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Sphingosylphosphorylcholine generates reactive oxygen species through calcium-, protein kinase Cδ- and phospholipase D-dependent pathways

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Abstract

Sphingosylphosphorylcholine (SPC) is a bioactive lipid molecule involved in numerous biological processes. Treatment of MS1 pancreatic islet endothelial cells with SPC increased phospholipase D (PLD) activity in a time- and dose-dependent manner. In addition, treatment of the MS1 cells with 10 μM SPC induced stimulation of phospholipase C (PLC) activity and transient elevation of intracellular Ca^{2+}. The SPC-induced PLD activation was prevented by pretreatment of the MS1 cells with a PLC inhibitor, U73122, and an intracellular Ca^{2+}-chelating agent, BAPTA-AM. This suggests that PLC-dependent elevation of intracellular Ca^{2+} is involved in the SPC-induced activation of PLD. The SPC-dependent PLD activity was also almost completely prevented by pretreatment with pan-specific PKC inhibitors, GF109203X and RO-31-8220, and with a PKCδ-specific inhibitor, rottlerin, but not by pretreatment with GO6976, a conventional PKC isozymes-specific inhibitor. Adenoviral overexpression of a kinase-deficient mutant of PKCδ attenuated the SPC-induced PLD activity. These results suggest that PKCδ plays a crucial role for the SPC-induced PLD activation. The SPC-induced PLD activation was preferentially potentiated in COS-7 cells transfected with PLD2 but not with PLD1, suggesting a specific implication of PLD2 in the SPC-induced PLD activation. SPC treatment induced phosphorylation of PLD2 in COS-7 cells, and overexpression of the kinase-deficient mutant of PKCδ prevented the SPC-induced phosphorylation of PLD2. Furthermore, SPC treatment generated reactive oxygen species (ROS) in MS1 cells and the SPC induced production of ROS was inhibited by pretreatment with U73122, BAPTA-AM, and rottlerin. In addition, pretreatment with a PLD inhibitor 1-butanol and overexpression of a lipase-inactive mutant of PLD2 but not PLD1 attenuated the SPC-induced generation of ROS. These results suggest that PLC-, Ca^{2+}-, PKCδ-, and PLD2-dependent pathways are essentially required for the SPC induced ROS generation.
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Keywords: PLD; PKCδ; Calcium; ROS; SPC

1. Introduction

Sphingosylphosphatidylcholine (SPC[1]) has been implicated in a number of biological processes, including proliferation, growth inhibition, smooth muscle contraction, wound healing, and angiogenesis [1]. SPC has been reported to activate G protein-coupled receptors (GPCRs) to induce phospholipase C (PLC)-mediated hydrolysis of

Abbreviations: SPC, sphingosylphosphorylcholine; GPCRs, G protein-coupled receptors; PLC, phospholipase C; PIP_2, phosphatidylinositol 4, 5-bisphosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+}; PLD, phospholipase D; LPA, lysophosphatidic acid; PKC, protein kinase C; ARF, ADP-ribosylation factor; ROS, reactive oxygen species; TLC, thin layer chromatography; PMA, 4β-phorbol 12-myristate 13-acetate; PBtOH, phosphatidylbutanol; DN-PKCδ, dominant negative PKCδ; H_2DCF-DA, dichlorodihydrofluorescein diacetate; DCFH, dichlorodihydrofluorescein; S1P, sphingosine-1-phosphate.

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phosphatidylinositol 4,5-bisphosphate (PIP_2) [1], although the molecular identity of specific receptors to which SPC binds with high affinity is still unclear [2]. PLC-mediated hydrolysis of PIP_2 generates inositol 1,4,5-trisphosphate and diacylglycerol, which increase intracellular Ca^{2+} ($[Ca^{2+}]_i$) and activate protein kinase C, respectively.

