphingosine; C $_2$ -dihydroceramide, *N*-acetyldihydroceramide; CL, cardiolipin; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; DG, 1,2-diacyl-*sn*-glycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PI, phosphatidylinositol; PKA, cAMP-dependent protein kinase; PLA $_2$, phospholipase A $_2$; PS, phosphatidylserine; SM, sphingomyelin; TNF α , tumour necrosis factor α .

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unaltered in these cells and that C₂-Cer stimulates mitochondrial phospholipase A₂ (PLA₂) activity in H9c2 cells.

A preliminary report of this study was previously published in abstract form [23].

EXPERIMENTAL

Materials

[1,3-³H]Glycerol, [U-¹⁴C]glycerol 3-phosphate, [2,8-³H]adenine and [*methyl*-³H]thymidine were obtained from Amersham Canada Limited (Oakville, Ontario, Canada) or DuPont Canada Inc. (Mississauga, Ontario, Canada). [¹⁴C]PG and [¹⁴C]PGP were prepared using the procedure of MacDonald and McMurray [24]. C₂-Cer, C₆-Cer, *N*-acetyldihydroceramide (C₂-dihydroceramide), sphingosine, SMase, 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), TNF α and all lipid standards were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Calyculin A and okadaic acid were from LC Laboratories (Woburn, MA, U.S.A.). Cell culture media and reagents were products of Canadian Life Technologies Inc. (Gibco) (Burlington, Ontario, Canada). Thin-layer chromatographic plates (silica gel G) were from Fisher Scientific (Edmonton, Alberta, Canada). All other biochemicals were of analytical grade and were obtained from Sigma Chemical Co.

Rat myoblastic H9c2 cells were obtained from the American Type Culture Collection. They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated newborn calf serum, 100 units/ml penicillin G, 10 g/ml streptomycin and 0.25 g/ml amphotericin B. Cell cultures were maintained at 37 °C, saturated with humidified air/5% CO₂. Each dish of cells was subcultured at a 1:5 ratio and confluency was usually obtained after 4 days of incubation. In all experiments, cells at confluence were made quiescent by incubation in a serum-free medium for 12 h (overnight) prior to addition of chemicals or labelling. All cell incubation procedures were performed at 37 °C. Cell viability was assessed by Trypan Blue exclusion and protein concentration. Treatment of cells with okadaic acid, calyculin A or CPT-cAMP for up to 2 h, or with C₂-Cer, C₆-Cer, sphingosine, SMase or TNF α for up to 4 h, did not affect Trypan Blue exclusion or protein concentration when compared with controls.

Treatment of cells with C₂-Cer, C₆-Cer, sphingosine, SMase, okadaic acid, calyculin A or TNF α , and cell labelling studies

C₂-Cer, C₆-Cer and sphingosine were dissolved in ethanol to obtain stock solutions (100 \times). SMase was dissolved in PBS to obtain a stock of 25 units/ml. Okadaic acid and calyculin A were dissolved in DMSO. CPT-cAMP was dissolved in Dulbecco's modified Eagle's medium, and TNF α was dissolved in PBS. Aliquots of the stock solution were added to quiescent cells maintained in serum-free Dulbecco's modified Eagle's medium. The final concentration of ethanol in the medium was 1% (v/v) or less, and that of DMSO was 0.25% (v/v) or less. H9c2 cells in 60 mm-diam. Petri dishes were incubated with 1 ml of medium containing 0.1 μ M okadaic acid, 0.1 μ M calyculin A or 0.5 mM CPT-cAMP for 2 h. The medium was removed and the cells were washed twice with 3 ml of fresh medium. The cells were then incubated with 2 ml of medium containing [1,3-³H]glycerol (5 μ Ci/dish) in the absence or presence of 30 μ M C₂-Cer for 4 h. In other experiments, H9c2 cells were incubated with 2 ml of medium containing [1,3-³H]glycerol (5 μ Ci/dish) in the absence or presence of 30 μ M C₂-Cer, 30 μ M C₆-Cer, 30 μ M sphingosine or 0.1 unit/ml SMase for 4 h. In some experiments, H9c2 cells

were incubated with 2 ml of medium in the absence or presence of various concentrations of C₂-Cer or 30 nM TNF α for up to 4 h. H9c2 cells were also incubated for 4 h with [³²P]P_i (10 μ Ci/dish) in the absence or presence of various concentrations of C₂-Cer. In other experiments, H9c2 cells were incubated in the absence or presence of 30 μ M C₂-Cer for 4 h and subsequently incubated with [2,8-³H]adenine or [³H]thymidine for 2 h. In some experiments, H9c2 cells were incubated for 12 h with 2 ml of medium containing [1,3-³H]glycerol (5 μ Ci/dish). The medium was removed and cells were then incubated with 2 ml of medium in the absence or presence of 30 μ M C₂-Cer for 1 h.

For cell permeabilization experiments, H9c2 cells were pre-incubated in the absence or presence of 30 μ M C₂-Cer for 4 h as described above. The cells were then permeabilized with saponin and incubated for up to 60 min with 0.1 mM [¹⁴C]glycerol 3-phosphate (0.5 μ Ci/dish) and 0.5 mM CDP-DG exactly as described in [25]. The radioactivity incorporated into PG was then determined as described [25]. In some experiments, H9c2 cells were incubated in the presence of 0.5 mM CPT-cAMP or 0.1 μ M okadaic acid for 1 h, followed by incubation with 30 μ M C₂-Cer for 4 h, and then were permeabilized with saponin and incubated as described above.

