Sphingosylphosphorylcholine Induces a Hypertrophic Growth Response Through the Mitogen-Activated Protein Kinase Signaling Cascade in Rat Neonatal Cardiac Myocytes

Kenichi Sekiguchi, Tomoyuki Yokoyama, Masahiko Kurabayashi, Fumikazu Okajima, Ryozo Nagai

Abstract—The sphingolipid metabolites, sphingosine (SPH), S1P (1-phosphate), and sphingosylphosphorylcholine (SPC), can act as intracellular as well as extracellular signaling molecules. These compounds have been implicated in the regulation of cell growth, differentiation, and programmed cell death in nonmyocytes, but the effects of sphingolipid metabolites in cardiac myocytes are not known. Cultured neonatal rat cardiac myocytes were stimulated with SPH (1 to 10 μmol/L), S1P (1 to 10 μmol/L), or SPC (0.1 to 10 μmol/L) for 24 hours to determine the effects of sphingolipid metabolites on the rates of protein synthesis and degradation. Stimulation with SPC led to an increase in the total amount of protein, an accelerated rate of total protein synthesis, and a decrease in protein degradation in a dose-dependent manner. However, S1P had little effect and SPH had no effect on total protein synthesis. In addition, stimulation with SPC led to a 1.4-fold increase in myocardial cell size and enhanced atrial natriuretic factor gene expression. Pretreatment of the cardiac myocytes with pertussis toxin or PD98059 attenuated the SPC-induced hypertrophic growth response. Further, stimulation with SPC increased phosphorylation of mitogen-activated protein kinase (MAPK) and stimulated MAPK enzyme activity. Finally, endothelin-1 stimulated the generation of SPC in cardiac myocytes. The observation that SPC induces a hypertrophic growth response in cardiac myocytes suggests that SPC may play a critical role in the development of cardiac hypertrophy. The effects of SPC could be mediated, in part, by activation of a G protein–coupled receptor and a MAPK signaling cascade. (Circ Res. 1999;85:1000-1008.)

Key Words: sphingolipid ■ cardiac myocyte ■ hypertrophy ■ receptor ■ mitogen-activated protein kinase

Sphingolipids were once thought to be the inert structural components of cell membranes. However, recent studies have shown that sphingolipids are important participants in the regulation of a variety of cellular processes.1 Sphingosine (SPH), a metabolite of ceramide, has been shown to stimulate cell proliferation2 and regulate intracellular Ca2+ mobilization.3,4 Exogenous SPH 1-phosphate (S1P) has also been reported to regulate Ca2+ mobilization,5,6 microfilament reorganization,7 cell migration,8 and fibroblast proliferation.9,10 Further, sphingosylphosphorylcholine (SPC), another metabolite of sphingomyelin, has been shown to be a potent mitogen in a variety of cell types.11,12 More recently, plasma membrane receptors coupled to G proteins with a high affinity for S1P and/or SPC have been described in various cell types.13 These sphingolipid metabolites can therefore act as intracellular and extracellular signaling molecules in the regulation of cell growth and differentiation.

In rat ventricular myocytes, SPH has been shown to modulate myocyte contractile behavior by inhibiting intracellular Ca2+ transients and L-type Ca2+ channel conductance.14,15 In contrast, SPC causes an increase in the diastolic Ca2+ concentration in rat cardiac myocytes by activating the ryanodine receptor and possibly another intracellular Ca2+ release channel.16 Further, both S1P and SPC are capable of activating muscarinic K+ current channels (Ik(Ach)) in guinea pig atrial myocytes.17 Activation of Ik(Ach) by SPC suggests that there is signaling via the pertussis toxin (PTX)–sensitive G protein–coupled pathway. Recently, Oral et al18 reported that the SPC content of adult feline cardiac myocytes increases with stimulation of tumor necrosis factor-α and that both SPH and S1P have negative inotropic effects on myocytes. However, the role of sphingolipid metabolites in the development of cardiac hypertrophy is not known.

