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Phytosphingosine and C2-phytoceramide induce cell death and inhibit carbachol-stimulated phospholipase D activation in Chinese hamster ovary cells expressing the *Caenorhabditis elegans* muscarinic acetylcholine receptor

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Abstract Sphingolipid metabolites, such as sphingosine and ceramide, are known to play important roles in cell proliferation, differentiation and apoptosis, but the physiological roles of phytosphingosine (PHS) and phytoceramide (PHC) are poorly understood. In this study we investigated the effects of PHS, C2-PHC (*N*-acetylPHS) and C6-PHC (*N*-hexanoylPHS) on cell growth and intracellular signalling enzymes. Treatment of Chinese hamster ovary (CHO) cells with PHS, C2-PHC or C6-PHC resulted in cell death in a time- and dose-dependent manner. C2-PHC induced internucleosomal DNA fragmentation, whereas PHS or C6-PHC had little if any effect on DNA fragmentation under the same experimental conditions. Both PHS and C2-PHC inhibited carbachol-induced activation of phospholipase D (PLD), but not of phospholipase C (PLC), in CHO cells expressing the *Caenorhabditis elegans* muscarinic acetylcholine receptor (mAChR). On the other hand, no significant effect of C6-PHC on PLD or PLC was observed. Our results show that PHS and C2-PHC exert strong cytotoxic effects on CHO cells and modulate the mAChR-mediated signal transduction pathway.

Key words: Phytosphingosine; Phytoceramide; Cell death; Phospholipase D; Muscarinic acetylcholine receptor

1. Introduction

Accumulating evidence indicates that sphingolipid metabolites, such as ceramide and sphingosine, serve as mediators of a variety of biological functions including the regulation of cell growth [1]. Ceramide, the basic building block of sphingolipids, has been a material of main interest. In response to cytokines, hormones, antigens, DNA-damaging agents, and many other chemicals, ceramide is generated from the hydrolysis of sphingomyelin by sphingomyelinase [2–4]. Ceramide has been postulated as an intracellular signalling molecule to modulate cell differentiation, apoptosis, protein secretion, and inflammatory responses [5].

Phospholipase D (PLD) plays an important role in signal transduction by catalyzing the hydrolysis of phosphatidylcholine to generate phosphatidic acid and choline in response to neurotransmitters, hormones, cytokines, and growth factors [6]. Phosphatidic acid can be dephosphorylated to diacylglycerol, which is an activator of certain isoforms of protein kinase C [7]. Like phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol, PLD is known to participate in diverse cellular functions. PLD has been implicated in senescence, differentiation, and apoptosis [8–10]. Sphingolipid metabolites have been shown to regulate PLD activity in many cell types [11–15], indicating crosstalk between sphingolipid and glycerophospholipid metabolic pathways.

Recently, we have cloned a muscarinic acetylcholine receptor (mAChR), termed GAR-3, from *Caenorhabditis elegans* [16]. Ligand binding specificity and effector coupling efficacy of the *C. elegans* mAChR are generally similar to those of mammalian counterparts [16], suggesting the evolutionary conservation of the receptor. More recently, we have shown that in Chinese hamster ovary (CHO) cells expressing the *C. elegans* mAChR, carbachol induces the activation of PLD, and that PLC, protein kinase C, Ca^{2+}/calmodulin-dependent kinase II and protein tyrosine kinase are involved in the PLD activation [17].

