Regulation of phospholipase C-\(\gamma\) activity by glycosphingolipids

Liming Shu, Lihsueh Lee, and James A. Shayman

Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan

Condensed titled: Glycolipids and phospholipase C

Key words: glycosphingolipids, glucosylceramide, phospholipase C, PDMP

Correspondence to: James A. Shayman
Nephrology Division, Department of Internal Medicine
University of Michigan
Box 0676, Room 1560 MSRB II
1150 West Medical Center Drive
Ann Arbor, Michigan 48109-0676
Telephone: 734-763-0992
Facsimile: 734-763-0982
Email: jshayman@umich.edu
Glycosphingolipid enriched domains are hot spots for cell signaling within plasma membranes and are characterized by the enrichment of glycosphingolipids. A role for glucosylceramide based glycosphingolipids in phospholipase C mediated inositol 1,4,5-trisphosphate formation has been previously documented. These earlier studies utilized a first generation glucosylceramide synthase inhibitor to deplete cells of their glycosphingolipids. Recently, more active and specific glucosylceramide synthase inhibitors, including $D$-threo-ethylendioxyphenyl-2-palmitoylamino-3-pyrrolidino-propanol ($D$-EtDO-P4), have been designed. $D$-EtDO-P4 has the advantage of blocking glucosylceramide synthase at low nanomolar concentrations but does not cause secondary elevations in cell ceramide levels. In the present study $D$-EtDO-P4 depleted cellular glucosylceramide and lactosylceramide in cultured ECV304 at nanomolar concentrations without obvious cellular toxicity. The expression of several signaling proteins was evaluated in glycosphingolipids depleted ECV304 cells in order to study the role of glycosphingolipids in phospholipase C mediated signaling. No difference was observed in the cellular expression of phospholipase C-$\gamma$ between controls and glycolipid depleted cells. Western blot analysis, however, revealed that depletion of endogenous glycosphingolipids in cultured ECV304 cells with $D$-EtDO-P4 induced tyrosine phosphorylation of phospholipase C-$\gamma$ in a concentration-dependent manner with maximum induction at 100 nM. The phosphorylation of phospholipase C-$\gamma$ induced by $D$-EtDO-P4 was abolished by exogenously added glucosylceramide, consistent with a specific glycosphingolipid-phospholipase C-$\gamma$ interaction. The phospholipase C-$\gamma$ phosphorylation was maximally enhanced by bradykinin when cells were exposed to 100
nM D-\textit{t}-EtDO-P4. The measurement of cellular activity of phospholipase C-\textgamma, by \textit{myo-inositol} 1,4,5-trisphosphate radioreceptor assay, demonstrated that depletion of glucosylceramide-based glycosphingolipids in cultured ECV304 cells with D-\textit{t}-EtDO-P4 resulted in significantly increased formation of inositol 1,4,5-trisphosphate above baseline, and an increased sensitivity of phospholipase C-\textgamma to bradykinin stimulation. Thus the activation of phospholipase C-\textgamma is negatively regulated by membrane glycosphingolipids in ECV304 cells.
**Introduction**

Caveolae are small invaginations in plasma membranes, recognized almost fifty years ago. (1,2). These invaginations depend on the expression of caveolin-1, an integral membrane protein. Caveolae are also characterized biochemically as low density membrane domains that are highly enriched in both cholesterol and glycosphingolipids. Cells that lack caveolin-1 also have low density domains commonly termed lipid rafts or glycosphingolipid-enriched microdomains.

Several investigators have provided support for the hypothesis that these microdomains are hot spots for cell signaling. Support for this view includes the observation that several signaling molecules are concentrated in these lipid domains. These molecules include EGF (3) and PDGF receptors (4), endothelin receptors (5), endothelial nitric oxide synthase (6), src family kinases (7), Grb2 (8), Shc (9), MAP kinase (10), and heterotrimeric and low molecular weight G proteins (11).

Phosphatidylinositol 4,5-bisphosphate (12,13) and sphingomyelin (14) are concentrated in caveolae. Both lipids are subject to hydrolysis following stimulation of cells with agonists. In addition, other receptors such as angiotensin II (15) and bradykinin (16) are recruited to caveolae following the stimulation of cells with agonists.