Harvesting of H9c2 cells, extraction and analysis of lipid

Cells were washed with 3 ml of ice-cold PBS. Then 2 ml of methanol/water (1:1, v/v) was added to the dish and the cells were harvested with a rubber policeman into screw-cap tubes. The suspension was vortexed twice and a 25 μ l aliquot was taken for the determination of protein by the method of Lowry et al. [26]. Lipids were extracted by the method of Folch et al. [27]. The organic phase was dried under nitrogen and resuspended in 100 μ l of chloroform/methanol (2:1, v/v). An aliquot of the lipid suspension was placed on to a TLC plate and phospholipids were separated in two dimensions as described [28]. The TLC plates were prepared by spraying them lightly with 0.4 M boric acid, blotting dry the excess and then air-drying the plates overnight. PG and CL standards were placed on the plate before chromatography, except when lipid phosphorus analysis was performed. The lipids were visualized with iodine vapour, removed and placed into scintillation vials. A 5 ml portion of Ecolite⁺ scintillant was added and the radioactivity was determined after a 24 h period. In some experiments the silica gel was removed for the determination of phospholipid phosphorus by the method of Rouser et al. [29].

Preparation of the H9c2 mitochondrial fraction

All isolation procedures were performed at 4 °C. H9c2 cells in 100 mm-diam. Petri dishes were incubated with 3 ml of medium in the absence or presence of 30 μ M C₂-Cer for 4 h. In other experiments, H9c2 cells were incubated with 2 ml of medium containing 0.1 μ M okadaic acid, 0.1 μ M calyculin A or 0.5 mM CPT-cAMP for 2 h, the medium was removed and the cells were washed twice with 5 ml of fresh medium and then incubated with 3 ml of medium in the absence or presence of 30 μ M C₂-Cer for 4 h. The cells were harvested into glass tubes with 4 ml of ice-cold PBS using a rubber policeman. These cells were centrifuged at 1000 *g* for 5 min. The PBS was removed and a 10% (w/v) homogenate was prepared in 0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, 0.145 M NaCl and 1 mM EDTA using 40 strokes of a tight-fitting Dounce A homogenizer. The homogenate was centrifuged at 1000 *g* (Sorvall RC-5 centrifuge with an SS-34 rotor) for 10 min. The resulting supernatant was centrifuged at 10000 *g*

for 15 min. The resulting pellet was resuspended in 1 ml of stabilization buffer [50 mM Tris-maleate, pH 6.5, 10% (v/v) glycerol, 0.1 M KCl, 10 mM MgCl₂ and 0.5% (v/v) Triton X-100] using 15 strokes of a Dounce A homogenizer. The mitochondrial fraction was used for the assay of PA:CTP cytidyltransferase, PGP synthase, PGP phosphatase and CL synthase activities. In other experiments, homogenates, mitochondrial fractions and microsomal fractions were prepared as described for the assay of PLA₂ activity towards [¹⁴C]PG [30]. Marker enzyme analysis indicated that the microsomal fraction was contaminated by approximately 10% with mitochondrial particles.

Assay of enzymes and other procedures

CL synthase was assayed as described by Schlame and Hostetler [31]. PA:CTP cytidyltransferase was assayed by the method of Carman and Kelley [32]. PGP synthase was assayed by the method of Carman and Belunis [33]. PGP phosphatase was assayed by the method of MacDonald and McMurray [24]. DNA and RNA synthesis were determined in cells by addition of 0.3 μM [2,8-³H]adenine (12 μCi/dish) or 0.3 μM [methyl-³H]thymidine (20 μCi/dish) to cells that were incubated for 4 h in the absence or presence of 30 μM C₂-Cer. After labelling, the radioactivity incorporated into DNA and RNA was determined as described [34]. PLA₂ activity towards [¹⁴C]PG was determined as described in [30].

Student's *t*-test was used for the determination of significance; the level of significance was defined as *P* < 0.05.

RESULTS

Short-chain ceramides stimulate PG and CL biosynthesis in H9c2 cells

To determine whether short-chain ceramides modulate PG and CL metabolism, H9c2 cells were incubated for up to 4 h with [1,3-³H]glycerol in the absence or presence of 30 μM C₂-Cer or C₆-Cer, and the incorporation of radioactivity into PG and CL was determined. C₂-Cer and C₆-Cer stimulated the incorporation of radioactivity into PG and CL at all times, and this was maximal at 4 h (results not shown). Incubation of cells with C₆-Cer for 4 h caused an approx. 2-fold increase in the incorporation of radioactivity into CL and PG compared with controls (Table 1). In cells incubated for 4 h with C₂-Cer, incorporation of radioactivity into CL and PG was elevated 3.5-fold and 4.2-fold respectively compared with controls. The presence of these short-

Table 1 Effects of C₂-Cer, C₆-Cer, sphingosine and SMase on the incorporation of [1,3-³H]glycerol into CL, PG, PE, PC and PI/PS in H9c2 cells

H9c2 cells were incubated for 4 h with [1,3-³H]glycerol (5 μCi/dish) in the absence or presence of 30 μM C₂-Cer, 30 μM C₆-Cer, 30 μM sphingosine or 0.1 unit/ml SMase. Radioactivity incorporated into CL, PE, PG, PC and PS/PI was then determined. Results are means ± S.D. of four determinations; **P* < 0.05 compared with control (no addition).