Phytosphingosine (PHS) and phytoceramide (PHC) are abundant in fungi and plants [18], and also found in animals including humans [19]. PHS and PHC are structurally similar to sphingosine and ceramide, respectively, with one major difference: PHS and PHC possess a hydroxyl group at C-4 of the sphingoid long-chain base while sphingosine and ceramide have a *trans* double bond between C-4 and C-5. In contrast to sphingosine and ceramide, the roles for PHS and PHC in cellular functions are unclear although recent experiments showed that PHS and PHC are involved in the heat stress response of the yeast *Saccharomyces cerevisiae* [20–22]. In the current work, we investigated the effects of PHS, C2-PHC (*N*-acetylPHS) and C6-PHC (*N*-hexanoylPHS) on the

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Abbreviations: CHO, Chinese hamster ovary; IP, inositol phosphate; mAChR, muscarinic acetylcholine receptor; PBS, phosphate-buffered saline; PHC, phytoceramide; PHS, phytosphingosine; PLC, phospholipase C; PLD, phospholipase D; PtdBut, phosphatidylbutanol

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regulation of cell growth and intracellular signalling enzymes, namely PLC and PLD, in CHO cells.

2. Materials and methods

2.1. Materials

PHS, C2-PHC and sphingosine were obtained from Doosan Biotech, and C2-ceramide was from Tocris. C6-PHC was synthesized by coupling PHS with hexanoic acid. The identity of C6-PHC was confirmed by nuclear magnetic resonance spectroscopy and matrix-assisted laser desorption ionization-time of flight mass spectrometry. The purity of the C6-PHC was >98% by high performance liquid chromatography. Carbamylcholine chloride (carbachol) was purchased from Sigma. Culture medium and fetal bovine serum (FBS) were from Gibco.

2.2. Cell culture and cell viability test

CHO-K1 cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS at 5% CO_2. To measure cell viability, the trypan blue exclusion method was used. Cells were cultured in 24-well plates with serum-free medium for 5 h and treated with various sphingolipids (solubilized in ethanol). Trypan blue solution (0.4%, Sigma) was added and viable cells were counted under a microscope (Olympus IMT-2).

2.3. Assay for DNA fragmentation

CHO cells were cultured in 24-well plates with serum-free medium for 5 h and further incubated in the presence of various sphingolipids. DNA was prepared using DNAzol (Molecular Research Center) and analyzed by electrophoresis on a 2% agarose gel.

2.4. PLD activity assay

The GAR-3/CHO cells stably expressing the *C. elegans* mAChR [16] were labeled overnight with 1 μCi/ml [^{3}H]myristic acid (NEN). The cells were washed and preincubated in HEPES buffer containing various sphingolipids for 20 min. After incubation in HEPES buffer containing 0.3% 1-butanol for 10 min, the cells were stimulated with 1 mM carbachol for 20 min. The reaction was terminated by removing the medium, washing on ice with 5 ml of ice-cold phosphate-buffered saline (PBS), and adding 1.5 ml of ice-cold methanol. The cell harvest, lipid extraction, and determination of [^{3}H]phosphatidylbutanol (PtdBut) production were performed as described [17].

2.5. PLC activity assay

GAR-3/CHO cells were seeded in six-well plates at 5×10^5 cells per well and cultured for 1 day. The cells were labeled overnight with 1 μCi/ml of *myo*-[2-^{3}H]inositol (Amersham). The cells were washed twice with PBS and incubated with PBS containing 10 mM LiCl (PBS-Li) for 15 min. The cells were preincubated with various sphingolipids for 20 min and treated with 1 ml of fresh PBS-Li containing 1 mM carbachol for 20 min. The cell harvest and measurement of [^{3}H]inositol phosphates (IPs) formation were performed as described [16].

3. Results

3.1. PHS, C2-PHC and C6-PHC exert lethal effects in CHO cells

To investigate the physiological roles of PHS, C2-PHC and C6-PHC, we first examined the effects of these sphingolipids on cell viability. All of these sphingolipids led to a time-dependent (Fig. 1A) and dose-dependent (Fig. 1B) death of CHO cells. PHS displayed a cytotoxic effect comparable to that of sphingosine. By contrast, C2-PHC showed a much stronger cytotoxic effect than C2-ceramide, implying that the hydroxyl group at C-4 of the sphingoid long-chain base is critical for the antiproliferative activity. The cytotoxic effect of C6-PHC, however, was significantly weaker than that of C2-PHC. This difference suggests that the size of the *N*-acyl group is also important for the cytotoxicity. In control experiments, ethanol (0.1%) treatment did not show any significant cytotoxic effect (Fig. 1A). Taken together, these results suggest the possibility that PHS, C2-PHC and C6-PHC, as well as sphingosine and C2-ceramide, act as mediators of cell death in CHO cells.