Cholesterol appears to be important for the regulation of signal transduction within these microdomains. For example, the depletion of cellular cholesterol with either filipin or lovastatin inhibits PDGF stimulated kinase activities. Similarly, the depletion of cholesterol with methyl β-cyclodextrin inhibits EGF and angiotensin II stimulated phosphatidylinositol hydrolysis (17). By contrast, less is known about the potential role of glycosphingolipids in the regulation of signaling events. In earlier work it had been
reported that glucosylceramide depletion of MDCK cells with a first generation
glucosylceramide synthase inhibitor, PDMP\(^1\), resulted in the enhanced formation of
inositol 1,4,5-trisphosphate following bradykinin stimulation (18). This observation had
several limitations. First, the glucosylceramide synthase inhibitor, PDMP, had limited
activity and specificity. Glucosylceramide depletion was also accompanied by an
increase in cell ceramide levels (19). Second, the mechanism of enhanced phospholipase
C activity was not delineated. Third, the little was known regarding the microdomains in
which phospholipase C activity was present to interpret the significance of this
observation.

A new generation of glucosylceramide synthase inhibitors has recently been
described (20,21). These inhibitors, typified by EtDO-P4, are active at low nanomolar
concentrations. These glucosylceramide synthase inhibitors also do not raise ceramide
levels at concentration at which near complete depletion of glucosylceramide and other
glucosylceramide based glycolipids occurs. EtDO-P4 and isotype specific antibodies to
phospholipase C-\(\gamma\) provide the basis for new studies on the mechanism of glycolipid
regulated inositol trisphosphate formation.

**Experimental procedures**

**Materials**

Protein A-agarose and bradykinin were purchased from Sigma Chemical Company (St.
Louis, MO). Mouse monoclonal antibodies to phospholipase C-\(\gamma\)1, annexin II,
phospholipase C-\(\delta\)1, PDGF-R\(\beta\), Ras, endothelial cell nitric oxide synthase III and c-Raf-
1 were obtained from Pharmingen/Transduction Laboratories (San Diego, CA). Rabbit
polyclonal anti-phosphotyrosine and monoclonal anti-bovine phospholipase C-β1 were from Upstate Biotechnology (Lake Placid, NY). The inositol 1,4,5-trisphosphate radioimmunoassay (1,4,5-IP$_3$) kit was acquired from NEN Life Science Products (Boston, MA). Horse radish peroxidase-conjugated goat anti-mouse IgG and prestained protein standards were purchased from Bio-Rad (Hercules, CA). [γ$^{-}$32P]-ATP was purchased from ICN (Costa Mesa, CA), and $sn$-1,2-diacylglycerol kinase was from CalBiochem (La Jolla, CA). D-[1-$^3$H]Galactose was obtained from Amersham Pharmacia Biotech (Buckinghamshire, England). D-$t$-EtDO-P4 was synthesized as previously described(21).

**Cell Culture**

Human ECV304 cells were routinely maintained in Medium 199 (M199) supplemented with 10% (v/v) newborn calf serum, 2 mM L-glutamine, 4.5 g/L D-glucose, 100 µg/mL streptomycin, and 100 U/mL penicillin. The cells were grown to subconfluence (90%) in either 100 mm or 150 mm culture dishes at 37°C in a 5% CO$_2$ enriched and humidified atmosphere. For treatment of cells with glucosylceramide synthase inhibitors, medium containing 10% serum was aspirated and replaced by serum-free M199 with or without D-$t$-EtDO-P4 for 48 h before each experiment. ECV304 cells co-incubated with glucosylceramide received 1 µM glucosylceramide or lactosylceramide administered as a liposomal preparation (18). Stock solutions of D-$t$-EtDO-P4 dissolved in 100% DMSO were diluted with serum-free M199 before use. The working solution of D-$t$-EtDO-P4/M199 contained 1% DMSO, and the final concentrations of DMSO in the incubation media were less than 0.03%. The normal morphology of cells and the total protein
content of cultured cells were unaffected by treatment with up to 3 µM D- t -EtDO-P4 for 48 hours.

