Sphingosylphosphorylcholine and Lysophosphatidylycholine Are Ligands for the G Protein-coupled Receptor GPR4*

Received for publication, September 4, 2000, and in revised form, August 29, 2001
Published, JBC Papers in Press, September 4, 2001, DOI 10.1074/jbc.M008057200

Kui Zhu‡, Linnea M. Baudhuin‡‡,†, Guifying Hong‡, Freager S. Williams‡,†, Kelly L. Cristina‡,†, Janusz H. S. Kabarowski‡*, Owen N. Witte‡, and Yan Xu‡†‡†‡‡‡

From the ‡Department of Cancer Biology and the ‡Department of Gynecology and Obstetrics, Cleveland Clinic Foundation, Cleveland, Ohio 44195, the ‡Department of Chemistry, Cleveland State University, Cleveland, Ohio 44115, and the ‡Department of Microbiology, Immunology and Molecular Genetics, Howard Hughes Medical Institute, University of California, Los Angeles, California 90095-1662

Sphingosylphosphorylcholine (SPC) and lysophosphatidylycholine (LPC) are bioactive lipid molecules involved in numerous biological processes. We have recently identified ovarian cancer G protein-coupled receptor 1 (OGR1) as a specific and high affinity receptor for SPC, and G2A as a receptor with high affinity for LPC, but low affinity for SPC. Among G protein-coupled receptors, GPR4 shares highest sequence homology with OGR1 (51%). In this work, we have identified GPR4 as not only another high affinity receptor for SPC, but also a receptor for LPC, albeit of lower affinity. Both SPC and LPC induce increases in intracellular calcium concentration in GPR4-expressing Swiss 3T3 cells, but not vector-transfected MCF10A cells. These effects are insensitive to treatment with RN52909, WEB-2170, and WEB-2096 (specific platelet-activating factor (PAF) receptor internalization). Swiss 3T3 cells expressing GPR4 respond to both SPC and LPC, but not sphingosine 1-phosphate (SIP), PAF, psychosine (Psy), glucosyl-β1-1-sphinogosine (Glu-Sph), galactosyl-β1-1-ceramide (Gal-Cer), or lactosyl-β1-1-ceramide (Lac-Cer) to activate extracellular signal-regulated kinase mitogen-activated protein kinase in a concentration- and time-dependent manner. SPC and LPC stimulate DNA synthesis in GPR4-expressing Swiss 3T3 cells. Both extracellular signal-regulated kinase activation and DNA synthesis stimulated by SPC and LPC are pertussis toxin-sensitive, suggesting the involvement of a Gi-heterotrimeric G protein. In addition, GPR4 expression confers chemotactic responses to both SPC and LPC in Swiss 3T3 cells. Taken together, our data indicate that GPR4 is a receptor with high affinity to SPC and low affinity to LPC, and that multiple cellular functions can be transduced via this receptor.

SPC is a bioactive lipid molecule involved in numerous biological processes, where it acts as a signaling molecule (1). We have recently identified a G protein-coupled receptor, OGR1, as the first specific high affinity receptor for SPC (2). OGR1 shares homology with several other G protein-coupled receptors, including GPR4, G2A, T cell death-associated G protein-coupled receptor 8 (TDAG8), and the platelet activating factor (PAF) receptor (3–8, 10). We have postulated that these receptors belong to a subfamily and their ligands may be lysolipids containing the phosphorylcholine moiety shared by SPC and PAF (2). Other than SPC and PAF, there are two naturally occurring sphingosylphosphorylcholine-containing lysolipids: LPC and lyso-PAF. LPC is an important lipid mediator involved in many cellular processes. In particular, LPC is believed to play an activation of serum response element reporter and receptor internalization. Swiss 3T3 cells expressing GPR4 respond to both SPC and LPC, but not sphingosine 1-phosphate (SIP), PAF, psychosine (Psy), glucosyl-β1-1-sphinogosine (Glu-Sph), galactosyl-β1-1-ceramide (Gal-Cer), or lactosyl-β1-1-ceramide (Lac-Cer) to activate extracellular signal-regulated kinase mitogen-activated protein kinase in a concentration- and time-dependent manner. SPC and LPC stimulate DNA synthesis in GPR4-expressing Swiss 3T3 cells. Both extracellular signal-regulated kinase activation and DNA synthesis stimulated by SPC and LPC are pertussis toxin-sensitive, suggesting the involvement of a G_i-heterotrimeric G protein. In addition, GPR4 expression confers chemotactic responses to both SPC and LPC in Swiss 3T3 cells. Taken together, our data indicate that GPR4 is a receptor with high affinity to SPC and low affinity to LPC, and that multiple cellular functions can be transduced via this receptor.