The involvement of sphingolipid metabolites in cell growth led us to hypothesize that sphingolipid metabolites trigger hypertrophic growth responses in cardiac myocytes. We therefore examined the effects of exogenous sphingolipid metabolites, SPH, S1P, and SPC, on the rate of protein synthesis and degradation in isolated rat neonatal cardiac myocytes. Additional studies were performed to evaluate whether the hypertrophic growth response induced by exogenous sphingolipid metabolites is mediated by activating a G protein–coupled receptor.
Figure 1. Effects of sphingolipid metabolites on total cellular protein content, total protein synthesis, and protein degradation in cardiac myocytes. A through D, Degrees of total cellular protein content and of [3H]phenylalanine incorporation in control cells were normalized to 100%, and results are expressed as percentage change (mean ± SEM) compared with time-matched control. Numbers of preparations studied are shown in parentheses. A, In comparison with control cells (CONT.; open column), treatment with SPC (solid columns) resulted in a concentration-dependent increase in total protein content. B, In comparison with control cells (open column), treatment with SPC (solid columns) resulted in a concentration-dependent increase in total protein synthesis. C, In comparison with control cells (open column), treatment with S1P (solid columns) resulted in a concentration-dependent increase in total protein synthesis. D, SPH did not have any significant effect on total protein synthesis. E and F, Values are mean ± SEM for 8 cultures in each group. E, In comparison with control cells (open columns), the release of incorporated [3H]phenylalanine was less for the SPC-treated cells (1 μmol/L SPC, hatched columns; 10 μmol/L SPC, solid columns). Two-way ANOVA indicated that there was a significant difference between groups (P < 0.013). F, In contrast, the release of incorporated [3H]phenylalanine in S1P-treated cells was not significantly different from that of control cells. *P < 0.05 in comparison with control values.
Materials and Methods

Materials

D-SPH, S1P, SPC, PTX, and calphostin C were obtained from Sigma Chemical Co. PD98059 (2’-amino-3’-methoxyflavone) was obtained from BIOMOL Research Laboratories, Inc. [3H]Phenylalanine, [N-methyl-14C]sphingomyelin, and [9,10(n)-3H]palmitic acid were obtained from Amersham Co.

Protein Synthesis and Degradation

Primary neonatal rat cardiac ventricular myocyte cultures were prepared as previously described.19 The amount of newly synthesized total protein and protein degradation were determined by the previously described methods.20

Measurements of Cell Surface Area

Cardiac myocyte cultures (2×10⁵ cells per 35-mm culture dish) were fixed with 3% paraformaldehyde and incubated with 3% BSA to block nonspecific binding and then with a monoclonal antibody against sarcomeric α-actinin (Sigma). Antibody binding proteins were visualized colorimetrically using a VECTATIN Elite ABC kit (Vector Laboratories, Inc). Planimetry was performed using NIH Image software, which automatically calculates cell area from a manual tracing of the cell outline.

Northern Blot Analysis of Atrial Natriuretic Factor (ANF) mRNA

Total RNA isolations and Northern hybridization with a restriction fragment probe for mouse ANF DNA21 were performed essentially as previously described.19

Identification of Threonine/Tyrosine–Phosphorylated Extracellular Signal–Regulated Kinases (ERKs) by Western Blot Analysis

To identify threonine/tyrosine–phosphorylated forms (which are active forms) of p44 ERK and p42 ERK in cardiac myocytes, Western blot analysis was performed using a polyclonal rabbit anti-human phospho-specific mitogen-activated protein kinase (MAPK) (Thr202/Tyr204) antibody, which detects phosphorylated threonine 202 and tyrosine 204 of p44 and p42 ERKs (New England Biolabs, Inc).22

MAPK Activity Assay

MAPK activity was assayed by using a commercially available kit (Amersham).

Assay of SPC Production

SPC generation was assessed by the method of Bowser and Gray,23 with some modifications. Cardiac myocyte cultures were labeled for 24 hours with 50 nmol of [N-methyl-14C]sphingomyelin (55 mCi/mmol) or 20 pmol of [9,10(n)-3H]palmitic acid (50 Ci/mmol). On day 2, the myocyte cultures were washed 3 times with cold PBS and then exposed to DMEM supplemented with 0.1% BSA. The cultures were then stimulated with diluent, endothelin-1 (ET-1; 0.1 μmol/L) or isoproterenol (1 μmol/L), for 24 hours. Culture medium and cells were collected separately. Samples were dried with evaporator and dissolved in 20 μL of chloroform:methanol (1:1, vol/vol). The autoradiography of TLC was quantified using a phosphor imager (Fuji BAS 2000).

Statistical Analysis

Values are mean±SE. One-way ANOVA was used to evaluate differences between groups. Where appropriate, post hoc multiple comparison tests were performed to evaluate differences between the control and experimental groups. A P value <0.05 was considered statistically significant.

Results

Effects of Sphingolipid Metabolites on Total Protein Synthesis in Cardiac Myocytes