	10 ⁻³ × Radioactivity incorporated (d.p.m./mg of protein)				
	CL	PG	PE	PC	PS/PI
No addition	3.5 ± 0.2	8.1 ± 0.6	6.5 ± 0.4	89 ± 10	5.7 ± 0.5
C ₂ -Cer	12.2 ± 1.0*	33.8 ± 2.8*	7.5 ± 0.8	75 ± 12	7.1 ± 0.8
C ₆ -Cer	6.3 ± 0.5*	16.9 ± 1.3*	7.0 ± 0.6	78 ± 7	6.9 ± 0.9
Sphingosine	3.4 ± 0.3	9.0 ± 0.6	6.6 ± 0.6	80 ± 8	6.6 ± 0.7
SMase	3.0 ± 0.2	7.8 ± 0.7	5.5 ± 0.8	87 ± 7	5.6 ± 0.4

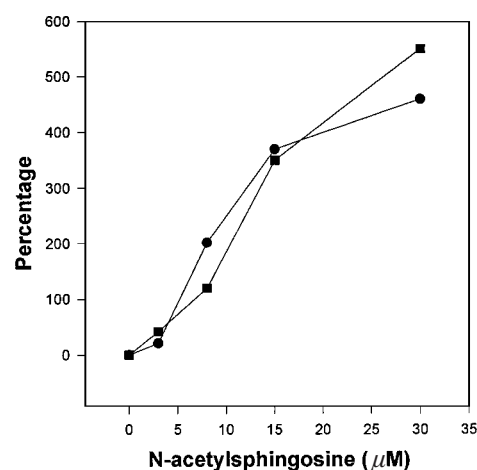


Figure 1 Concentration-dependent effect of C₂-Cer on incorporation of [1,3-³H]glycerol into CL and PG in H9c2 cells

H9c2 cells were incubated with [1,3-³H]glycerol (5 μCi/dish) for 4 h in the presence of 0–30 μM C₂-Cer. Radioactivity incorporated into CL (●) and PG (■) was determined. The control value was assigned as 0%. In controls, radioactivity incorporated into CL and PG was 3500 ± 200 and 8100 ± 600 d.p.m./mg of protein respectively. Results represent means of three determinations.

chain ceramides did not markedly affect the synthesis from glycerol of the other major membrane phospholipids, i.e. phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS)/phosphatidylinositol (PI).

Addition of C₂-dihydroceramide to H9c2 cells did not affect the incorporation of radioactivity into PG or CL (results not shown). H9c2 cells were then incubated for 4 h with [1,3-³H]glycerol in the absence or presence of various concentrations of C₂-Cer, and the incorporation of radioactivity into PG and CL was determined. At all concentrations examined, C₂-Cer stimulated [1,3-³H]glycerol incorporation into these phospholipids (Figure 1). Thus the C₂-Cer-mediated stimulation of CL biosynthesis from [1,3-³H]glycerol was concentration-dependent. Finally, cells were incubated for 4 h with [³²P]P_i in the absence or presence of 30 μM C₂-Cer. The radioactivity incorporated into PG and CL was 1018 ± 35 and 915 ± 109 c.p.m./mg of protein respectively in control cells (means ± S.D. of three determinations). In the presence of C₂-Cer, incorporation of radioactivity into PG and CL was elevated over 2-fold (*P* < 0.05) to 2805 ± 104 and 1931 ± 143 c.p.m./mg of protein respectively. The C₂-Cer-mediated stimulation of CL biosynthesis from [³²P]P_i was also concentration-dependent (results not shown). H9c2 cells were then incubated for 4 h with [1,3-³H]glycerol in the absence or presence of 0.1 unit/ml SMase or 30 μM sphingosine, and the incorporation of radioactivity into PG and CL was determined. The presence of sphingosine or SMase did not affect PG or CL biosynthesis from [1,3-³H]glycerol (Table 1). In cells incubated with SMase, the pool size of SM was decreased by 53%, from 17.2 ± 2.3 to 8.0 ± 0.6 nmol/mg of protein, but the masses of other phospholipids were not affected [35]. Thus treatment of H9c2 cells with short-chain ceramides elevated PG and CL biosynthesis, and C₂-Cer was more potent than C₆-Cer.

C₂-Cer does not affect the CL pool size, synthesis of high-energy nucleotides or DNA synthesis in H9c2 cells

H9c2 cells were incubated for 4 h in the absence or presence of 30 μM C₂-Cer, and the phospholipid phosphorus pool sizes of

Table 2 Effects of C₂-Cer on the pool sizes of individual phospholipids in H9c2 cardiac myoblasts

H9c2 cardiac myoblasts were incubated for 4 h in the absence or presence of 30 μ M C₂-Cer, and the pool sizes of phospholipids were determined. Results are means \pm S.D. of three determinations.