In the present study, we sought to identify the ligand(s) for GPR4. We tested SPC, LPC, PAF, lyso-PAF, and psychosine (Psy; a recently identified glycosphingolipid ligand of TDAG8 (15)) as potential ligands for GPR4. GPR4 exhibits the highest homology with OGR1 (51% identity and 64% similarity in amino acid sequence) (2). Similarly to OGR1, GPR4 responded to SPC, but also responded to LPC, mediating an increase in intracellular calcium concentration, SRE activation, receptor internalization, ERK activation, and stimulation of cell migration. LPC bound to GPR4, albeit with lower affinity compared with SPC, and competed with SPC for specific binding to GPR4. GPR4 did not bind or respond to PAF, lyso-PAF, Psy, Glu-Sph, Gal-Cer, or Lac-Cer. Our results indicate that SPC is a high affinity and LPC is a lower affinity ligand for GPR4, and its activation by SPC and LPC mediates biological functions.

The abbreviations used are: SPC, sphingosylphosphorylcholine; ERK, extracellular signal-regulated kinase; CHO, Chinese hamster ovary; Glu-Sph, glucosyl-β1-1-sphinogosine; Gal-Cer, galactosyl-β1-1-ceramide; Lac-Cer, lactosyl-β1-1-ceramide; PAF, platelet activating factor; Psy, psychosine; S1P, sphingosine 1-phosphate; SRE, serum response element; TDAG8, T cell death-associated gene 8; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; HA, hemaggutinin; GFP, green fluorescent protein; BSA, bovine serum albumin; MAP, mitogen-activated protein; [Ca^{2+}], intracellular calcium.

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
EXPERIMENTAL PROCEDURES

MATERIALS—LPCs (14:0, 16:0, 18:0, and 18:1), lysophosphatidylcholine from liver, 80% 18:0), 18:1-LPA, 16:0-PAF, 16:0-lyso-PAF, psychosine, glucosyl-β1-1-sphinogol, galactosyl-β1-1-C8-ceramide, and lacteol-β1-1-C8-ceramide were from Avanti Polar Lipids, Inc. (Alabaster, AL). Sphingomyelin (bovine brain, mainly 18:0), C6 sphingomyelin, sphingosine-1-phosphate (S1P), and SPC were from Toronto Research Chemicals (Toronto, ON) or Matreya, Inc. ( Pleasant Gap, PA). t-Erythro-13-threeo-SPC were from Matreya, Inc. ( Pleasant Gap, PA). pDNA1-C3 (encoding the C3-oxo-enzyme) was a kind gift from Dr. A. Wolfman, Cleveland Clinic Foundation. The PAF receptor antagonist, BN52012, was from Biomol (Plymouth Meeting, PA). WEB-2170 and WEB-2086 were from Boehringer Ingelheim (Ridgefield, CT). [H]Spc or [H]18:0-LPC were custom synthesized by Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom (68 Ci/mmol, 1 mCi/ml for [H]SPC and 102 Ci/mmol, 1 mCi/ml for [H]18:0-LPC). [H]16:0-LPC (60 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO).

Cell Culture—MCF10A cells (passage 34) were purchased from the Barbara Ann Karmanos Cancer Institute (Detroit, MI) and cultured as recommended by the provider. Experiments were performed using MCF10A cells from passage 40–46. Other cells were obtained from ATCC and were cultured either in RPMI 1640 with 10% FBS or DMEM with 5% FBS (CHO and Swiss 3T3 cells).

Human RNA Master Blot Probed with GPR4—Human RNA Master Blot (CLONTECH, Palo Alto, CA) was probed with radiolabeled full-length GPR4. Briefly, the full-length GPR4 was gel purified and 25 ng was used for the synthesis of a StripAble DNA α-32P-labeled probe (Ambion, Austin, TX) as per the manufacturer’s instructions. The radiolabeled probe (20 ng, 20 × 106 cpm) was hybridized to the Master Blot in ExpressHyb hybridization solution (CLONTECH) overnight with continuous agitation at 65 °C. The following day, the Master Blot was washed following the manufacturer’s instructions and exposed to a PhosphoScreen (Molecular Dynamics, Sunnyvale, CA).