We first examined the effects of sphingolipid metabolites, SPC, S1P, and SPH on the total protein contents of neonatal rat cardiac myocytes. As shown in Figure 1A, stimulation with SPC for 24 hours increased the total cellular protein content in a concentration-dependent manner. The increase in the total protein content caused by incubation with SPC at a concentration of 5 μmol/L was greater than in control cells. In contrast, stimulation for 24 hours with neither S1P nor SPH had effects on the total cellular protein content (data not shown). Second, the effects of sphingolipid metabolites on [3H]phenylalanine incorporation in cardiac myocytes was examined. Figure 1B demonstrates that stimulation with SPC for 24 hours increased the rate of [3H]phenylalanine incorporation in myocytes in a concentration-dependent manner. Treatment with ≤0.5 μmol/L SPC had no significant effect on [3H]phenylalanine incorporation, whereas stimulation with ≥1 μmol/L SPC resulted in a significant increase in the rate of [3H]phenylalanine incorporation. Stimulation with S1P for 24 hours also increased [3H]phenylalanine incorporation in myocytes in a concentration-dependent manner. However, the effect of S1P on [3H]phenylalanine incorporation was less than that seen with SPC (Figure 1C). Only a high concentration (10 μmol/L) of S1P resulted in a significant increase in the rate of [3H]phenylalanine incorporation. In contrast, incubation with SPH had no effect on either [3H]phenylalanine incorporation or total protein content (Figure 1D). These data indicate that SPC increases total protein synthesis as well as total protein content. However, S1P had little effect and SPH had no effect on total protein synthesis.
Effect of Sphingolipid Metabolites on Protein Degradation in Cardiac Myocytes

Figure 3 shows that upon 24 and 48 hours of stimulation with SPC, the release of incorporated [\(^{3}\)H]phenylalanine was lower for SPC-treated cardiac myocytes than for control cells, suggesting that SPC stimulation decreases protein turnover. Two-way ANOVA indicated that there were both significant time-dependent (\(P<0.0001\)) and group-dependent (\(P<0.013\)) differences in the release of incorporated [\(^{3}\)H]phenylalanine. In contrast, stimulation with S1P for 24 and 48 hours had no significant effect on the release of incorporated [\(^{3}\)H]phenylalanine (Figure 1F). In the present study, SPC increased the rate of phenylalanine incorporation and reduced the rate of release of incorporated phenylalanine. These facts show that SPC not only quickens amino acid metabolism but also works to accumulate protein in cardiac myocytes.

Effect of SPC on Cardiac Myocyte Size

After treatment with SPC (10 \(\mu\)mol/L) for 48 hours, the cell area increased significantly compared with control cells (Figure 2). This increase in cell area after stimulation with SPC was similar to the increase in area seen with ET-1 (0.1 \(\mu\)mol/L). ET-1 is known to induce hypertrophy in cultured neonatal rat myocytes.

Effect of SPC on the Embryonic-Fetal Type Gene Expression in Cardiac Myocytes

To determine whether sphingolipid metabolites induce expression of embryonic-fetal type genes, the level of ANF mRNA expression was determined by Northern blot analysis. As shown in Figure 3, stimulation with SPC (10 \(\mu\)mol/L) or ET-1 (0.1 \(\mu\)mol/L) for 24 hours increased ANF mRNA expression in cardiac myocytes. However, ANF mRNA expression in myocytes incubated with S1P (10 \(\mu\)mol/L) was not different from ANF mRNA expression in control cells.

Effect of PTX on Sphingolipid Metabolite-Induced Hypertrophic Response in Cardiac Myocytes

To investigate the possibility that G proteins may be involved in the hypertrophic growth response induced by SPC or S1P in cardiac myocytes, myocyte cultures were treated with PTX (50 ng/mL) before the addition of SPC (10 \(\mu\)mol/L) or S1P (10 \(\mu\)mol/L). PTX pretreatment inhibited the SPC- or S1P-induced increase in total protein synthesis by 60% to 78% (Figure 4). Furthermore, PTX partially, but significantly, inhibited SPC-induced increase in cell size and ANF mRNA expression (Figures 5 and 6). In the absence of SPC, PTX had no effects on total protein synthesis (Figure 4) and cell size (108% of control). These results suggest that the increase in cardiac myocyte hypertrophy induced by SPC or S1P requires, at least in part, PTX-sensitive G proteins.

Effect of PD98059 on the SPC-Induced Hypertrophic Response in Cardiac Myocytes

To investigate the possibility that the MAPK signaling cascade may be involved in the hypertrophic growth response induced by SPC, myocyte cultures were treated with PD98059 (50 \(\mu\)mol/L), an inhibitor of p44 and p42 ERK, before the addition of SPC (10 \(\mu\)mol/L). ERKs are protein serine/threonine kinases and are members of the MAPK family. PD98059 pretreatment inhibited 74% of the SPC-induced increase in total protein synthesis (Figure 4). As shown in Figures 5 and 6, PD98059 pretreatment inhibited 64.5% of the SPC-induced increase in cell size and signifi-
cantly inhibited SPC-induced increase in ANF mRNA expression in cardiac myocytes. In the absence of SPC, PD98059 had no effects on total protein synthesis (Figure 4) and cell size (103 ± 5% of control). These results suggest that the increase in protein synthesis, cell size, and ANF mRNA expression caused by SPC involves, at least in part, the MAPK signaling cascade. Furthermore, both PTX and PD98059 pretreatment for 24 hours inhibited SPC-induced decrease in release of incorporated phenylalanine by 58.5% and 53.8%, respectively (Figure 7). In the absence of SPC, neither pretreatment with PTX nor PD98059 for 24 hours had an effect on the release of incorporated phenylalanine (315.7 ± 10.7 and 302.7 ± 6.1 cpm/μg protein, respectively).