**Immunoprecipitation and Immunoblotting**

ECV304 cell stimulation was conducted at 37°C following treatment with D- t -EtDO-P4. Quiescent cultures of ECV304 cells in 100 mm dishes (90% confluence) were stimulated with bradykinin (2 µM) for various times. Following stimulation, plates were washed twice with ice-cold phosphate-buffered saline containing 1 mM Na3VO4. The cells were lysed by the addition of 1 ml of ice-cold lysis buffer. Lysis buffer consisted of 25 mM Tris-HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 20 mM NaF, 2 mM EDTA, 2 mM Na3VO4, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupetin, and 10 µg/ml aprotinin. Plates were then left on ice for 10 min. The cells were harvested by scraping followed by sonication for 2 seconds twice with a probe sonicator. The homogenates were clarified by centrifugation at 16,000 x g for 15 min. Samples were normalized for equal amounts of protein using bicinchoninic acid assay (Sigma) with bovine serum albumin as a standard. For immunoprecipitation studies, cell lysates (600 µg of protein) were incubated with anti-phosphotyrosine (4 µg/ml) for 2 hours at 4°C with gentle rotation. After this, protein A-agarose beads (60 µl of a 50% suspension) were then added and the samples were rotated at 4°C for an additional 1-hour. The immune complexes were recovered by centrifugation at 16,000 x g for 30 seconds and washed four times with ice-cold buffer containing 25 mM Tris-HCl (pH, 7.4), 150 mM NaCl, 0.1% Triton X-100, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupetin, and 10 µg/ml aprotinin. The immunoprecipitates were recovered with Laemmlli
sample buffer (22) and heated for 5 min at 90°C. The immune precipitates or 10-50 µg protein of total cell lysates were then subjected to a 4-13% gradient SDS-PAGE and transferred to a nylon membrane (Life Technologies). The membranes were incubated with selected antibodies. Immunoblots were developed and visualized with the enhanced chemiluminescence-plus system (ECL-plus) (NEN Life Science Products).

**Extraction and Measurement of 1,4,5-IP₃**

Following treatment with D-ᵢ-EtDO-P₄, stimulated cells were exposed to 2 µM bradykinin at various times. The incubations were stopped with 100% ice-cold trichloroacetic acid. The dishes were left on ice for 10 minutes and the cells were scraped. The cell extracts were centrifuged for 1 min in a microcentrifuge at 12,000 x g. The trichloroacetic acid was removed from extracts by adding 2 mL of trioctylamine/1,1,2-trichloro-1,2,2-trifluoroethane mixture (1:3). After vigorous shaking the supernatants were partitioned, and a clear aqueous upper layer that contained water soluble 1,4,5-IP₃ was carefully removed and stored on ice for assay. The level of 1,4,5-IP₃ was determined by a competitive ligand-binding assay according to the manufacturer’s instructions (Amersham).

**Lipid extraction and analysis**

Cells cultured in 150 mm dishes were deprived of serum and incubated in the absence or presence of D-ᵢ-EtDO-P₄ (concentrations from 0.1 nM to 1 µM) for 48 hours. Cells were then washed with ice-cold phosphate buffered saline two times, fixed with 2 mL of ice-cold methanol and harvested by scraping. The lipids were extracted with chloroform-
methanol-water at a ratio of 1:2:0.8 (v/v/v). The samples were sonicated for 15 minutes in
a bath sonicator and centrifuged at 2200 \( x \) \( g \) for 30 minutes. The supernatants were
transferred into new glass tubes and pellets were reextracted with 3 ml of
chloroform/methanol (2:1, v/v). After a brief sonication, samples were centrifuged at
2200 \( x \) \( g \) for another 30 minutes. The resultant supernatants were pooled with the first
extracts and partitioned into aqueous and organic phases by the addition of chloroform
and water. The ratio of chloroform/methanol/water was adjusted to 2:1:0.8 (v/v/v). The
upper aqueous phase was removed and discarded after centrifugation at 500 \( x \) \( g \) for 5
minutes, and the lower organic phase was dried under a stream of nitrogen gas. The
residues were resuspended in 600 µl of chloroform/methanol (2:1, v/v). After
determination of lipid phosphate (23), a portion of the lipids (150 nmol total phospholipid
phosphate) was subjected to base hydrolysis by incubation with 2 mL of chloroform and
1 mL of 0.2 N NaOH in methanol at 37°C for 1 hour. The incubation was terminated by
the addition of 0.8 ml of 0.3 M acetic acid. The lower organic phase was washed with
methanol/water (1:0.8, v/v) two times and evaporated under a stream of nitrogen gas. The
residues were dissolved into 60 µl of chloroform/methanol (80:20, v/v) and analyzed by
high performance thin layer chromatography with a solvent system consisting of
chloroform/methanol/water (65:25:4, v/v/v). The glucosylerceramide and lactosylerceramide
levels were determined by densitometric scanning using NIH Image 1.62 software and
compared with authentic standards run in parallel on the same plates. The quantitative
measurement of ceramide was performed by the diacylglycerol kinase assay (24). Briefly,
lipid samples containing 50 nmol of total phospholipid phosphate were evaporated under
a steam of nitrogen gas. The ceramide in the lipid extract was converted to \(^{[32}P\)-
ceramide-1-phosphate by enzymatic reaction with diacylglycerol kinase. A set of ceramide standards was included with each experiment. The products were separated by high performance thin layer chromatography followed by autoradiography. The radioactive spots were scraped and counted by liquid scintillation spectrometry.