NOVEMBER 7, 2005

WYDRUN

Dr. J. DiDonato, Cleveland Clinic Foundation. The resulting 3HA-GPR4 was subsequently cloned into the mammalian expression vector pIRE-Shyg-GPR4, using primer 5′-CAGATGCATTAAAGGCCTCAACTTGGG and the T7 primer (inserted into the NsiI and Nol sites of pIRE-Shyg-GPR4). pGPR4-GFP was generated by cloning the T3 primer using 5′-GTCGCCATCTGGCTGCGGACGATC (stop codon was deleted and the resulting GPR4 was cloned into HindIII and KpnI sites of pEGFP-N1; CLONTECH). pSRE-Luc was purchased from Stratagene (La Jolla, CA). MCF10A cells were transiently transfected with pGPR4-GFP and used for calcium assays. CHO cells were transfected with pIRE-Shyg-GPR4 (LipofectAMINE reagent; Life Technologies, Inc., Rockville, MD) and stable clones were selected with 100 μg/ml hygromycin in DMEM/F-12 plus 5% FBS. HEK293 cells were transfected with pGPR4-GFP and stable clones were selected with 400 μg/ml G418 in RPMI 1640 plus 10% FBS. Swiss 3T3 cells expressing GPR4 were derived by infection with retroviruses encoding receptor (MSCV GPR4-ires-GFP) followed by fluorescence-activated cell sorter sorting of GFP positive cells (16).

Calcium Assays—Measurement of [Ca2+]i, was performed as described previously (2). Briefly, pGP4-GFP-transfected MCF10A cells were grown in specialized glass-bottom dishes (Biotech, Inc., Butler, PA) and loaded with fura-2 in HEPES-buffered saline. Using a dual-wavelength spectrophotofluorometer (RFK-6002, Photon Technology Int., S. Brunswick, NJ) coupled to an inverted fluorescence microscope (Olympus, IX-70, Lake Success, NY), GFP-positive cells were identified using an excitation wavelength of 488 nm, a dichroic 505 nm long-pass filter, and an emitter filter at band pass of 535 nm (Chroma Technology, Brattleboro, VT). Measurements of [Ca2+]i, were performed on individual GPR4-GFP positive cells at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Conversion of the 340/380 ratio value into [Ca2+]i, in nanomolar was estimated by comparing the cellular fluorescence ratio with ratios acquired using fura-2 (free acid) in buffers containing known Ca2+ concentrations. [Ca2+]i, was then calculated as described by Grynkiewicz et al. (17). All calcium assays were performed in the presence of 1 μM EGTA in the assay buffers.

Downloaded from http://www.jbc.org/ by guest on July 26, 2018
experimental Procedures

Human RNA Master Blot Probed with GPR4—GPR4 has been shown to be expressed in many human tissues (18). For a wider analysis of GPR4 expression in human tissues, we probed the Human RNA Master Blot (CLONTECH) containing RNAs from 50 different human tissues with the full-length human GPR4 clone labeled with \[^{32}\text{P}]\text{dCTP}\) ("Experimental Procedures"). GPR4 showed the highest expression in ovary, gland, and heart, but not in other tissues including some areas of the brain, colon, bladder, uterus, stomach, pancreas, salivary gland, mammary gland, peripheral blood leukocytes, fetal brain, and fetal heart (Fig. 1).

**Both SPC- and 16:0-LPC-induced Transient Increases in Intracellular Calcium Concentration ([Ca\(^2+\)]\text{i}) in GPR4-transfected MCF10A Cells**—We have shown that OGR1 is a high affinity receptor for SPC (2). To test whether GPR4, which shares 51% sequence homology with OGR1, is also a receptor for SPC, MCF10A cells were transiently transfected with pGPR4-GFP. MCF10A cells were chosen because these cells do not respond to either SPC or 16:0-LPC in calcium assays and they express very low levels of endogenous GPR4 among many human cell lines tested (Fig. 2).

The GFP receptor fusion was used to identify positively transfected cells, and single-cell calcium assays were performed as described in our previous studies (2). SPC (1 \(\mu\text{M}\)) stimulated an increase in \([\text{Ca}^{2+}]\text{i}\), in GPR4-, but not vector-transfected MCF10A cells (Fig. 3A, first and second panels), suggesting that GPR4 is a receptor for SPC. This is further confirmed by the stereoselectivity of GPR4 favoring \(\text{n}-\text{erythro}-\text{SPC}\) (the bioactive form of SPC) versus \(\text{l}-\text{threo}-\text{SPC}\) (Fig. 3A, third panel). Interestingly, unlike OGR1, which is specific for SPC as its ligand (2), GPR4-transfected cells were stimulated to produce increased \([\text{Ca}^{2+}]\text{i}\), by an additional phosphorylcholine-containing lysolipid, 16:0-LPC (Fig. 3A, fourth panel).

**RESULTS**

**Human RNA Master Blot Probed with GPR4**—GPR4 has been shown to be expressed in many human tissues (18). For a
[\text{Ca}^{2+}], response to LPC in GPR4-transfected cells were higher than those of SPC at greater concentrations of LPC (up to 10 \mu M) (Fig. 3B).