Effect of SPC on the Threonine/Tyrosine Phosphorylation of ERKs and MAPK Activity in Cardiac Myocytes

To determine whether SPC activates p44 and/or p42 ERK in cardiac myocytes, we examined the content of threonine/tyrosine–phosphorylated p44 and p42 ERK with Western blot analysis using a specific antibody recognizing only threonine/tyrosine–phosphorylated p44 and p42 ERK. As shown in Figure 8, stimulation with SPC (10 μmol/L) or ET-1 (0.1 μmol/L) for 5 or 15 minutes significantly increased the contents of threonine/tyrosine–phosphorylated p44 and p42 ERK. Thus, SPC stimulated threonine/tyrosine phosphorylation of both ERK isoforms in cardiac myocytes. As shown in Figure 9A, stimulation with SPC (10 μmol/L) for 5 or 15 minutes significantly increased the MAPK activity. These results were similar to the effect of SPC on threonine/tyrosine phosphorylation of ERKs. Thus, SPC stimulates a MAPK cascade in cardiac myocytes with increasing phosphorylation of ERKs and enzyme activity of MAPK.

Effect of PD98059 on the SPC-Induced Increase in MAPK Activity in Cardiac Myocytes

To confirm that 50 μmol/L of PD98059 inhibited MAPK activity in cardiac myocytes, we examined the effect of SPC-elicited increase in MAPK activity by Western blot analysis using a specific antibody recognizing only threonine/tyrosine–phosphorylated p44 and p42 ERK. As shown in Figure 9B, stimulation with SPC (10 μmol/L) for 5 or 15 minutes significantly increased the contents of threonine/tyrosine–phosphorylated p44 and p42 ERK. Thus, SPC stimulated threonine/tyrosine phosphorylation of both ERK isoforms in cardiac myocytes. As shown in Figure 9A, stimulation with SPC (10 μmol/L) for 5 or 15 minutes significantly increased the MAPK activity. These results were similar to the effect of SPC on threonine/tyrosine phosphorylation of ERKs. Thus, SPC stimulates a MAPK cascade in cardiac myocytes with increasing phosphorylation of ERKs and enzyme activity of MAPK.

Effect of PD98059 on the SPC-Induced Increase in MAPK Activity in Cardiac Myocytes

To confirm that 50 μmol/L of PD98059 inhibited MAPK activity in cardiac myocytes, we examined the effect of PD98059 on SPC-elicited increase in MAPK activity. As shown in Figure 9B, 50 μmol/L of PD98059 decreased the SPC-elicited increase in MAPK activity by 47.0% of the SPC-induced increase in MAPK activity. Although 50 μmol/L of PD98059 should be enough to inhibit MAPK activity, 50 μmol/L of PD98059 did not completely inhibit the activation of MAPK by SPC. These results were similar to a previous study in which PD98059 inhibited ~50% of the nerve growth factor–elicited increase in MAPK activity in PC-12 cells.25 Also, the degree of inhibition of MAPK activity in Swiss 3T3 cells by PD98059 differed by the type of agonists.26 Thus, we could not exclude the possibility that the present results, in which PD98059 inhibited 64% to 74% of the SPC-induced increase in protein synthesis and cell size, were due to a partial inhibitory effect of PD98059 on MAPK.
activity. This is a potential limitation of studies using PD98059. It is also possible that MAPK-independent pathways are also involved in SPC-induced increase in protein synthesis and cell size.

**Effect of Calphostin C on the SPC-Induced Hypertrophic Response and Increase in MAPK Activity in Cardiac Myocytes**

To investigate the possibility that the protein kinase C (PKC)-dependent signaling cascade may be involved in the hypertrophic growth response induced by SPC, myocyte cultures were treated with calphostin C (1 micromol/L), an inhibitor of PKC, before the addition of SPC (10 micromol/L). Calphostin C pretreatment inhibited 55.5% of SPC-induced increase in cell size (Figure 5). However, the effect of calphostin C on SPC-induced increase in ANF mRNA expression was not significant (Figure 6). Further, calphostin C and PTX inhibited the SPC-induced increase in MAPK activity by 29.9% and 37.4%, respectively (Figure 9B). From these results, SPC stimulates MAPK activity in part via a sphingolipid receptor coupled to PTX-sensitive G proteins and PKC in cardiac myocytes. However, SPC induces hypertrophic growth responses via both PKC-dependent and PKC-independent signaling pathways.