Phospholipase D (PLD) catalyzes phosphatidylcholine hydrolysis to produce phosphatidic acid and choline in response to receptor activation induced by various extracellular agonists. Phosphatidic acid can further be metabolized to lysophosphatidic acid (LPA) and diacylglycerol by phospholipase A_2 and phosphatidic acid phosphohydrolase, respectively [3,4]. These reactions are involved in receptor-mediated physiological responses, such as membrane trafficking, cell growth and differentiation, cytoskeletal reorganization, respiratory burst, and apoptosis [5–7]. It has been reported that PLD activity is regulated by several signaling molecules, including protein kinase C (PKC), ADP-ribosylation factor (ARF), RhoA, PIP_2, Ca^{2+}, and unsaturated fatty acids [5,8–14]. Up to date, two isoforms of PLD, PLD1 and PLD2, have been isolated and characterized from human, rat, and mouse cDNA libraries [15–17]. PLD1 has low basal activity and is potently activated by phosphorylation with conventional types of PKC isoforms-dependent phosphorylation and direct interaction with ARF and RhoA [8,9,13,18], whereas PLD2 has high basal activity and its activity is stimulated by PKCδ-dependent phosphorylation, interaction with ARF and several unsaturated fatty acids, and elevation of $[Ca^{2+}]_i$ [11,12,14,16,19–23]. Although SPC induces the elevation of $[Ca^{2+}]_i$ and activation of PKC, it still remains unclear whether SPC stimulates PLD activity through Ca^{2+}- and PKC-dependent pathways and which of the isozymes of PLD is linked to the SPC-induced cell signaling.

Reactive oxygen species (ROS) are known to be produced during ligand-induced activation of nonphagocytic cells as well as phagocytic cells [24], and is involved in a variety of physiological responses, such as proliferation, apoptosis, atherogenesis, and hypertrophy [25]. In vascular endothelial and smooth muscle cells, ROS contributes to stimulation of smooth muscle cell growth, oxidation of low density lipoprotein, and endothelial cell injury [24–26]. In addition, PLD has been shown to play a role in the formation of NADPH-dependent formation of ROS in neutrophiles and vascular smooth muscle cells [27–29]. However, it is still unknown whether SPC induces ROS generation and whether PLD is involved in the SPC-induced generation of ROS in endothelial cells.

In the current study, we demonstrate for the first time to the best of our knowledge that SPC stimulates PLD activity by the PLC-, Ca^{2+}-, and PKCδ-dependent pathways in MS1 pancreatic endothelial cells, and we also show that the SPC-induced signaling pathways play a role in the production of ROS.

2. Experimental procedures

2.1. Materials

The Silica Gel 60 thin layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). [^{3}H]Myristic acid (54 Ci/mmol), myo-[^{3}H]inositol, and the chemiluminescence kit (ECL) were obtained from Amersham Biosciences (Buckinghamshire, UK). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Lab. (West Grove, PA). Dulbecco's modified Eagle's medium and LipofectAMINE were from Invitrogen (Carlsbad, CA), and fetal bovine serum was from HyClone (Logan, UT). 4β-phorbol 12-myristate 13-acetate (PMA), U73122, BAPTA-AM, GF109203X, Ro-31-8220, Go6976, and rottlerin were from BIOMOL (Plymouth Meeting, PA). Anti-PKCδ monoclonal antibody and anti-phospho-PKCδ (Thr^{505}) antibodies were purchased from Pharmingen (San Diego, CA) and Cell Signaling Tech. (Beverly, MA), respectively. Dichlorodihydrofluorescein diacetate (H_2DCF-DA) and fluo-3-AM were purchased from Molecular Probes (Eugene, OR). S1P and SPC were purchased from Avanti Polar Lipids (Alabaster, AL) and solubilized in 1 mg/ml fatty acid-free bovine serum albumin. All the other reagents were from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture and transient transfection

MS1 cells, pancreatic islet endothelial cells transformed by infection with a temperature sensitive SV40 large T antigen (tsA-58-3), were purchased from American Type Culture Collection (Manassas, VA). MS1 and COS-7 cells were maintained in a high glucose Dulbecco's modified Eagle's medium that was supplemented with 25 mM $NaHCO_3$, 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in a 5% CO_2/95% O_2 humidified incubator at 37 °C. For transient overexpression of PLD isoforms, a mammalian expression vector (pcDNA3.1) bearing wild type rat PLD1, wild type human PLD2, lipase-inactive mutants of rat PLD1 (K860R) and human PLD2 (K758R) was transfected into COS-7 cells with LipofectAMINE, as was suggested by the manufacturer (Invitrogen).

2.3. Measurement of PLD activity

PLD activity was assayed by measuring the formation of phosphatidylbutanol (PBtOH), the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol. MS1 and COS-7 cells were seeded onto 6-well tissue culture plates at 1×10^6 cells/well, they were serum-starved for 16 h, and then loaded with [^{3}H]myristic acid (3 μCi/ml) for 4 h. The [^{3}H]myristic acid-labeled cells were treated with SPC in the presence of 0.5% 1-butanol (v/v) for the indicated times at 37 °C. After the incubation, the medium was

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