LPC has been shown to activate cellular responses in a PAF receptor-dependent manner (19–21). However, LPC and SPC were not able to induce an increase in calcium through the endogenous PAF receptor in parental cells (Fig. 3A, upper panel). Therefore, it is unlikely that the increase in calcium induced by LPC was mediated by a PAF receptor. Nevertheless, and to delineate the structural specificity of ligands for GPR4, we tested the effect of Psyc, Glu-Sph, Gal-Cer, and Lac-Cer to increase [\text{Ca}^{2+}], in MCF10A cells. We found that at 1 \mu M, Psyc, Glu-Sph, and Gal-Cer did not stimulate increases in [\text{Ca}^{2+}], in either MCF10A parental cells or GPR4-expressing cells (Fig. 3H). Lac-Cer (1 \mu M) induced the same level of increased [\text{Ca}^{2+}], in both parental and GPR4-expressing MCF10A cells (Fig. 3H). These data suggest that these glycosphingolipids are unlikely to be ligands of GPR4.

\textit{Withdrawn}

\textbf{November 7, 2005}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Expression of GPR4 in human cell lines. Real-time quantitative PCR was utilized to determine relative expression levels of GPR4 expressed in cells, as described under \textit{Experimental Procedures}. All PCR reactions were performed in triplicate. The comparative \textit{C}_\text{T} method was used to calculate the relative expression levels of GPR4 in different cell lines as described under \textit{Experimental Procedures}. HEY, OCC1, NIH-Ovca3, SKOV3, Ovca429, Ovca432, and Ovca433 are ovarian cancer cells. MCF7 is a breast cancer cell line. MCF10A is an immortalized breast cell line. HeLa is a cervical cell line. All cell lines shown, except Swiss 3T3, are human cell lines.}
\end{figure}
GPR4 as a Receptor for SPC and LPC

GPR4 induced by either SPC or 16:0-LPC (Fig. 5, D and E).

Similarly, WEB-2170 and WEB-2086 did not affect the internalization of GPR4 induced by either SPC or 16:0-LPC (data not shown).

LPC and SPC Activated the SRE Reporter System in HEK293 Cells—The serum-response element (SRE) reporter system is a sensitive assay for receptors of lipid factors (27, 28). Using the luciferase assay, vector-transfected HEK293
cells transfected with the SRE reporter system responded to SPC (1 μM), but not 16:0-LPC, with ≤1.5-fold activation (Fig. 6A). Activation was increased 3.1- and 4-fold, respectively, in response to 16:0-LPC (1 μM) and SPC (1 μM) in GPR4-transfected HEK293 cells that were also transfected with the SRE reported system (Fig. 6B). These increases were statistically significant (p < 0.001) when compared with the responses in vector-transfected cells (Fig. 6A). In contrast, although LPA and S1P induced significant transcriptional activation of SRE in vector-transfected HEK293 cells, this activation was not altered by GPR4 transfection. In addition, we tested other phosphorylcholine-containing lipids, including 16:0-PAF, 16:0-lyso-PAF, and 18:0-sphingomyelin, and found that none of them induced significant transcriptional activation of SRE (Fig. 6A).

The SRE transcriptional activity in response to SPC, but not

Fig. 4. Binding of SPC and 16:0-LPC to GPR4. A and B, time dependence of specific [3H]SPC and [3H]LPC binding. Cell homogenates (100 μl, equivalent to 10⁵ cells) from vector or GPR4 stably transfected CHO cells were incubated with [3H]SPC (1 nM) or [3H]16:0-LPC (1 nM) for the indicated times. Specific binding is shown. C and D, saturation isotherm of specific binding of [3H]SPC and [3H]16:0-LPC to GPR4-transfected CHO cells. Cell homogenates (100 μl) were incubated with the indicated concentrations of [3H]SPC or [3H]16:0-LPC in the presence or absence of unlabeled SPC (100-fold excess) or unlabeled 16:0-LPC (100-fold excess). Specific binding is presented. E and F, structural specificity of binding of [3H]SPC and [3H]16:0-LPC to GPR4. GPR4-transfected CHO cells were incubated with [3H]SPC (1 nM) or [3H]16:0-LPC (1 nM) in the presence or absence of 100 nM of different unlabeled lipids. Total binding is presented. All binding experiments were performed in triplicate in 96-well plates.

Data are mean ± S.D. from three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001; as compared with the control (Student’s t test).