**Effect of ET-1 and Isoproterenol on SPC Generation in Cardiac Myocytes**

To confirm whether SPC generation and release is increased in cardiac myocytes in response to agonists, we assayed SPC generation in cardiac myocytes and culture medium using 14C-labeled sphingomyelin. SPC generation was assayed using radio-TLC. As shown in Figure 10, the cellular lysates labeled with 14C-sphingomyelin were spotted on silica gel plates, and autoradiograph showed specific bands corresponding to SPC. Radioactivity quantified by BAS 2000.
phosphor imager system revealed that the samples treated with ET-1 had increased \( \approx 1.8 \) fold in radioactive SPC in comparison with that in control cells. Isoproterenol was not increased in radioactive SPC. Further, there were no bands corresponding to SPC in cellular lysates labeled with \( ^{14} \text{C} \)palmitic acid (data not shown). These results demonstrated that cardiac myocytes were capable of producing SPC, and ET-1 stimulated the generation of SPC in myocytes. The observation that cellular lysates labeled with \( ^{14} \text{C} \)palmitic acid did not contain any SPC band suggests that SPC is generated by the degradation of sphingomyelin but is not produced by the pathway mediating SPH. We also attempt to determine the release of SPC in culture medium from cardiac myocytes. However, there was no band corresponding to SPC in medium labeled with \( ^{14} \text{C} \)sphingomyelin or \( ^{14} \text{C} \)palmitic acid (data not shown). This may be a technical limitation of studies using radio-TLC assay.

**Discussion**

This simple experimental study shows for the first time that SPC induces a hypertrophic growth response in cardiac myocytes. The following 4 lines of evidence support this statement. First, when cardiac myocytes were stimulated with SPC, there were increases both in the rate of total protein synthesis and in the total cellular content of protein. Second, the stimulation of myocyte cultures with SPC resulted in a significant decrease in protein degradation to diluent-treated control cultures. Third, SPC stimulation produced a significant increase in cardiac myocyte cell size. Fourth, SPC induced the expression of ANF, which is one of the embryonic-fetal type genes in cardiac myocytes. Furthermore, the SPC-induced hypertrophic growth response was inhibited by a p44 and p42 ERK inhibitor, PD98059. SPC also stimulated threonine/tyrosine phosphorylation of ERKs and MAPK enzyme activity.

In previous studies, SPC has been shown to induce DNA synthesis and cellular proliferation in various cell lines. SPC-induced cellular proliferation is similar to the proliferation caused by serum and is much greater than that induced by insulin, fibroblast growth factor, epidermal growth factor, and 12-\( \text{O-tetradecanoylphororbol-13-acetate} \) (TPA) in fibroblasts. Recently, it has been shown in Swiss 3T3 fibroblast that SPC transiently activates p42 ERK and 90-kDa ribosomal S6 kinase (p90\( \text{rsk} \)) through a pathway dependent on PKC activity. Also, SPC stimulates PKC-mediated activation of p44 and p42 ERK in freshly isolated smooth muscle cells. Furthermore, SPC, as well as S1P, stimulates the DNA binding activity of the transcription factor protein AP-1.

AP-1, which is one of the transcription factors activated by the Ras pathway and the MAPK cascade, consists of homo- and/or heterodimers of the fos and jun gene products and controls genes that are required for cell growth. In cardiac myocytes, the activation of the MAPK cascade is believed to be involved in the initiation of the hypertrophic response induced by ET-1, fibroblast growth factor, or mechanical stress. Furthermore, expression of the proto-oncogenes c-fos and c-jun is increased in cardiac myocytes stimulated with isoproterenol or stretch. The enhanced DNA binding activity of the AP-1 transcriptional factor complex is believed to regulate the hypertrophic response of myocytes. These previous studies support our present observations that SPC is a potent inducer of hypertrophic growth in ventricular myocytes.

The existence of sphingolipid receptors has been hypothesized on the basis of observations that the actions of SPH, S1P, or SPC can be attenuated by treatment with PTX. PTX prevents receptor coupling to \( G \) or \( G \) proteins, thereby blocking cellular signaling by these receptors. In Swiss 3T3 fibroblasts, SPC-induced activation of p42 ERK requires a PTX-sensitive G protein. On the basis of these previous studies, we examined the effect of PTX on S1P- or SPC-induced increase in protein synthesis, cell size, and ANF mRNA expression in cardiac myocytes. PTX attenuated 60\% to 80\% of the sphingolipid metabolite-induced effects. Therefore, exogenous administration of S1P or SPC may stimulate cardiac hypertrophy, at least in part, by activating sphingolipid receptors that are coupled to PTX-sensitive G proteins.