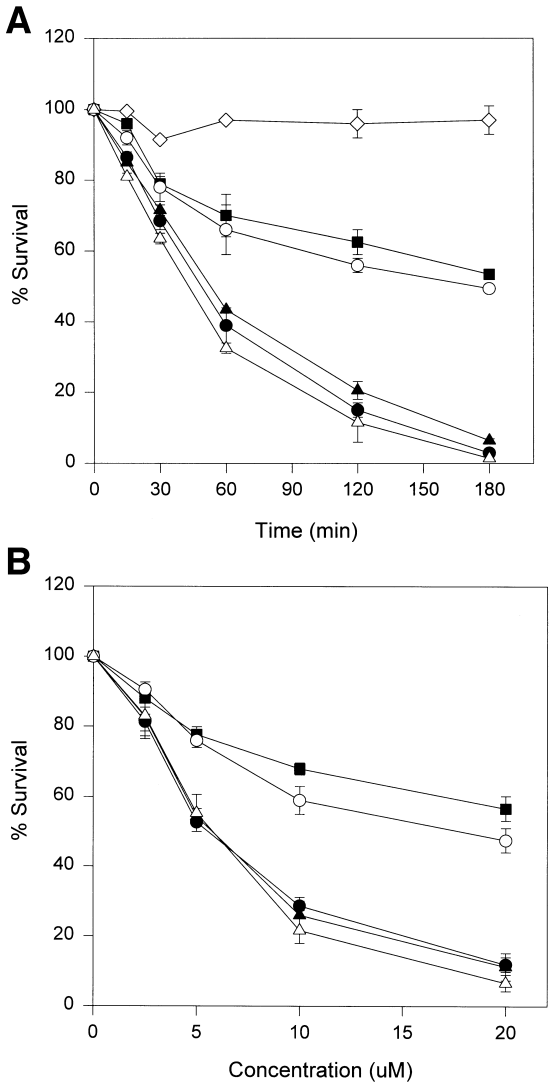

Fig. 1. PHS, C2-PHC and C6-PHC induce cell death. (A) CHO cells were grown in a serum-free medium for 5 h and treated with 20 μM of PHS (closed triangles), C2-PHC (closed circles), C6-PHC (closed squares), sphingosine (open triangles), C2-ceramide (open circles), or ethanol (diamonds) for the indicated times. (B) CHO cells were grown in a serum-free medium for 5 h and treated with various concentrations of PHS (closed triangles), C2-PHC (closed circles), C6-PHC (closed squares), sphingosine (open triangles), or C2-ceramide (open circles) for 3 h. Cells were then stained with trypan blue and the viable cells were counted. The data are mean ± S.E.M. values of triplicate determinations. Similar results were obtained from three separate experiments.

3.2. C2-PHC induces internucleosomal DNA fragmentation

We next examined whether PHS, C2-PHC or C6-PHC induces DNA fragmentation in CHO cells. C2-PHC (20 μM) elicited DNA fragmentation after 3 h of treatment, whereas neither PHS nor C6-PHC induced DNA fragmentation under the same experimental conditions (Fig. 2A). No DNA fragmentation was observed by sphingosine or C2-ceramide treat-