Fig. 5. Internalization of GPR4 induced by SPC and LPC. A, HEK293 cells stably expressing pGPR4-GFP. B, pGPR4-GFP stably expressing cells were treated with SPC (1 μM) at 37 °C for 2 h. C, pGPR4-GFP-expressing cells were treated with 16:0-LPC (1 μM) at 37 °C for 2 h. D and E, as in B and C, except cells were pretreated with BN52021 (200 μM) for 5 min. F, pGPR4-GFP-expressing cells were treated with PAF (1 μM). All experiments were repeated at least three times. Representative data are shown.
FIG. 6. SPC and LPC activate SRE in a GPR4-dependent manner. A, the SRE-luciferase responses to different lipids in vector- and GPR4-transfected HEK293 cells. 18:1-LPA, 16:0-LPC, SPC, S1P, 18:0-sphingomyelin, 16:0-PAF, and 16:0-lyso-PAF (1 μM each) were used. The experiments were conducted as described under “Experimental Procedures” and represent data are shown. Con.: control; *, p < 0.05; **, p < 0.001; as compared with the control. #, p < 0.001 when compared with SPC- or 16:0-LPC induced activity in vector-transfected cells. The Student’s t test was performed using the GraphPad InStat software (San Diego, CA). p < 0.05 was considered to be statistically significant.
LPC, in parental HEK293 cells (Fig. 6, A and B), can be explained by the endogenous expression of GPR4 in HEK 293 cells and the relatively lower affinity of GPR4 for LPC compared with SPC. GPR4 transfection enhanced the activation of SRE reporter by both SPC and LPC (Fig. 6, A and B). EC₅₀ values for the activation of SRE were 63 nM for SPC and 160 nM for 16:0-LPC. The differences in EC₅₀ values obtained using SRE activation from those using the calcium assay (105 nM and 1.1 μM for SPC and LPC, respectively) are possibly derived from different coupling efficiencies of distinct signaling pathways and/or different cellular environments.

To determine which G protein and other signaling intermediates might be involved in the activation of SRE by SPC and 16:0-LPC, we pretreated cells with PTX (100 ng/ml) for 16 h, or co-transfected the specific Rho inhibitor C3-exoenzyme (1.5 μg of pcDNA3-C3), with the reporter system. Both PTX and C3-exoenzyme partially inhibited SRE-reporter activation (Fig. 6C). When the two inhibitors were added together, SRE-reporter activation in response to either SPC or 16:0-LPC was almost completely blocked, suggesting that Gi and Rho signaling pathways were involved in SRE activation through GPR4.

**SPC and LPC Activated ERK MAP Kinase in a GPR4-dependent Manner**—MAP kinases are key signaling intermediates of DNA synthesis and cell proliferation. To determine whether GPR4 mediates ERK MAP kinase activation in response to SPC and LPC, we conducted Western blot analyzes of GPR4-ires-GFP-Swiss 3T3 and GFP-Swiss 3T3 cells treated with SPC and 16:0-LPC. Cells were treated with SPC (100 nM) or LPC (100 nM) for 5 min in the absence or presence of PTX (100 ng/ml, 16 h pretreatment).

**Fig. 7.** DNA synthesis stimulated by SPC and 16:0-LPC in GPR4 overexpressing cells. DNA synthesis was measured by [3H]thymidine incorporation as described under "Experimental Procedures" in both GFP- and GPR4-ires-GFP-Swiss 3T3 cells. PTX was added to selected groups at 100 ng/ml for 16 h prior to lipid treatment. The data shown represent the mean ± S.D. from three independent experiments.
and found that at final solvent concentrations of 70–100% ethanol, and 100% methanol did not alter any parameters tested.

The higher potency of SPC over LPC was further reflected in the concentration- and time-dependent ERK activation (Fig. 7, B and C). ERK activation induced by SPC compared with that by LPC was evident at a lower concentration (~10 nM versus 100 nM), at earlier time points (1 min versus 5 min), and was maintained for a longer time. These results strengthen the notion that both SPC and LPC are ligands for GPR4, but SPC has a higher affinity than LPC for GPR4.

In GPR4-infected Swiss 3T3 cells, SPC-induced ERK activation was sensitive to PTX, suggesting involvement of G_i signaling (Fig. 7D). This is in contrast to our previous studies where SPC induced ERK activation via a PTX-insensitive pathway in OGR1-transfected HEK293 cells (2). To determine whether this difference was due to receptor subtype or different cell lines used, we tested the PTX sensitivity of SPC-induced ERK activation in OGR1-infected Swiss 3T3 cells. Our results showed that in Swiss 3T3 cells, SPC-induced ERK activation via OGR1 was PTX-insensitive (Fig. 7D). Thus, although GPR4 and OGR1 are highly homologous, the same high affinity ligand (SPC) induces activation of ERK through a different G protein pathway for each receptor.