Although sphingolipid receptors have not yet been characterized at the molecular level, 2 different G protein-coupled receptors for sphingolipid metabolites have been cloned. S1P activates the G protein-coupled receptor EDG-1, which was originally cloned as an immediate-early gene induced during differentiation of human endothelial cells. Other studies have shown that overexpression of the G protein-coupled receptors H218 and EDG-3, but not EDG-1, in Jurkat cells results in responsiveness to both S1P and SPC. There are also functional data supporting a pharmacological classification of the sphingolipid receptor family. The first class of receptors is characterized by their comparable sensitivities to nanomolar concentrations of both S1P and SPC. The second class includes receptors that are only activated by nanomolar concentrations of S1P but not by micromolar concentrations of SPC. The third class includes receptors that are only activated by nanomolar to low micromolar concentrations of SPC and not activated by micromolar concentrations of S1P. In our study, SPC induced cardiac hypertrophy at a concentration of 1 \( \mu \text{mol/L} \). However, only high concentrations of S1P stimulated cardiac hypertrophy. Therefore, the sphingolipid receptors on cardiac myocytes that induce cardiac hypertrophy may belong to the third class of sphingolipid receptors.

Despite their structural relationship, the increase in total protein synthesis induced by S1P was less than that induced by SPC, and SPH had no effect on protein synthesis. Similar discrepant cellular responses have been reported. Specifically, SPC stimulates cellular proliferation of quiescent Swiss 3T3 fibroblasts to a greater extent than S1P or SPH. Furthermore, SPC elicits a rapid Ca\( ^{2+} \) release from rat brain microsomes, whereas SPH and S1P do not cause Ca\( ^{2+} \) release. Of the various sphingolipid metabolites, SPC is the only agent that can accelerate cutaneous wound healing in vivo in a diabetic mouse model. The effects of SPC that are distinct from those of other sphingolipid metabolites may be the result of the following 2 factors. First, our observations suggest that sphingolipid receptors of the third class are present on cardiac myocytes. This class of sphingolipid receptor has different sensitivities to SPC and S1P. Second, SPC may act via both PKC-dependent and PKC-independent
signaling pathways, whereas the mitogenic activities of SPH and S1P are clearly independent of PKC activation. Further, in contrast to the effects of SPH, SPC causes activation of one protein kinase and inhibition of another. Therefore, SPC may act through activation of signaling pathway(s) distinct from those utilized by SPH and S1P. In this study, SPC induces hypertrophic growth responses via both PKC-dependent and PKC-independent signaling pathways.

It is well known that SPC is present in patients with Niemann-Pick disease, a lipid storage disorder. Recent studies based on nuclear magnetic resonance spectroscopy confirmed the existence of SPC in normal mouse tissue and other tissues under pathophysiological conditions, and identifying the signaling molecules. Further studies demonstrating quantitative studies of SPC are ongoing.

In conclusion, the addition of exogenous SPC, a sphingolipid metabolite, induces a hypertrophic growth response in cardiomyocytes similar to that seen with ET-1. Furthermore, the effects of SPC may be mediated by a sphingolipid receptor coupled to PTX-sensitive G proteins and the MAPK pathway. Although we could not detect the release of SPC from cardiomyocytes, cardiomyocytes were capable of producing SPC by the degradation of sphingomyelin, and ET-1 stimulated the generation of SPC. Thus, our findings are the first to suggest a role for sphingolipid metabolites in the development of cardiac hypertrophy. Because SPC may potentially be used for clinical applications, such as a wound-healing agent, we believe that sphingolipid metabolites may play a critical role in the progression of cardiac hypertrophy in both extracellular and intracellular signaling molecules. Further studies demonstrating quantitative changes in the concentration of SPC in cardiomyocytes or heart tissue under pathophysiological conditions, and identifying the source of extracellular SPC, are now in progress to confirm our hypothesis.

References


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Materials and Methods

Materials

Cell culture reagents and fetal bovine serum were obtained from Life Technologies Inc. (Gaithersburg, MD). d-Sphingosine, sphingosine 1-phosphate, sphingosylphosphorylcholine, pertussis toxin and calphostin C were obtained from Sigma Chemical Co. (St. Louis, MO). PD98059 (2'-amino-3'-methoxyflavone) was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). [3H]Phenylalanine, [α-32P]dCTP, [N-methyl-14C]sphingomyelins and [9,10(n)-3H]palmitic acid were obtained from Amersham Co. (Buckinghamshire, UK).

Preparation of neonatal rat cardiac myocytes

Primary neonatal rat cardiac ventricular myocyte cultures were prepared as previously described\(^\text{19}\). Briefly, hearts were excised from 1- to 2-day-old Wistar rats anesthetized with ethyl ether, the ventricles were minced with scissors in Ca\(^{2+}\)-free Krebs-Henseleit buffer (KHB) solution (118 mM NaCl, 4.0 mM KCl, 1.2 mM MgCl\(_2\), 1.1 mM KH\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), 5.0 mM glucose, 20 mM HEPES, pH 7.4). The cells were dissociated with 0.05% trypsin and 0.05% collagenase type II in KHB. The cells were stirred with the use of a small magnetic stirrer bar for 10 min at 37°C, and the supernatants were saved in cold D-MEM containing 10% fetal calf serum. Digestion was repeated four times. The resulting cell suspension was centrifuged, and the pellet was resuspended in D-MEM containing 10% fetal calf serum.