For the radiolabeling studies, cells were treated with vehicle or with 100 nM D-\textit{t}-EtDO-P4 for 12 h at 37°C. The cellular lipids were then radiolabeled by the addition of D-[\textit{1-3}H]galactose (0.5 mCi/ml, 6.1 Ci/mmol) for an additional 24 h before the extraction of total cellular lipids or before the isolation and extraction of the caveolar fractions. The glycosphingolipid-enriched fractions were isolated as previously described (25),

**Results**

D-\textit{t}-EtDO-P4 was first solubilized in 100% dimethyl sulfoxide and then diluted in culture medium prior to addition to achieve a final DMSO concentration of less than 0.03%. ECV304 cells grown in the medium containing up to 0.1% DMSO alone for 48 hours did not show any significant changes in cell morphology, cell viability, protein and total phospholipid content compared to control cells (data not shown). The cytotoxicity induced by D-\textit{t}-EtDO-P4 on the ECV304 cells was measured by trypan blue exclusion and lactate dehydrogenase release. The concentration at which toxicity was observed in 50% of the cultured cells (TC\textsubscript{50} value) was 6 ± 0.25 \mu M (n = 9) after a 48 hour incubation with D-\textit{t}-EtDO-P4.

The concentration dependent depletion of glucosylceramide and lactosylceramide in ECV304 cells by D-\textit{t}-EtDO-P4 was next determined (Figs. 1A and 1B). Cells were incubated with D-\textit{t}-EtDO-P4 for 48 hours at varying concentrations of inhibitor (0.1, 1,
10, 25, 50, 75, 100, and 150 nM) in serum-free medium. Under these conditions, treatment of cells with 100 nM D-\(\text{t}\)-EtDO-P4 resulted in maximal decrements of 99.9% glucosylceramide mass and 99.7% lactosylceramide mass, respectively. The concentrations at which half maximal depletion of glucosylceramide and lactosylceramide occurred were 0.2 ± 0.05 nM, and 0.4 ± 0.05 nM respectively. The ratio of TC\(_{50}/IC_{50}\) or selective index (SI) was >12,000. The toxicities associated with PDMP and earlier homologues of the glucosylceramide synthase inhibitor were associated with elevations in cell ceramide content.

To confirm the high selective index observed with D-\(\text{t}\)-EtDO-P4 treatment of the ECV304 cells occurred in the absence of changes in cell ceramide levels, ceramide content was determined using the diglyceride kinase assay. At concentrations where D-\(\text{t}\)-EtDO-P4 was highly effective in depleting glucosylceramide and lactosylceramide, cell ceramide levels were not significantly elevated in ECV304 cells (Fig. 2). Only a 10% increase in ceramide content was seen when ECV304 cells were incubated with 600 nM D-\(\text{t}\)-EtDO-P4 for 48 hours. No changes in sphingomyelin content were detected in cells treated with D-\(\text{t}\)-EtDO-P4 (data not shown). These results confirm that D-\(\text{t}\)-EtDO-P4 is both an active and specific inhibitor of glucosylceramide synthase.

The lipid composition of the intact ECV304 cells and the glycosphingolipid-enriched fractions were compared (Table I). As previously observed in NIH 3T3 cells, a significant enrichment of the major cellular sphingolipids was observed (25). A moderate enrichment of cholesterol was also seen. Triglyceride, on the other hand, was markedly deenriched in the sphingolipid enriched fractions. The effect of glucosylceramide synthase inhibition on the glycosphingolipids from these fractions was evaluated by
radiolabeling the ECV304 cells with D-[1-3H]galactose (Fig. 3). The major glycolipids labeled in the intact cells were glucosylceramide, lactosylceramide, globotriaosylceramide and ganglioside GM3. Globotriaosylceramide and ganglioside GM3 were not detected by charring of the thin layer chromatography plates. Their identification by radiolabeling was aided by the incorporation of the tritiated galactose into both glucose and galactose by the conversion of the radiolabeled substrate, UDP-glucose, to UDP-galactose by a cellular epimerase (18). The radiolabeling of glucosylceramide, lactosylceramide and globotriaosylceramide was increased in the sphingolipid enriched fractions. However, ganglioside GM3 was significantly deenriched in these fractions. The absence of ganglioside GM3 in these fractions is consistent with previous observations (26).