**SPC Stimulated DNA Synthesis in GPR4-infected Swiss 3T3 Cells**—To determine whether SPC and LPC affect DNA synthesis in a GPR4-dependent fashion, we measured [3H]thymidine incorporation into GPR4-ires-GFP-Swiss 3T3 and GFP-Swiss 3T3 cells. SPC stimulated DNA synthesis in both parental and GFP-infected cells (~6.3-fold increase with 3 μM SPC). These results are qualitatively consistent with changes

---

**Fig. 9.** SPC and LPC stimulate cell migration in GPR4 overexpressing Swiss 3T3 cells. Cell migration was measured in a modified Boyden chamber assay as described under “Experimental Procedures.” The cell numbers on the lower faces of the membranes were determined and are presented as the mean ± S.D. of three independent experiments. **, *p < 0.01; ***, *p < 0.001, compared with the control. Student’s t test was performed using the GraphPad Instat software (San Diego, CA). p < 0.05 was considered to be statistically significant.

used in this study was 10 μM, the solvent content was ≤0.1% in any experiment. We routinely performed solvent controls and found that at final solvent concentrations of ≤0.1%, significantly to 16:0-LPC, whereas [3H]thymidine incorporation increased 1.6-fold in GPR4-infected Swiss 3T3 cells in response to 3 μM 16:0-LPC (Fig. 8B). Higher concentration of lipids did not further increase [3H]thymidine incorporation stimulated by SPC or LPC (data not shown).

**SPC and LPC Induced Cell Migration in a GPR4-dependent Manner**—As a major component of oxidized low-density lipoprotein, LPC has been proposed to play a role in atherosclerotic lesion development (30, 31). One of the roles of LPC potentially related to atherosclerosis is as a chemoattractant for monocytes, T lymphocytes, and smooth muscle cells (32–34). We used Swiss 3T3 cells infected with GFP or GPR4-ires-GFP as a model system to compare the effects of SPC and 16:0-LPC on cell migration. GPR4 overexpression in Swiss 3T3 fibroblasts increased cell migration in response to 16:0-LPC (Fig. 8A). Higher concentration of lipids did not further increase [3H]thymidine incorporation stimulated by SPC or LPC (data not shown).

Concentration response studies (Fig. 9B) indicate that SPC and LPC were effective at inducing cell migration in the 1–100 nM concentration range. To determine whether this effect was chemotactic or chemokinetic, we measured cells that migrated from the upper to the lower chambers in Boyden chamber assays, conducted with lipids (at 100 nM) in both upper and lower chambers. SPC or 16:0-LPC did not significantly change cell motility when compared with controls (without lipid in

---

**Withdrewn**

November 7, 2005
GPR4 bind SPC with similar affinities (33 and 36 nM, respectively) and pathological roles. We have shown that OGR1 and speculated that these two receptors may have overlapping

LPC increases intracellular Ca\(^{2+}\)/H\(_{11349}\) concentrations (\(\text{LPC acts through membrane receptors: (a) at relatively low}

fore, it is possible that some of the LPC effects

levels, in the liver, kidney, and ovary (Fig. 1), OGR1 is not expressed, whereas GPR4 is expressed at relatively high levels, in these tissues. Whereas GPR4 is expressed at high levels, in the testis, small intestine, and peripheral leukocytes (8, 18), whereas GPR4 is not expressed, or is expressed at relatively low levels, in these tissues. Whereas GPR4 is expressed at high levels, in the liver, kidney, and ovary (Fig. 1), OGR1 is not expressed in these tissues (8, 18). The physiological and pathological roles of these receptors remain to be further investigated.

Another significant finding from this study is the identification of GPR4 as the second G protein-coupled receptor for LPC (the first LPC receptor, G2A, was recently described (14)). GPR4 binds to LPC (in addition to SPC), but not PAF or lyso-PAF, to mediate an increase in intracellular calcium, receptor internalization, SRE activation, MAP kinase activation, and cytoskeleton. Both LPA and S1P are able to affect cytoskeleton (Fig. 5), suggesting that SPC and LPC may affect the cellular cytoskeleton. Both LPA and S1P are able to affect cytoskeleton (Fig. 5), suggesting that SPC and LPC may affect the cellular cytoskeleton. Either chamber) in either GFP or GFP-GPR4 expressing Swiss 3T3 cells (Fig. 9C). S1P slightly inhibited, PAF slightly enhanced, and LPA did not show a significant effect on cell migration in treated versus untreated GFP or GFP-GPR4 expressing cells (Fig. 9C). These results suggest that the effect of SPC and 16:0-LPC on cell migration was chemotactic, not chemokinetic, and that the chemotactic effect was mediated through GPR4.