The cells were plated in culture flasks for 1.5 hours to remove noncardiac myocytes. The unattached cells were removed and seeded (8x10⁵ cells) in 35-mm gelatin-coated culture dishes in D-MEM containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. After incubation for 48 hours at 37°C with 5% CO\(_2\), the medium was replaced by D-MEM containing 10
μg/ml insulin, 5.5 μg/ml sodium transferrin, 6.7 ng/ml sodium selenite, 2.0
μg/ml ethanolamine, 0.1% BSA and the above antibiotics, and the cells were
incubated for another 24 hours. Using this method, we routinely obtained
cultures containing more than 95% cardiac myocytes, as determined by
immune histochemical staining with a monoclonal antibody against sarcomeric
α-actinin (Sigma Chemical Co., St. Louis, MO).

**Protein synthesis**

The amount of newly synthesized total protein was determined by the
previously described methods²⁰ with minor modifications. Briefly, neonatal rat
cardiac myocyte cultures were exposed for 24 hours to diluent, SPC (0.1 - 10
μM), S1P (1 - 10 μM) or SPH (1 - 10 μM). To determine whether these
sphingolipid metabolites increased the rate of protein synthesis in isolated
cardiac myocytes, the cells were pulse-labeled for 6 hours with 2 μCi/ml
[^3H]phenylalanine. At the conclusion of the study, the incorporation of radio-
labeled phenylalanine was stopped by washing the cultures with cold-
phosphate buffered saline (PBS) containing 10 mmol/L dl-phenylalanine.
Cardiac myocyte proteins were then solublized with 500 μl of 0.5% SDS, 1 mM
DTT, 50 mM Tris-HCl, pH7.5. A portion of the solublized sample was used for
the determination of protein concentration using a commercially available
assay kit (BCA; Pierce, Rockford, IL), using BSA as a standard. The extent of
radiolabel incorporation was determined after acid precipitation of proteins for
30 min with cold (4°C) 10% trichloroacetic acid (TCA). The precipitates were
collected on 16-mm glass filters, and the filters were washed sequentially with
10% TCA, 5% TCA, and 95% ethanol. The glass filters were air-dried, and
subjected to liquid scintillation counting.[^3H]Phenylalanine incorporation is
expressed as cpm/μg myocyte protein. To facilitate the comparison between
different myocyte cultures, the rate of protein synthesis in control cells
(diluent-treated cells) was normalized to 100% in each experiment. Results are expressed as the percent change in the rate of protein synthesis of sphingolipid metabolites treated cells compared to time-matched control experiments.

**Protein degradation**

Protein degradation was determined by previously described method\(^\text{20}\) using minor modifications. Briefly, cardiac myocyte cultures were labeled for 24 hours with 2 \(\mu\)Ci/ml \(^3\)Hphenylalanine. On day 2, the myocyte cultures were washed three times with cold PBS containing 10 mmol/L dl-phenylalanine and then exposed to D-MEM supplemented with 10 mmol/L dl-phenylalanine. The cultures were then stimulated with diluent, SPC (1 - 10 \(\mu\)M) or S1P (1 - 10 \(\mu\)M), and the amount of \(^3\)Hphenylalanine released into the culture medium at 6, 24 or 48 hours was used as a measure of the rate of protein turnover. The results are expressed as cpm/\(\mu\)g myocyte protein.

**Measurements of cell surface area**

Cardiac myocyte cultures (2x10\(^5\) cells per 35-mm culture dish) were incubated with SPC (10 \(\mu\)M), endothelin-1 (ET-1, 0.1 \(\mu\)M) or diluent for 48 hours at 37°C in D-MEM supplemented with 0.1% BSA. After incubation, cells were rinsed with PBS, fixed with 3% paraformaldehyde, incubated with 3% BSA to block nonspecific binding, and then with a monoclonal antibody against sarcomeric \(\alpha\)-actinin. Antibody-binding proteins were visualized colorimetrically using a VECTATIN\(^\text{R}\) Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA). The cells stained with \(\alpha\)-actinin were identified by a light microscopy (Nikon, Tokyo, Japan) at a 200-fold magnification, and images were recorded with a CCD-video camera (Sankei, Tokyo, Japan). Planimetry was performed using NIH Image software, which automatically calculates cell area from a manual tracing of the cell outline.