The expression of several signaling proteins including phospholipase C-γ, phospholipase C-β, phospholipase C-δ, eNOS, c-Raf-1 and Ras as well as annexin II was evaluated by immunoblot analysis following glycosphingolipid depletion with D-η-EtDO-P4 (Fig. 4). Because phospholipase C-γ has been identified as a key mediator of PDGF-dependent cellular transformation (27), the expression of PDGF-Rβ, therefore, was also examined. ECV304 cells were exposed for 48 hours to concentrations of D-η-EtDO-P4 ranging from 50 nM to 300 nM. Cell lysates containing equal amounts of protein were subjected to SDS-PAGE and immunoblotted. No significant differences were observed in the cellular expression of phospholipase C-γ1, phospholipase C-β1, phospholipase C-δ1, annexin II, eNOS III, c-Raf-1, PDGF-Rβ and Ras between control cells and cells treated with EtDO-P4. These results demonstrate that depletion of glucosylceramide-based
glycosphingolipids with EtDO-P4 has no effect on the expression of these proteins in cultured ECV304 cells.

Immunoprecipitation using anti-phosphotyrosine antibody followed by Western blot analysis with anti-phospholipase C-γ1 antibody revealed that greater than 99% depletion of glucosylceramide-based glycosphingolipids with 100 nM D-\(t\)-EtDO-P4 induced a significant increase in the tyrosine phosphorylation of phospholipase C-\(\gamma\) (Fig. 5A). The tyrosine phosphorylation was concentration dependent and paralleled the depletion of glucosylceramide and lactosylceramide. Concentrations of D-\(t\)-EtDO-P4 in excess of 100 nM, however, caused a reduction of phospholipase C-\(\gamma\)1 phosphorylation to basal levels. When ECV304 cells were simultaneously incubated with D-\(t\)-EtDO-P4 and exogenously added glucosylceramide (1 \(\mu\)M), the effect of D-\(t\)-EtDO-P4 on the induction of tyrosine phosphorylation of phospholipase C-\(\gamma\) was abrogated (Figs. 5B), consistent with a functional role of glycosphingolipids in the mediation of transient tyrosine phosphorylation of phospholipase C-\(\gamma\)1.

To determine whether the induction of phospholipase C-\(\gamma\)1 phosphorylation by D-\(t\)-EtDO-P4 resulted in a change in phospholipase C activity, the hydrolysis of phosphatidylinositol 4,5-bisphosphate in the glycosphingolipid depleted ECV304 cells was measured as inositol 1,4,5-trisphosphate formation. The concentration and time dependent effects of EtDO-4 on bradykinin stimulated inositol 1,4,5-trisphosphate formation were studied (Figs 6A-C). The peak formation of 1,4,5-IP\(_3\) formation was observed in cells were exposed to 100 nM EtDO-P4. At this concentration of inhibitor glucosylceramide content was maximally depleted and peak phosphorylation of phospholipase C-\(\gamma\)1 were noted. Cells were also stimulated with 2 \(\mu\)M bradykinin. A
markedly increased 1,4,5-IP₃ accumulation above baseline was observed in bradykinin stimulated cells treated with inhibitor. The peak effect was noted to occur at 100 nM EtDO-P4 (Fig. 6A).

The relationship between inhibitor concentration and peak 1,4,5-IP₃ formation was determined. The stimulation with bradykinin resulted in a more sustained generation of 1,4,5-IP₃ as the concentration of D-ß-EtDO-P4 increased (Figs. 6B and 6C). When control cells were stimulated by 2 µM bradykinin in the absence of inhibitor pretreatment, a peak of 1,4,5-IP₃ formation was observed at 30 second. In cells treated with 50 nM EtDO-P4 for 48 hours the peak formation of 1,4,5-IP₃ shifted to 1 min (Fig. 6B). Exposure of cells to 100 nM and 200 nM D-ß-EtDO-P4 for 48 hours resulted in a delay in the maximal formation of 1,4,5-IP₃ to 2 and 3 min, respectively (Figs. 6C).