Phospholipid	Pool size (nmol/mg of protein)	
	Control	C ₂ -Cer
CL	7.49 \pm 0.23	7.62 \pm 0.89
PC	81.34 \pm 3.67	80.55 \pm 3.25
PE	51.12 \pm 1.35	51.62 \pm 3.73
PG	6.84 \pm 0.22	6.92 \pm 0.35
PS	8.25 \pm 0.33	8.69 \pm 0.83
PI	6.29 \pm 0.58	6.66 \pm 0.34
SM	23.94 \pm 3.20	24.2 \pm 2.30

PG and CL were determined. The presence of C₂-Cer in the incubation medium did not affect the phospholipid phosphorus pool sizes of CL and PG, or of other phospholipids (Table 2).

We next examined whether DNA and RNA synthesis was affected by C₂-Cer treatment in H9c2 cells. Cells were incubated for 4 h with either [2,8-³H]adenine or [methyl-³H]thymidine in the absence or presence of 30 μ M C₂-Cer, and the incorporation of radioactivity into these nucleotides was determined. The rates of total nucleic acid synthesis from [2,8-³H]adenine and DNA synthesis from [methyl-³H]thymidine were unaltered by treatment of cells with C₂-Cer (Table 3). Since CL biosynthesis in H9c2 cells may be regulated by CTP levels [34], we examined whether C₂-Cer treatment of H9c2 cells affected levels of high-energy nucleotides. The pool sizes of ATP, CTP, GTP and UTP in H9c2 cells were 3666, 333, 517 and 708 μ M respectively, and these were unaltered when cells were incubated with C₂-Cer (means of two determinations). Thus alterations in DNA and RNA synthesis or in levels of high-energy nucleotides were not responsible for the elevated *de novo* synthesis of PG and CL.

C₂-Cer stimulates mitochondrial PLA₂ activity in H9c2 cells

Since C₂-Cer treatment of H9c2 cells increased [1,3-³H]glycerol incorporation into PG and CL, but did not affect the pool sizes of these lipids, we examined the possibility that mitochondrial phospholipid turnover was elevated in these cells. Mitochondrial and microsomal fractions were prepared from homogenates of control and C₂-Cer-treated cells. The fractions were incubated with [¹⁴C]PG, and the formation of lyso[¹⁴C]PG was determined.

Table 3 DNA and RNA synthesis in H9c2 cardiac myoblasts treated with C₂-Cer

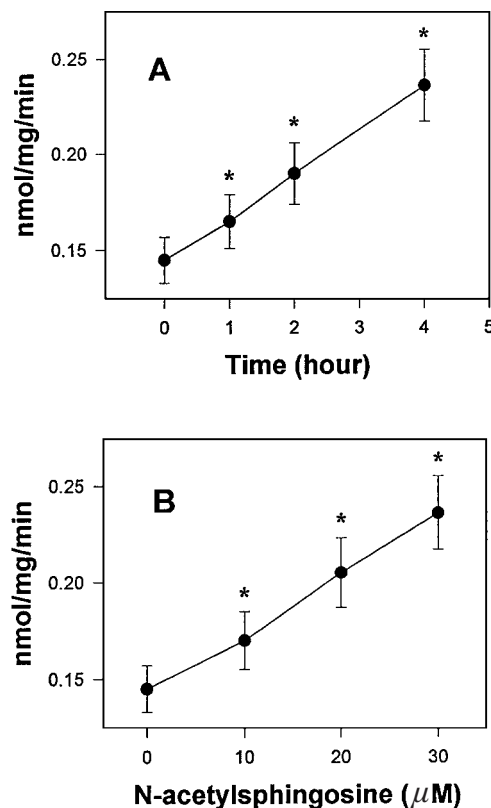
H9c2 cardiac myoblasts were incubated for 4 h in the absence or presence of 30 μ M C₂-Cer, and subsequently incubated with [2,8-³H]adenine or [³H]thymidine for 2 h, and the radioactivity incorporated into DNA and RNA was determined. Results are means of two determinations.

Cells	Radioactivity incorporated (d.p.m./mg of protein)		
	[2,8- ³ H]Adenine		[³ H]Thymidine
	DNA	RNA	DNA
Control	36 794	181 206	200 484
C ₂ -Cer-treated	33 455	165 506	191 089

Table 4 Effect of C₂-Cer on PLA₂ activity in H9c2 cells

H9c2 cells were incubated for 4 h in the absence or presence of 30 μ M C₂-Cer. The cells were harvested and homogenized, and then mitochondrial and microsomal fractions were prepared from the homogenates. PLA₂ activity directed towards [¹⁴C]PG was determined in mitochondrial and microsomal fractions. Results are means \pm S.D. of four determinations; **P* < 0.05 compared with control.

	Formation of lyso[¹⁴ C]PG (nmol/min per mg of protein)	
	Control	C ₂ -Cer
Mitochondrial fraction	0.15 \pm 0.01	0.24 \pm 0.02*
Microsomal fraction	0.18 \pm 0.02	0.21 \pm 0.03

**Figure 2** Time- and concentration-dependent effects of C₂-Cer on mitochondrial PLA₂ activity in H9c2 cells

(A) H9c2 cells were incubated for up to 4 h in the presence of 30 μ M C₂-Cer, and PLA₂ activity was determined. (B) H9c2 cells were incubated for 4 h in the absence or presence of various concentrations of C₂-Cer (μ M), and PLA₂ activity was determined. Values are means \pm S.D. of six determinations; **P* < 0.05 compared with control.