**DISCUSSION**

GPR4 shares ∼50% homology with OGR1. We therefore speculated that these two receptors may have overlapping ligand specificity. Indeed, the results presented here show that GPR4 is a second high affinity receptor for SPC. OGR1 and GPR4 may play both overlapping and distinct physiological and pathological roles. We have shown that OGR1 and GPR4 bind SPC with similar affinities (33 and 36 nM, respectively) and both receptors mediate SPC-induced increases in intracellular calcium and ERK activation. However, GPR4 and OGR1-mediated ERK activation is PTX-sensitive and -insensitive, respectively (Fig. 7A), suggesting that GPR4 and OGR1 couple to different G proteins to activate ERK. More importantly, these differential couplings appear to lead to differential effects on cell proliferation. Whereas OGR1 mediates PTX-insensitive growth inhibition by SPC in a number of cells tested (2), GPR4 mediates PTX-sensitive DNA synthesis by SPC in Swiss 3T3 cells. Together, these data suggested that the endogenous receptor(s) for SPC in Swiss 3T3 cells was GPR4-like, rather than OGR1-like, because parental Swiss 3T3 cells respond to SPC to activate ERK and

**WTIHDAWN**

**NOVEMBER 7, 2005**

GPR4 as a Receptor for SPC and LPC

2 Y.-J. Xiao and Y. Xu, unpublished results.
lines tested. This cell line does not respond to either SPC or LPC in calcium assays (2). Therefore, calcium assays described here were performed in these cells. Because the transfection efficiency of MCF10A cells is very low (2), we were unable to establish stably expressing lines for conducting other assays. Despite their relatively high level of GPR4 expression, HEK293 cells were chosen for the internalization and SRE reporter assays, mainly because they are human in origin, and also yielded a high transfection efficiency (Fig. 1). The internalization assays utilized transfected receptor-GFP fusion proteins and the transcriptional responses in SRE reporter assays were compared with those in parental or vector-transfected cells. Therefore, the effects of the exogenous GPR4 receptor were readily separable from those of the endogenous receptor(s). CHO cells were chosen for binding assays, because they exhibit low responses to SPC and LPC in calcium assays and are readily transfected. We detected SPC- and LPC-induced MAP kinase activation through GPR4 in Swiss 3T3, but not HEK293 and CHO cells (Fig. 7 and data not shown). Hence, Swiss 3T3 cells were chosen for MAP kinase activation and mitogenic studies. It is well known that receptor-mediated signaling transduction is dependent on multiple cellular factors. The molecular basis for the differential activation of GPR4 in different cells remains to be further explored.

In summary, our results indicate that SPC is a high affinity, and LPC a lower affinity, ligand for GPR4. This conclusion is directly derived from the results of ligand binding assays (Kd values of 36 versus 159 nM for SPC and 16-0-LPC, respectively). This is also supported by results from assays of different signaling pathways activated by SPC and LPC, including

lipoprotein, which accumulates in atherosclerotic lesions (11), plays pathological roles in the development of atherosclerosis and other chronic inflammatory diseases (11, 12). LPC also plays other important biological roles. For example, LPC functions as a fatty acid and choline carrier and delivers fatty acids more specifically to brain than other tissues (22). The identification of GPR4 as a receptor for LPC and SPC solidifies the assignment of a new lysosphospholipid receptor subfamily (OGR1, GPR4, and G2A). Further studies should address possible functional redundancy among these receptors and add important information to our understanding of inflammatory diseases.

Acknowledgments—We thank Dr. Bryan Williams and Dr. Guy Chisolm for critical reading of this manuscript.