The phosphorylation of phospholipase C-γ₁ was further assessed to determine whether the change in peak 1,4,5-IP₃ formation corresponded to a change in the tyrosine phosphorylation of the lipase. A peak in tyrosine phosphorylation of the phospholipase was noted to occur at 100 nM EtDO-P4 in the bradykinin stimulated cells (Fig. 7A), the same concentration noted in the unstimulated cells (Fig. 5). No change in total phospholipase C-γ₁ was observed. When the phosphorylation was evaluated as a function of time following bradykinin exposure, cells that were not treated with inhibitor demonstrated a peak tyrosine phosphorylation at 30 sec (Fig. 7B). In contrast, cells pretreated with 100 nM EtDO-P4 demonstrated a peak of tyrosine phosphorylation at 2 min. The time course was comparable to that observed for 1,4,5-IP₃ formation (Fig. 7C).
Discussion

The present study supports and extends earlier observations reported on the role of glucosylceramide-based glycolipids in the regulation of agonist-stimulated phospholipase C activity. In earlier studies the treatment of MDCK cells with PDMP, a first generation glucosylceramide synthase inhibitor, increased hormone stimulated 1,4,5-IP$_3$ levels significantly over baseline (18). The effect increased as a function of PDMP exposure and could be replicated in isolated plasma membranes. Furthermore, the increased 1,4,5-IP$_3$ occurred in membranes exposed to GTP$\gamma$S, consistent with an increased activity of phospholipase C. No changes in phosphatidylinositol 4,5-bisphosphate were observed. As in the present study the PDMP effect was reversed with exogenous glucosylceramide but not galactosylceramide addition. In a subsequent study, increases in endogenous glucosylceramide were shown to have the opposite effect (28). The incubation of MDCK cells with conduritol B epoxide, an inhibitor of β-glucocerebrosidase, inhibited phospholipase dependent 1,4,5-IP$_3$ formation. The conduritol B epoxide effect was both time and concentration dependent.

PDMP represents the prototypical glucosylceramide synthase inhibitor. This compound has found wide spread application as a tool for the cellular depletion of glucosylceramide based glycosphingolipids(20). PDMP contains two chiral carbons and thus four enantiomers. Only the D-threo enantiomer of this compound is active conferring a relative high degree of specificity. However, its use is limited in two respects. First, the inhibitory activity of PDMP against the cerebroside synthase is in the middle micromolar range. Second, PDMP treatment of cells results in the accumulation
of ceramide, a bioactive sphingolipid. The ceramide elevating effect was originally believed to be secondary to the accumulation of substrate for glucosylceramide formation. However, with the development of more active PDMP homologues, specifically 1-phenyl-2-palmitoyl-3-pyrrolidinopropanol, the ceramide elevation was shown to result from the inhibition of a second enzyme, 1-\(O\)-acylceramide synthase (29). This enzyme has phospholipase A2 activity and catalyzes the transacylation of \(sn-2\) fatty acids from phosphatidylethanolamine or phosphatidylcholine to the 1 hydroxyl of ceramide. Both PDMP and P4 inhibit the transacylase at micromolar concentrations.

The recognition of this second site of PDMP activity and the ability to dissociate ceramide accumulation from glucosylceramide depletion led to the design and synthesis of more active PDMP homologues by Hansch analysis (21). Phenyl group substitutions resulted in two compounds that were significantly more active against glucosylceramide synthase but retained limited activity against the transacylase. These compounds included the ethylendioxyphenyl- and 4’-hydroxyphenyl-P4 homologues. Both compounds significantly deplete glucosylceramide-based glycosphingolipids at concentrations between 10 and 100 nM without observable changes in ceramide content. Because the inhibitors have the ability to deplete glycosphingolipids without inducing cell toxicity, they have been proposed for use in the treatment of glucosylceramide-based glycosphingolipidoses, including Fabry disease (30).

In the present study, it has been demonstrated that glucosylceramide depletion in the absence of changes in ceramide content increases the activity of Phosphatidylinositol 4,5-bisphosphate specific phospholipase \(C_\gamma\). The increase in phospholipase activity is the result of changes in tyrosine phosphorylation and not the result of changes in recoverable
phospholipase Cγ. This conclusion is supported by the close relationships between the biphasic nature of the phosphorylation and the time course of phosphorylation following bradykinin stimulation.

These results extend those of other investigators that have implicated glycosphingolipid containing membrane domains and their unique biochemical composition in the regulation of phosphoinositide signaling. Pike and Casey first demonstrated that phosphatidylinositol 4,5-bisphosphate turnover was localized to caveolae (12). Subsequently it was demonstrated that EGF and bradykinin stimulated phosphatidylinositol turnover was inhibited by cholesterol depletion (31). This basic finding has been confirmed in more recent studies.