As seen in Table 4, there was a 60% increase (*P* < 0.05) in lysoPG formation in the mitochondrial fraction of C₂-Cer-treated cells compared with controls. In addition, there was a slight (but not significant) increase in the formation of lysoPG in the microsomal fraction of C₂-Cer-treated cells. We attribute this to contamination of the mitochondria with microsomal particles. The C₂-Cer-mediated increase in mitochondrial PLA₂ activity in H9c2 cells was time- and concentration-dependent (Figure 2).

Table 5 Effects of C₂-Cer on the activities of enzymes involved in CL biosynthesis in mitochondrial fractions prepared from H9c2 cells

H9c2 cells were incubated for 4 h in the absence or presence of 30 μ M C₂-Cer. The cells were harvested, mitochondria were isolated and enzyme activities were determined. Results represent means of two determinations.

	Enzyme activity (pmol/min per mg of protein)	
	Control	+ C ₂ -Cer
PA:CTP cytidylyltransferase	2.5	2.6
PGP phosphatase	250	262
CL synthase	4.3	3.9

Table 6 Effects of C₂-Cer, calyculin A, okadaic acid and CPT-cAMP on mitochondrial PGP synthase activity

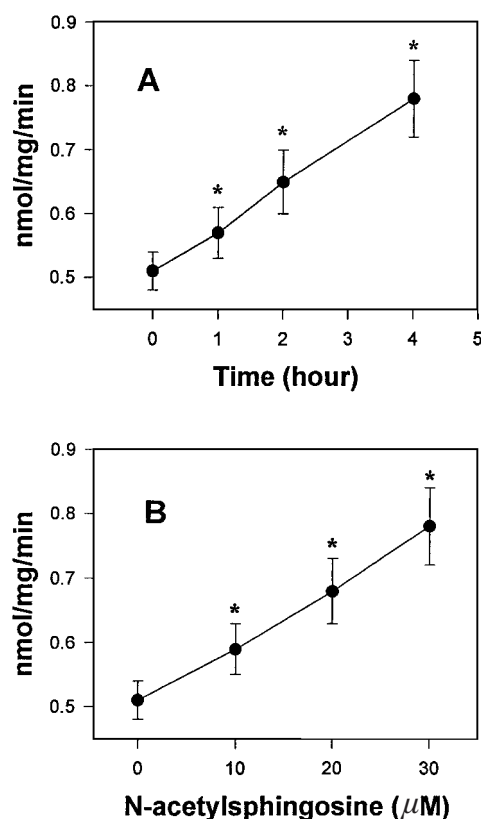
H9c2 cells were preincubated for 2 h in the absence or presence of 0.1 μ M calyculin A, 0.1 μ M okadaic acid or 0.5 mM CPT-cAMP and subsequently incubated in the absence or presence of 30 μ M C₂-Cer for 4 h. The mitochondrial fraction was prepared as described in the Experimental section and PGP synthase activity was determined. Results are means \pm S.D. of four determinations; **P* < 0.05 compared with control; †*P* < 0.05 compared with C₂-Cer alone.

Treatment	PGP synthase activity (nmol/min per mg of protein)
No addition	0.51 \pm 0.03
+ C ₂ -Cer	0.78 \pm 0.02*
+ C ₂ -Cer + okadaic acid	0.56 \pm 0.02†
+ C ₂ -Cer + calyculin A	0.54 \pm 0.03†
+ CPT-cAMP	0.65 \pm 0.04*
+ C ₂ -Cer + CPT-cAMP	0.92 \pm 0.04†

Next, H9c2 cells were incubated with [1,3-³H]glycerol for 12 h and then incubated in the absence or presence of 30 μ M C₂-Cer for 1 h, and the radioactivity in PG was determined. The radioactivity incorporated into PG was 7348 d.p.m./mg of protein (mean of two determinations) prior to incubation with C₂-Cer, and this was decreased to 6835 d.p.m./mg of protein by a 1 h incubation with C₂-Cer. Thus addition of C₂-Cer to H9c2 cells stimulated mitochondrial PLA₂ activity.

C₂-Cer stimulates PGP synthase activity

We examined the mechanism behind the increase in [1,3-³H]glycerol incorporation into PG and CL in C₂-Cer-treated cells. The activities of the enzymes of the CDP-DG pathway of PG and CL biosynthesis were examined. H9c2 cells were incubated for 4 h in the absence or presence of 30 μ M C₂-Cer and mitochondrial fractions were prepared from homogenates. PA:CTP cytidylyltransferase, PGP synthase, PGP phosphatase and CL synthase were assayed in these mitochondrial fractions. Treatment of cells with C₂-Cer did not affect PA:CTP cytidylyltransferase, CL synthase or PGP phosphatase activities compared with controls (Table 5). In contrast, mitochondrial PGP synthase activity was increased by 53% in C₂-Cer-treated H9c2 cells compared with controls (Table 6). Since PGP synthase activity is also localized to microsomes, the enzyme was also assayed in this subcellular fraction. Microsomal PGP synthase activity was also increased, by 55%, in C₂-Cer-treated H9c2 cells compared with controls;

**Figure 3** Time- and concentration-dependent effects of C₂-Cer on mitochondrial PGP synthase activity in H9c2 cells

(A) H9c2 cells were incubated for up to 4 h in the presence of 30 μ M C₂-Cer, and PGP synthase activity was determined. (B) H9c2 cells were incubated for 4 h in the absence or presence of various concentrations of C₂-Cer (μ M), and PGP synthase activity was determined. Values are means \pm S.D. of four determinations; **P* < 0.05 compared with control.

however, this accounted for only 10% of the total cellular PGP synthase activity (results not shown). The C₂-Cer-mediated increase in mitochondrial PGP synthase activity in H9c2 cells was time- and concentration-dependent (Figure 3). To determine whether C₂-Cer stimulates PGP synthase directly, mitochondrial fractions from control H9c2 cells were prepared and PGP synthase activity was assayed in these fractions in the presence of 0–1000 μ M C₂-Cer. The presence of C₂-Cer did not stimulate PGP synthase activity directly (results not shown). Thus the stimulation of PGP synthase activity by C₂-Cer seems to be indirect.