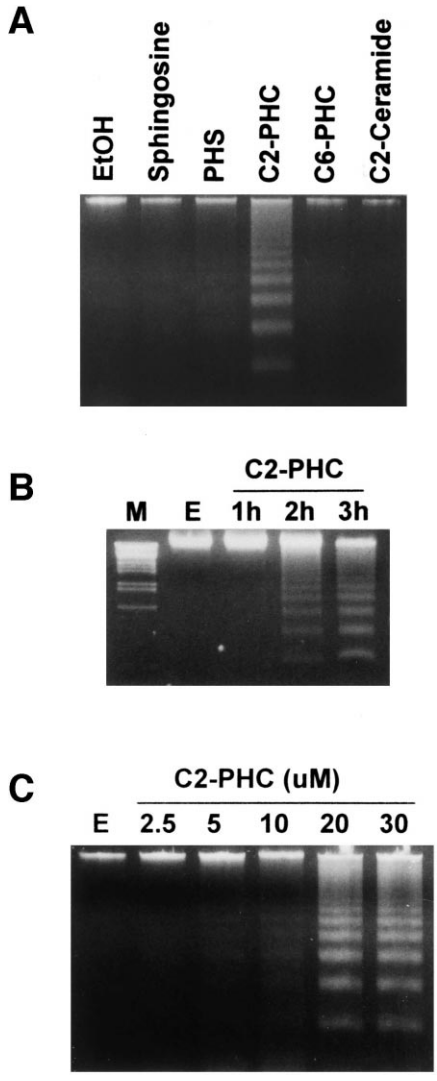

Fig. 2. C2-PHC induces internucleosomal DNA fragmentation. (A) CHO cells were grown in a serum-free medium for 5 h and treated with various sphingolipids (20 μM each) for 3 h. DNA was isolated and analyzed by electrophoresis on a 2% agarose gel. Control sample was treated with 0.1% ethanol (EtOH). (B) CHO cells were treated with C2-PHC (20 μM) for the indicated times and the time-dependent DNA fragmentation pattern was analyzed by agarose gel electrophoresis. Lambda DNA digested with *Hin*dIII and *Eco*RI was used as the molecular weight marker (M). E, EtOH. (C) CHO cells were treated with various concentrations of C2-PHC for 3 h and the dose-dependent DNA fragmentation pattern was analyzed by agarose gel electrophoresis. E, EtOH.

ment (Fig. 2A). We were unable to observe any significant level of DNA fragmentation even after 12 h incubation with PHS, C6-PHC, sphingosine, or C2-ceramide (data not shown). The differential effects of C2-PHC and C6-PHC on DNA fragmentation may indicate that the mechanisms by which these two sphingolipids induce cell death are distinct.

The effect of C2-PHC on DNA fragmentation was time-dependent (Fig. 2B). Internucleosomal DNA fragmentation was barely detectable after 1 h treatment of 20 μM C2-PHC, but was evident after 2 h treatment. After 3 h treatment, the DNA degradation was even greater. We also observed that C2-PHC induced internucleosomal DNA fragmentation in a dose-dependent fashion (Fig. 2C). After 3 h incubation, a very low level of DNA degradation was detectable in the presence of 5 μM or 10 μM PHC. In the presence of 20 μM or 30 μM C2-PHC, the internucleosomal DNA fragmentation pattern became distinct.

3.3. PHS and C2-PHC reduce PLD activation, but not PLC activation, mediated by mAChRs

PLD is considered to be a critical cell growth-regulating enzyme whose activity is controlled by many sphingolipids [6,8]. We thus asked whether PHS, C2-PHC or C6-PHC affects agonist-induced activation of PLD. For this purpose, we used the CHO cells stably expressing GAR-3, the *C. elegans* mAChR (GAR-3/CHO) [16]. GAR-3/CHO cells have been shown to possess an endogenous PLD enzyme (PLD1) and to activate the PLD by treatment with carbachol, a muscarinic agonist [17]. PHS and C2-PHC greatly inhibited the PLD activity stimulated by the *C. elegans* mAChR, while C6-PHC had little, if any, effect on the mAChR-stimulated PLD activity (Fig. 3A). The inhibitory effects of sphingosine