The mechanism of glycosphingolipid mediated regulation of phospholipase C activity remains undefined. Tyrosine phosphorylation of the phospholipase C predictably represents the balance between both kinase and phosphatase activities. Strong support exists for the direct association of ganglioside GD3 with the Src family tyrosine kinase Lyn (7). Whether glucosylceramide based glycosphingolipids interact with and regulate the activities of one or both of these enzymes or modulate the tyrosine phosphorylation of phospholipase Cγ by direct interaction with the lipase itself will require additional study.
Footnotes

* This work was supported by National Institutes of Health grant RO1 DK55823 (J.A.S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed.

1. Abbreviations used in this paper: GlcCer, glucosylceramide; D-\textit{t}-EtDO-P4, D-\textit{threo}-ethylendioxyphenyl-2-palmitoylamino-3-pyrrolidino-propanol; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-propanol.
References

1. Stan, R. V., Roberts, W. G., Predescu, D., Ihida, K., Saucan, L., Ghitescu, L., and Palade, G. E. (1997) Mol Biol Cell 8, 595-605.

2. Yamada, E. (1955) Journal of Biophysical and Biochemical Cytology 1, 455-457.

3. Mineo, C., James, G. L., Smart, E. J., and Anderson, R. G. (1996) J Biol Chem 271, 11930-11935.

4. Liu, P., Ying, Y., Ko, Y. G., and Anderson, R. G. (1996) J Biol Chem 271, 10299-10303.

5. Chun, M., Liyanage, U. K., Lisanti, M. P., and Lodish, H. F. (1994) Proc Natl Acad Sci U S A 91, 11728-11732.

6. Feron, O., Belhassen, L., Kobzik, L., Smith, T. W., Kelly, R. A., and Michel, T. (1996) J Biol Chem 271, 22810-22814.

7. Myers, S. J., and Stanley, K. K. (1999) Atherosclerosis 143, 389-397.

8. Wu, C., Butz, S., Ying, Y., and Anderson, R. G. (1997) J Biol Chem 272, 3554-3559.

9. Smith, R. M., Harada, S., Smith, J. A., Zhang, S., and Jarett, L. (1998) Cell Signal 10, 355-362.

10. Furuchi, T., and Anderson, R. G. (1998) J Biol Chem 273, 21099-21104.

11. Toselli, M., Taglietti, V., Flati, S., Pavan, A., Guzzi, F., and Parenti, M. (2001) J Physiol 536, 361-373.

12. Pike, L. J., and Casey, L. (1996) J Biol Chem 271, 26453-26456.
13. Liu, Y., Casey, L., and Pike, L. J. (1998) _Biochem Biophys Res Commun_ **245**, 684-690.

14. Dobrowsky, R. T., and Gazula, V. R. (2000) _Methods Enzymol_ **311**, 184-193

15. Ishizaka, N., Griendling, K. K., Lassegue, B., and Alexander, R. W. (1998) _Hypertension_ **32**, 459-466.

16. de Weerd, W. F., and Leeb-Lundberg, L. M. (1997) _J Biol Chem_ **272**, 17858-17866.

17. Ushio-Fukai, M., Hilenski, L., Santanam, N., Becker, P. L., Ma, Y., Griendling, K. K., and Alexander, R. W. (2001) _J Biol Chem_ **3**, 3

18. Shayman, J. A., Mahdiyoun, S., Deshmukh, G., Barcelon, F., Inokuchi, J., and Radin, N. S. (1990) _J Biol Chem_ **265**, 12135-12138.

19. Rani, C. S., Abe, A., Chang, Y., Rosenzweig, N., Saltiel, A. R., Radin, N. S., and Shayman, J. A. (1995) _J Biol Chem_ **270**, 2859-2867.

20. Shayman, J. A., Lee, L., Abe, A., and Shu, L. (2000) _Methods Enzymol_ **311**, 373-387

21. Lee, L., Abe, A., and Shayman, J. A. (1999) _J Biol Chem_ **274**, 14662-14669.

22. Laemmli, U. K. (1970) _Nature_ **227**, 680-685.

23. Ames, B. N. (1966) _Methods in Enzymology_ **8**, 115-188

24. Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E., and Bell, R. M. (1986) _J Biol Chem_ **261**, 8597-8600.
25. Shu, L., Lee, L., Chang, Y., Holzman, L. B., Edwards, C. A., Shelden, E., and Shayman, J. A. (2000) *Arch Biochem Biophys* **373**, 83-90.