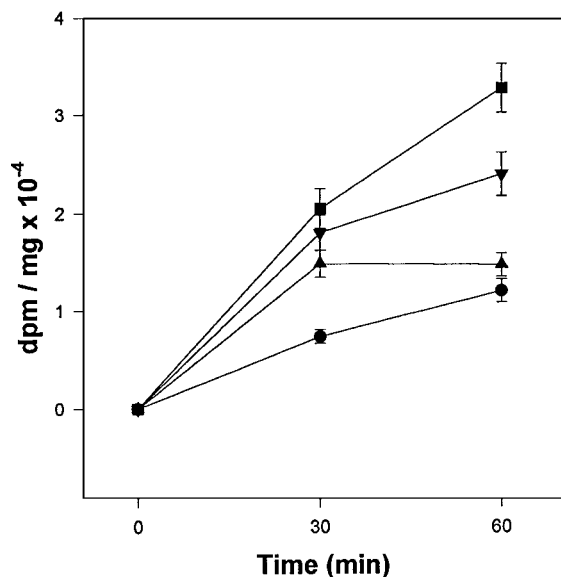
PGP synthase activity in H9c2 cells may be modulated by ceramide-activated protein phosphatase and cAMP-dependent protein kinase (PKA)

Since the stimulation of PGP synthase activity by C₂-Cer seemed to be indirect, we examined whether this effect is mediated through protein kinases or protein phosphatases, since ceramides are known to modulate these activities [15,16]. Cells were incubated in the presence of 30 μ M C₂-Cer in the absence or presence of 0.1 μ M calyculin A or 0.1 μ M okadaic acid. Calyculin A and okadaic acid are potent inhibitors of type 1 and type 2A protein phosphatases. The presence of calyculin A or okadaic acid inhibited the C₂-Cer-mediated stimulation of PGP synthase (Table 6). These data indicate that, in H9c2 cells, PGP

Table 7 Effects of C₂-Cer, calyculin A, okadaic acid and CPT-cAMP on the incorporation of [1,3-³H]glycerol into CL and PG in H9c2 cells

H9c2 cells were preincubated for 2 h in the absence or presence of 0.1 μ M calyculin A, 0.1 μ M okadaic acid or 0.5 mM CPT-cAMP, and subsequently incubated with [1,3-³H]glycerol (5 μ Ci/dish) in the absence or presence of 30 μ M C₂-Cer for 4 h. Radioactivity incorporated into CL and PG was then determined. Results are means \pm S.D. of four determinations; * P < 0.05 compared with control; † P < 0.05 compared with C₂-Cer alone.

Treatment	10 ⁻³ × Radioactivity incorporated (d.p.m./mg of protein)	
	CL	PG
No addition	3.5 \pm 0.2	8.1 \pm 0.6
+ C ₂ -Cer	12.2 \pm 1.0*	33.8 \pm 2.8*
+ C ₂ -Cer + okadaic acid	3.2 \pm 0.2†	8.0 \pm 0.6†
+ C ₂ -Cer + calyculin A	3.4 \pm 0.2†	7.9 \pm 0.6†
+ CPT-cAMP	5.4 \pm 0.4*	16.1 \pm 0.8*
+ C ₂ -Cer + CPT-cAMP	14.6 \pm 1.2†	41.9 \pm 3.6†

**Figure 4** Effects of C₂-Cer, okadaic acid and CPT-cAMP on the incorporation of *sn*-[¹⁴C]glycerol 3-phosphate into PG in permeabilized H9c2 cells

H9c2 cells were preincubated in the absence (●) or presence (▼) of 30 μ M C₂-Cer for 4 h or the presence of 0.5 mM CPT-cAMP (■) for 1 h or the presence of 0.1 μ M okadaic acid for 1 h followed by incubation with 30 μ M C₂-Cer for 4 h (▲). The cells were then permeabilized and incubated for up to 60 min with *sn*-[¹⁴C]glycerol 3-phosphate (0.4 μ Ci/dish) and the radioactivity incorporated into PG was determined. Results are means \pm S.D. of three determinations.

synthase activity may be modulated through a ceramide-activated protein phosphatase(s) which could be inhibited by calyculin A or okadaic acid.

We next examined whether PMA, a potent stimulator of protein kinase C, or CPT-cAMP, a non-hydrolysable cAMP analogue which stimulates PKA, altered PGP synthase activity. Cells were incubated in the absence or presence of 1 μ M PMA or 0.5 mM CPT-cAMP. The presence of CPT-cAMP stimulated PGP synthase activity (Table 6), indicating an apparent stimulation of enzyme activity by the PKA pathway. PMA did not affect enzyme activity (results not shown). Furthermore, the

Table 8 Effects of TNF α on mitochondrial PGP synthase and PLA₂ activities in H9c2 cells

H9c2 cells were incubated with 30 nM TNF α for up to 4 h. The mitochondrial fraction was prepared and mitochondrial PGP synthase and PLA₂ activities were determined. Values are means \pm S.D. of four determinations; * P < 0.05 compared with control (0 h).