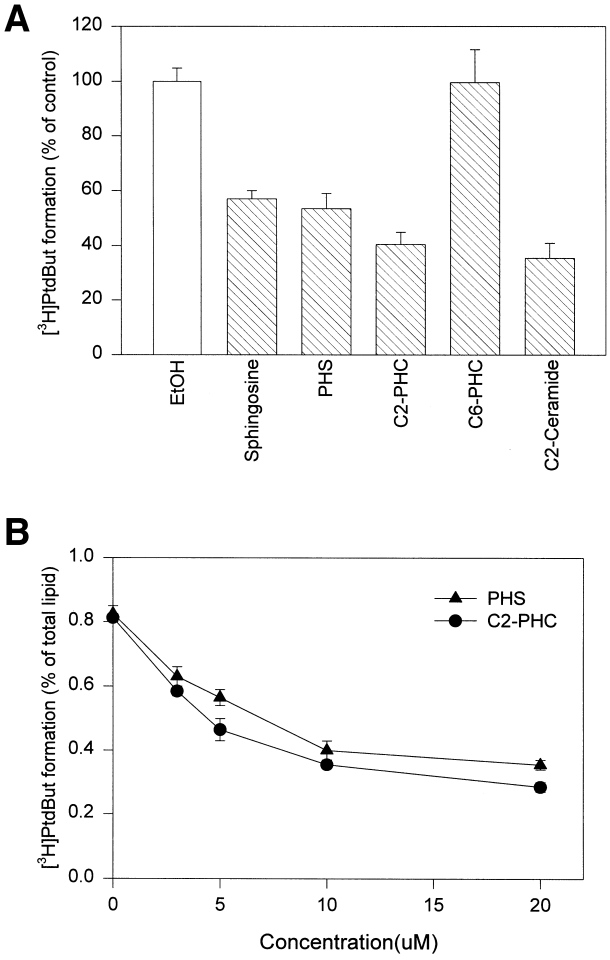

Fig. 3. PHS and C2-PHC inhibit mAChR-mediated PLD activation. (A) GAR-3/CHO cells were serum-deprived and labeled with [^{3}H]myristic acid. The cells were pretreated with various sphingolipids (10 μM each) for 20 min and 0.3% 1-butanol for 10 min. Following incubation with 1 mM carbachol for 20 min, lipids were extracted and the production of [^{3}H]PtdBut was analyzed. The production of [^{3}H]PtdBut with ethanol (EtOH) treatment was set to be 100%. (B) Dose-dependent inhibition of carbachol-induced PLD activation by PHS (triangles) and C2-PHC (circles). The data are mean ± S.E.M. values from three separate experiments, each performed in duplicate.

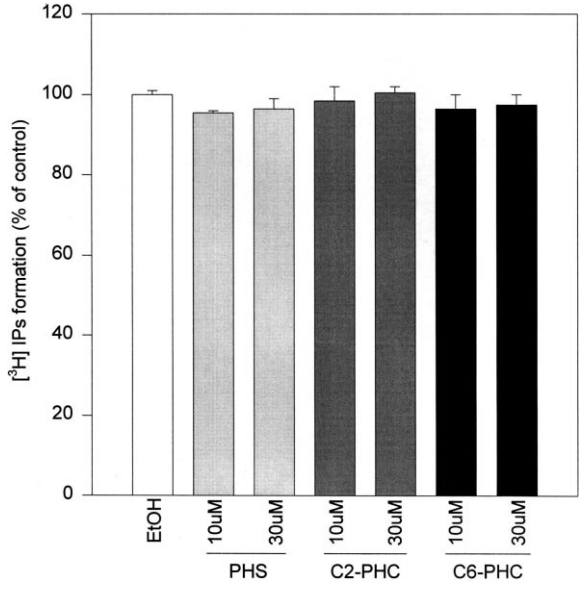

Fig. 4. PHS, C2-PHC or C6-PHC does not affect mAChR-mediated PLC activation. GAR-3/CHO cells were labeled with *myo*-[2-^{3}H]-inositol for 1 day and pretreated with the indicated concentrations of PHS, C2-PHC, or C6-PHC for 20 min. Following incubation with 1 mM carbachol for 20 min, the formation of [^{3}H]IPs was analyzed by anion exchange chromatography. The production of [^{3}H]IPs with ethanol (EtOH) treatment was set to be 100%. The data are mean ± S.E.M. values from three separate experiments, each performed in triplicate.

and C2-ceramide were similar to those of PHS and C2-PHC (Fig. 3A).