**Northern blot analysis of ANF mRNA**
Cardiac myocyte cultures (5x10⁶ cells per 100-mm culture dish) were incubated with SPC (10 μM), S1P (10 μM), ET-1 (0.1 μM) or diluent for 24 hours at 37°C in D-MEM supplemented with 0.1% BSA. After incubation, the cells were harvested and total cellular RNA was isolated using the acid guanidium thiocyanate-phenol-chloroform extraction method. RNA (20 μg) was fractionated on 1.2% formaldehyde-agarose gels and transferred onto nylon membranes. The blots were prehybridized for 4 hours at 42°C in 40% formamide, 0.1% SDS, 5x SCC, 5x Denhardt’s (0.1% Ficoll, 0.1% BSA and 0.1% polyvinylpyrrolidone) and 0.01 mg/ml denatured salmon sperm DNA. Hybridization was carried out at 42°C for 12 hours using a restriction fragment probe for mouse atrial natriuretic peptide (ANF) DNA. The probe was labeled via nick translation using a random primer DNA labeling kit obtained from Boehringer Mannheim (Mannheim, Germany). The nylon membranes were stained with methylene blue after transfer and photographed. After hybridization, the blots were washed 2 or 3 times with 0.1% SDS-2x SCC at 42°C and exposed to Kodak X-ray Omat AR film (Rochester, NY) at -80°C.

Identification of threonine/tyrosine-phosphorylated ERKs by Western blot analysis

To identify threonine/tyrosine-phosphorylated forms (=active forms) of p44 extracellular signal-regulated kinase (ERK) and p42 ERK in cardiac myocytes, Western blot analysis was performed using specific antibodies recognizing only threonine/tyrosine-phosphorylated p44 and p42 ERK. Cardiac myocyte cultures (5x10⁶ cells per 100-mm culture dish) were incubated with SPC (10 μM), ET-1 (0.1 μM) or diluent for 5 or 15 min at 37°C in D-MEM supplemented with 0.1% BSA. After incubation, the medium was removed, and the cells were washed with ice-cold PBS, solubilized in lysis buffer (25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 0.5 mM EGTA, pH 8.0, 1 mM
sodium orthovanadate, 10 nM okadaic acid, 1 mM phenyl methyl sulfonyl fluoride, 0.8 μg/ml leupeptin, 10 μg/ml aprotinin) and frozen at -20°C. Samples were thawed and heated for 5 min at 100°C, and 10-μl aliquots corresponding to 20 μg protein were loaded onto a 12% acrylamide-SDS gel. Electrophoresis was performed at 40 mA. For Western blotting, the proteins were transferred to a nitrocellulose membrane (2 mA/cm², 120 min) using a semidry electrophoresis transfer system (ATTO, Tokyo, Japan). The nitrocellulose membranes were blocked with 3% BSA in PBS for overnight at 4°C. The membranes were incubated for overnight at 4°C with a polyclonal rabbit anti-human phospho-specific MAP kinase (Thr202/Tyr204) antibody, which detects the phosphorylated threonine 202 and tyrosine 204 of p44 and p42 ERKs (New England Biolabs, Inc., Beverly, MA). Membranes were washed three times in PBS, and antibody-binding proteins were visualized using an ECL Western blotting detection kit (Amersham).

**MAPK activity assay**

Cardiac myocyte cultures (5x10⁶ cells per 100-mm culture dish) were incubated with SPC (10 μM) for 5 or 15 min at 37°C in D-MEM supplemented with 0.1% BSA. After incubation, the medium was removed, and the cells were washed with ice-cold PBS, solubilized in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 1 mM sodium orthovanadate, 2 mM DTT, 1 mM phenyl methyl sulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Samples were centrifuged at 15000 rpm for 20 min and the supernatant was retained to obtain cytoplasmic mitogen-activated protein kinase (MAPK). MAPK activity was assayed by using a commercially available kit (Amersham).

**Assay of SPC production**
SPC generation was assessed by the method of Bowser and Gray\textsuperscript{24} with some modifications. Cardiac myocyte cultures were labeled for 24 hours with 50 nmol of [N-methyl-\textsuperscript{14}C] sphingomyelin (55 mCi/mmol) or 20 pmol of [9,10(n)-\textsuperscript{3}H] palmitic acid (50 Ci/mmol). On day 2, the myocyte cultures were washed three times with cold PBS and then exposed to D-MEM supplemented with 0.1% BSA. The cultures were then stimulated with diluent, ET-1 (0.1 \textmu M) or isoproterenol (1 \textmu M) for 24 hours. Culture medium and cells were collected separately. Samples were dried with evaporator and dissolved in 20 \textmu l of chroloform:methanol (1:1, v/v). The sample solution was subjected to thin layer chromatography (TLC, HPTLC plate silica gel 50, Merck, Darmsted, Germany), and developed twice with methanol:0.5% NaCl:13N ammonia solution (50:50:1, v/v). The autoradiography of TLC was quantified using a phosphoimager (Fujix BAS 2000, Tokyo, Japan).

\textit{Statistical analysis}

Values are expressed as the mean \pm SE. One-way analysis of variance was used to evaluate differences between groups. Where appropriate, \textit{post-hoc} multiple comparison tests were performed to evaluate differences between the control and experimental groups. A \textit{P} value <.05 was considered statistically significant.