Incubation time (h)	Enzyme activity (nmol/min per mg of protein)	
	PGP synthase	PLA ₂
0	0.51 \pm 0.03	0.15 \pm 0.01
1	0.75 \pm 0.06*	0.23 \pm 0.02*
4	0.55 \pm 0.04	0.17 \pm 0.02

stimulatory effect of CPT-cAMP on PGP synthase activity appeared to be additive to that of C₂-Cer (Table 6).

We then examined if okadaic acid or calyculin A could block, or if CPT-cAMP could augment, the C₂-Cer-mediated stimulation of *de novo* PG and CL biosynthesis. H9c2 cells were preincubated for 2 h with 0.1 μ M calyculin A, 0.1 μ M okadaic acid or 0.5 mM CPT-cAMP and subsequently incubated for 4 h with [1,3-³H]glycerol in the absence or presence of 30 μ M C₂-Cer, and the radioactivity incorporated into PG and CL was determined. The presence of C₂-Cer stimulated PG and CL biosynthesis, and this was reversed by both okadaic acid and calyculin A (Table 7). In addition, CPT-cAMP stimulated PG and CL biosynthesis, and this effect was additive to the stimulatory effect of C₂-Cer.

C₂-Cer stimulates PG synthesis in permeabilized H9c2 cells

Previously we demonstrated that PG could be synthesized from *sn*-[¹⁴C]glycerol 3-phosphate and exogenous CDP-DG in permeabilized rat liver hepatocytes [25]. To provide another model to demonstrate that C₂-Cer treatment of H9c2 cells stimulates PGP synthase activity via a ceramide-activated protein phosphatase(s), we preincubated cells in the absence or presence of C₂-Cer for 4 h, and then permeabilized the cells with saponin and incubated them for up to 60 min with [¹⁴C]glycerol 3-phosphate and CDP-DG. We subsequently determined the incorporation of radioactivity into PG. In permeabilized cells, incorporation of radioactivity into PG increased with time (Figure 4). In cells preincubated with C₂-Cer, incorporation of radioactivity into PG was increased compared with controls, and preincubation with okadaic acid attenuated the C₂-Cer-mediated increase in PG biosynthesis. In addition, when cells were preincubated with CPT-cAMP and then permeabilized, PG biosynthesis from [¹⁴C]glycerol 3-phosphate was increased compared with controls.

TNF α stimulates mitochondrial PLA₂ and PGP synthase activities

Since addition of C₂-Cer to H9c2 cells would mimic the generation of endogenous ceramide, it was important to demonstrate that an inducer of endogenous ceramide had a similar effect to that of C₂-Cer in elevating mitochondrial PGP synthase and PLA₂ activities. Cells were incubated in the absence or presence of 30 nM TNF α for up to 4 h and mitochondrial fractions were prepared. As seen in Table 8, there was a 53% increase (P < 0.05) in lysoPG formation in the mitochondrial fraction of cells treated with TNF α for 1 h compared with controls. In contrast, cells treated with TNF α for 4 h did not exhibit an increase in lysoPG formation. This was not surprising, since in HL-60 cells the effects TNF α on SM hydrolysis and ceramide generation

were shown to be rapid (within 1 h), and by 4 h ceramide and SM levels were shown to have returned to control values [10]. Treatment of cells with TNF α for 1 h resulted in a 47% elevation in PGP synthase activity, whereas treatment for 4 h did not affect this activity. Thus TNF α addition to H9c2 cells resulted in a stimulation of both mitochondrial PLA₂ and PGP synthase activities, similar to that produced by C₂-Cer. Taken together, all of the above results suggest that, in H9c2 cells, PG and CL metabolism may be regulated by intracellular ceramide signalling.

DISCUSSION

In the present study, the effects of short-chain ceramides on PG and CL biosynthesis in H9c2 cells were investigated. Our results demonstrate that addition of short-chain ceramides to H9c2 cardiac myoblasts resulted in the stimulation of PG and CL biosynthesis, and the mechanism was an increase in mitochondrial PGP synthase activity. The increase in PGP synthase activity, and hence in PG and CL biosynthesis, might be a compensatory mechanism for a ceramide-mediated elevation in mitochondrial PLA₂ activity, since the pool sizes of PG and CL were unaltered. The results with both intact and permeabilized cells provide evidence that PGP synthase activity in H9c2 cells may be regulated by a mechanism involving a ceramide-activated protein phosphatase(s). To our knowledge this study is the first demonstration that mammalian PGP synthase, and hence PG and CL biosynthesis, may be regulated by intracellular ceramide signalling.