The inhibition of carbachol-stimulated PLD activity by PHS and C2-PHC was dose-dependent (Fig. 3B). The inhibition appeared to be incomplete (57–65% inhibition), suggesting that both sphingolipid-sensitive and -insensitive pathways for the PLD activation may exist in CHO cells.

In GAR-3/CHO cells, carbachol activates PLC [16,17] and this PLC activation is a prerequisite for the activation of PLD [17]. We examined whether PHS or C2-PHC inhibits mAChR-mediated PLC activation. Neither PHS nor C2-PHC significantly altered the PLC activity stimulated by carbachol (Fig. 4). C6-PHC had little effect on the PLC activity as well.

4. Discussion

Extensive evidence suggests that sphingosine and ceramide are critical regulators of cell proliferation and apoptosis [1,2]; however, the roles of PHS and PHC in cellular functions are poorly characterized. In the current work, we found that PHS and C2-PHC exert lethal effects in CHO cells and inhibit mAChR-stimulated PLD activation. Our findings suggest that PHS and C2-PHC might regulate cell growth and the agonist-induced signal transduction pathway in mammalian cells.

Sphingolipid metabolites control cell growth presumably by altering enzyme activities involved in signal transduction. In particular, reduction in PLD activity has been postulated to be responsible for cell death caused by ceramide treatment [23,24]. In agreement with this postulation, our results showed that PHS and C2-PHC, as well as sphingosine and C2-ceramide, suppress receptor-stimulated PLD activity in the same concentration range that induces cell death. However, C6-PHC, which exhibited a strong cytotoxic effect (Fig. 1), did not significantly alter the receptor-stimulated PLD activity (Fig. 3). In fact, some controversy exists about the relationship between PLD and mitogenesis [25,26]. Further study is required to define the role of PLD in the regulation of cell growth.

Treatment of GAR-3/CHO cells with carbachol activates PLC and PLD [16,17]. The specific PLC inhibitor, U73122, almost completely blocks the PLD activation [17], suggesting that PLC is the upstream signal for the PLD activation. In this study, PHS and C2-PHC were shown to inhibit the carbachol-stimulated PLD activation (Fig. 3) without affecting the PLC activation (Fig. 4), providing evidence that both sphingolipids interfere with the mAChR-mediated signal transduction pathway downstream from PLC and upstream of PLD. It is possible that PHS and C2-PHC act on protein kinase C, protein tyrosine kinase, or Ca^{2+}/calmodulin-dependent kinase II, since these protein kinases have been postulated to be involved in the PLC-dependent activation of PLD in GAR-3/CHO cells [17]. All these kinases have been shown to be regulated by ceramide and sphingosine [2]. Alternatively, PLD itself could be the direct target of the sphingolipids.

Our data suggest that the three PHS-based sphingolipids examined in this study (PHS, C2-PHC and C6-PHC) affect cellular functions via distinct mechanisms. Although all three sphingolipids exerted cytotoxic effects (Fig. 1), only C2-PHC effectively evoked internucleosomal DNA fragmentation (Fig. 2). While PHS and C2-PHC substantially inhibited mAChR-mediated PLD activation, C6-PHC had little effect on the enzyme activity (Fig. 4). Taken together, it seems likely that the three sphingolipids perform different roles in cell signalling.

In the current study we used PHCs with short acyl chains (C2 and C6), which are supposed to be cell-permeable. Interestingly, these two short-chain PHCs exhibited distinct cellular activities. Presumably CHO cells have the ability to recognize the length of the acyl chain in the PHC. It will be of interest to compare the cellular functions of PHCs with different acyl chain lengths, including C16-PHC and C18-PHC.

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