REFERENCES

1. Spiegel, S., and Milstien, S. (1995) J. Membr. Biol. 146, 225–237
2. Xu, Y., Zhu, K., Hong, G., Wu, W., Baudhuin, L. M., Xiao, Y., and Damron, D. S. (2000) Nat. Cell Biol. 2, 261–267
3. Heber, M., Docherty, J. M., Shah, G., Nguyen, T., Cheng, R., Heng, H. H., Marchese, A., Tsui, L. C., Shi, X., and George, S. R. (1995) DNA Cell Biol. 14, 25–35
4. Mahadevan, M. S., Baird, S., Bailly, J. E., Shutler, G. G., Sabourin, L. A., Tsiflidis, C., Neville, C. E., Narang, M., and Kornebich, R. G. (1995) Genoms 36, 84–88
5. Choi, J., Lee, S., and Choi, Y. (1996) Cell. Immunol. 168, 78–84
6. Kawan, H., Zeng, Z., Su, K., Fan, P., Shell, B., Carter, K., and Li, Y. (1998) DNA Cell Biol. 17, 493–500
7. Weng, Z., Fluckiger, A. C., Nisitani, S., Wahl, M. I., Le, L. Q., Hunter, C. A., Fernal, A. A., Le Beau, M. M., and Witte, O. N. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12334–12339
8. Xu, Y., and Caussy, G. (1996) Genoms 35, 397–402
9. Murphy, A. A., Santamum, N., Morales, A. J., and Parthasarthathy, S. (1998) J. Clin. Endocrinol. Metab. 83, 2110–2113
10. Choi, J. W., Lee, S. Y., and Choi, Y. (1996) Cell. Immunol. 168, 78–84
11. Chisolm, G. M., III, and Penn, M. (1996) Oxidized Lipoproteins and Atherosclerosis, Lippincott-Raven Publishers, Philadelphia, PA
12. Yokota, T., and Hansson, G. (1996) J. Intern. Med. 238, 479–489
13. Murugasu, G., and Fox, P. L. (1996) J. Clin. Invest. 97, 2736–2744
14. Kabarovski, J. H. S., Zhu, K., Le, L. Q., Witte, O. N., and Xu, Y. (2001) Science 293, 702–705
15. Im, D. S., Heise, C. E., Nguyen, T., O’Dowd, B. F., and Lynch, K. R. (2001) J. Cell Biol. 153, 429–433
16. Kabarowski, J. H. S., Fennemose, J. D., Le, L. Q., Gu, J. L., Luoh, S. W., Simon, M. I., and Witte, O. N. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12109–12114
17. Gryniewicz, G., Pocien, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
18. An, S., Tsai, C., and Goetzl, E. J. (1995) FEBS Letters 375, 121–124
19. Ogita, T., Tanaka, Y., Nakasaka, T., Matsuoka, R., Kira, Y., Nakamura, M., Shihmu, T., and Fujita, T. (1997) Am. J. Physiol. 272, H17–H24
20. Gomez-Munoz, A., O’Brien, L., Hundal, R., and Steinbrecher, U. P. (1999) J. Lipid Res. 40, 988–993
21. Huang, Y. H., Schaeffer, L., Wu, R., Claesson, H. E., and Frostegard, J. (1999) Clin. Exp. Immunol. 116, 326–331
22. Croset, M., Brossard, N., Polette, A., and Lagarde, M. (2000) Biochem. J. 345, 61–67
23. Carson, M. J., and Lo, D. (2001) Science 293, 618–619
24. Mukherjee, S., Ghosh, R. N., and Maxfield, P. R. (1997) Physiol. Rev. 77, 261–262

 withdrawing

NOVEMBER 7, 2005

Copyright © 2005 American Society for Biochemistry and Molecular Biology, Inc.

J. Biol. Chem. 280: 8007–8018

Downloaded from http://www.jbc.org/ by guest on July 26, 2018
Sphingosylphosphorylcholine and Lysophosphatidylcholine Are Ligands for the G Protein-coupled Receptor GPR4

Kui Zhu, Linnea M. Baudhuin, Guiying Hong, Freager S. Williams, Kelly L. Cristina, Janusz H. S. Kabarowski, Owen N. Witte and Yan Xu

J. Biol. Chem. 2001, 276:41325-41335.
doi: 10.1074/jbc.M008057200 originally published online September 4, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M008057200

Alerts:
  • When this article is cited  
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 15 of which can be accessed free at http://www.jbc.org/content/276/44/41325.full.html#ref-list-1
**VOLUME 276 (2001) PAGES 41325–41335**

**Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4.**

Kui Zhu, Linnea M. Baudhuin, Guiying Hong, Freager S. Williams, Kelly L. Cristina, Janusz H. S. Kabarowski, Owen N. Witte, and Yan Xu

This paper has been withdrawn.

---

**VOLUME 278 (2003) PAGES 48890–48897**

**Direct observation of G-protein binding to the human δ-opioid receptor using plasmon-waveguide resonance spectroscopy.**

Isabel D. Alves, Zdzislaw Salamon, Eva Varga, Henry I. Yamamura, Gordon Tollin, and Victor J. Hruby

PAGE 48891:

In the right column, under the heading "Lipid Bilayer Formation, hDOR Incorporation, and G-protein Addition," “methanol (0.05:0.95:0.5, v/v)” should read “methanol (0.05:9.5:0.5, v/v).”

---

**ADDITIONS AND CORRECTIONS**

This paper is available online at www.jbc.org

We suggest that subscribers photocopy these corrections and insert the photocopies in the original publication at the location of the original article. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.