The products of the SMase-catalysed reaction, i.e. ceramide and sphingosine, may be involved in the regulation of protein kinase C activity in cells (for reviews, see [36,37]). Our finding that the addition of sphingosine to H9c2 cells did not affect PG and CL metabolism suggests that such metabolism in these cells is not regulated through protein kinase C. A previous study had indicated that PMA induced retinoblastoma gene product phosphorylation in Molt-4 cells and that C₆-Cer inhibited this effect by inhibiting the protein kinase C pathway [38]. The finding that PMA did not affect PGP synthase activity in H9c2 cells suggests that the effect of C₂-Cer on this enzyme in these cells is probably not mediated by the protein kinase C pathway. Furthermore, incubation of cells with SMase did not affect PG or CL biosynthesis from [1,3-³H]glycerol or PGP synthase activity, whereas C₂-Cer and TNF α were effective in stimulating PGP synthase activity. These data suggest that the pool of ceramide produced from hydrolysis of SM in the outer leaflet of the plasma membrane does not participate in the regulation of PG and CL metabolism. Several studies support the existence of a signal-transducing pool of SM that is distinct from the pool that is accessible to exogenous SMase (for a review, see [8]). In U987 cells the biological actions of ceramide were shown to depend upon its topology of production [39]. Ceramide generated by membrane-associated neutral SMase activated pathways different from those activated by ceramide generated by endosomal acidic SMase. When Molt-4 leukaemia cells were transfected with *Bacillus cereus* SMase, the resultant increases in SMase activity and cellular levels of ceramide resulted in cleavage of poly(ADP-ribosyl)polymerase, and hence apoptosis [40]. In contrast, addition of exogenous *Bacillus cereus* SMase to these cells did not induce such changes. Thus it is likely that the intracellularly generated ceramide pool, but not the ceramide pool generated at the outer leaflet of the plasma membrane, is biologically active for the regulation of PG and CL metabolism in H9c2 cells. The C₂-Cer-mediated regulation of PGP synthase is likely to be physiological rather than pharmacological, since C₆-Cer, as well as C₂-Cer, was active in stimulating PG and CL

biosynthesis in H9c2 cells, and the C₂-Cer-mediated stimulation of PG and CL biosynthesis was concentration-dependent. The concentration-dependent effects of short-chain cell-permeant ceramides on cellular metabolic processes are well documented [41,42]. Furthermore, addition of TNF α to H9c2 cells resulted in stimulation of PGP synthase activity.

Ceramide was shown to directly activate highly purified forms of heterotrimeric protein phosphatase 2A [15]. In H9c2 cells, calyculin A or okadaic acid attenuated the C₂-Cer-mediated elevation of PGP synthase activity and of PG and CL biosynthesis. Thus it is likely that PGP synthase activity, and hence PG and CL biosynthesis, in H9c2 cells are modulated by a ceramide-activated protein phosphatase(s). As an alternative hypothesis, the phosphatase inhibitors could target a component of the C₂-Cer response pathway that is necessary to initiate the increase in PG and CL labelling, but is not directly related to PGP synthase. This is unlikely, since preincubation with C₂-Cer resulted in an elevation of PG biosynthesis from exogenous *sn*-[¹⁴C]glycerol 3-phosphate in permeabilized H9c2 cells, and this could be reversed by preincubation with okadaic acid. Since CL synthase activity was unaltered in H9c2 cells incubated with C₂-Cer, it was possible that the elevated *de novo* synthesis of CL observed was due to an increase in the specific radioactivity of PG. Although we cannot exclude this possibility, newly synthesized PG has been shown to be preferentially utilized for CL biosynthesis [30]. Addition of CPT-cAMP to H9c2 cells stimulated PG and CL biosynthesis from [1,3-³H]glycerol and PGP synthase activity. In addition, preincubation with CPT-cAMP stimulated PG biosynthesis from *sn*-[¹⁴C]glycerol 3-phosphate in permeabilized cells. These two findings implicate PKA as being involved in the regulation of PG and CL biosynthesis. The stimulation of PGP synthase activity and of PG and CL biosynthesis by C₂-Cer in combination with CPT-cAMP was additive. A previous study in HEL 299 cells indicated that down-regulation of the M₂ muscarinic receptor is mediated by synergy between activation of both the ceramide and PKA pathways [43].

Rapid alterations in CL metabolism have been reported previously [44]. In that study, intraperitoneal injection of aged rats with acetylcarnitine resulted in a rapid (within 3 h) elevation of cardiac mitochondrial CL levels. In our study, C₂-Cer treatment of H9c2 cardiac cells for 4 h resulted in rapid *de novo* synthesis of PG and CL, but the pool sizes of these phospholipids were not significantly altered. Since mitochondrial PLA₂ activity was elevated in H9c2 cells treated with C₂-Cer or TNF α , it is likely that the rapid elevation of mitochondrial CL levels in acetylcarnitine-treated aged rats is different from that involved in C₂-Cer signalling. In addition, the increase in mitochondrial PLA₂ activity in C₂-Cer-treated cells would explain why we did not observe an accumulation of PG or CL mass that would normally accompany an elevation in PGP synthase activity [45]. Finally, it is possible that the observed increase in mitochondrial phospholipid turnover mediated by C₂-Cer or TNF α in H9c2 cells may be the primary event that drives the ceramide-mediated stimulation of PG and CL biosynthesis. Thus the elevation of PGP synthase activity may simply be a compensatory mechanism to maintain the appropriate levels of these glycerophospholipids in the mitochondrial membrane during ceramide generation. A previous study indicated that phospholipid turnover may play an important role in phospholipid regulation in eukaryotic cells [46]. In conclusion, our results support the notion that PG and CL metabolism in H9c2 cells may be regulated by intracellular ceramide signalling.

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