26. Iwabuchi, K., Handa, K., and Hakomori, S. (2000) *Methods Enzymol* **312**, 488-494

27. DeMali, K. A., Whiteford, C. C., Ulug, E. T., and Kazlauskas, A. (1997) *J Biol Chem* **272**, 9011-9018.

28. Mahdiyoun, S., Deshmukh, G. D., Abe, A., Radin, N. S., and Shayman, J. A. (1992) *Arch Biochem Biophys* **292**, 506-511.

29. Abe, A., Shayman, J. A., and Radin, N. S. (1996) *J Biol Chem* **271**, 14383-14389.

30. Abe, A., Gregory, S., Lee, L., Killen, P. D., Brady, R. O., Kulkarni, A., and Shayman, J. A. (2000) *J Clin Invest* **105**, 1563-1571.

31. Pike, L. J., and Miller, J. M. (1998) *J Biol Chem* **273**, 22298-22304.
Figure legends

Fig. 1. Dose-dependent depletions of GlcCer and LacCer in D-t-EtDO-P4 treated ECV304 cells. Cultured cells were untreated or treated with D-t-EtDO-P4 at indicated concentrations for 48 hours. Total cellular lipids were extracted. Glucosylceramide and lactosylceramide mass were analyzed by high performance thin layer chromatography using a solvent system consisting of chloroform/methanol/water (65:25:4, v/v/v). The levels of glucosylceramide and lactosylceramide were determined by comparison to authentic standards run in parallel on the same plates. A. Dose-dependent changes on glucosylceramide content. B. Dose-dependent changes on lactosylceramide content. Results represent the mean ± S.E. of three independent experiments.

Fig. 2. Quantitative measurements of ceramide levels in the absence and presence of D-t-EtDO-P4. Whole cellular lipid extracts were normalized by lipid phosphate assay. The ceramide in the lipid extract (50 nmol of total lipid phosphate (l.p.)) was converted to $[^{32}\text{P}]$-ceramide-1-phosphate by enzymatic reaction with diacylglycerol kinase as described in Methods. The levels of $[^{32}\text{P}]$-ceramide were counted by liquid scintillation spectrometry and quantified according to a standard curve (n = 6).

Fig. 3. Autoradiography of the total cellular and caveolar lipids in the presence or absence of D-t-EtDO-P4. ECV304 cells were treated with 100 nM D-t-EtDO-P4 or vehicle alone for a total of 36 h. After an initial 12 h exposure to inhibitor, the cells were radiolabeled with D-[1-$^{3}$H]galactose for an additional 24 h prior to fractionation. Radiolabeled lipids were identified by autoradiography after separation by high
performance thin layer chromatography with a solvent system consisting of chloroform/methanol/water (65:25:4, v/v/v). The results are representative of four separate experiments.

**Fig. 4. Effects of D-t-EtDO-P4 on expressions of signaling enzymes in cultured** human ECV304 cells. Triton X-100-soluble cell lysates (10-50 µg protein) from control and treated cells with various concentrations of D-t-EtDO-P4 were separated by 4-13% gradient SDS-PAGE. Phospholipase C-γ1 (10 µg), phospholipase C-β1 (30 µg), PLC-δ1 (20 µg), PDGF-Rβ (20 µg), Annexin II (10 µg), eNOS III (50 µg), c-Raf-1 (20 µg), and Ras (20 µg) were detected by Western blot (WB) with their specific antibodies. The blots are representative of experiments performed using three independent cells cultures.

**Fig. 5. Effect of D-t-EtDO-P4 on tyrosine phosphorylation of phospholipase C-γ1 in** human ECV304 cells. The human ECV304 cells were incubated either with D-t-EtDO-P4 alone or simultaneously with D-t-EtDO-P4 and 1 µM glucosylceramide or lactosylceramide in liposomes for 48 h. Cell lysates (600 µg protein) were immunoprecipitated (IP) with anti-phosphotyrosine antibody (4 µg/ml). Immunoprecipitated proteins were subjected to 4-13% gradient SDS-PAGE, transferred to a nylon membrane, and probed with anti-phospholipase C-γ1 antibody. Results shown are representative of three separate experiments.

**Fig. 6. Effect of D-t-EtDO-P4 on phospholipase C-γ1 enzyme activity in human** ECV304 cells. A. Cells either untreated or treated with D-t-EtDO-P4 were stimulated
with or without 2 µM bradykinin for 2 min. B and C. Cells were incubated with D-\(t\)-EtDO-P4 at various concentrations. At the end of treatments, cells were stimulated by 2 µM bradykinin for the indicated time intervals. Bradykinin stimulation was stopped with 100% TCA, and 1,4,5-IP\(_3\) was extracted. The level of 1,4,5-IP\(_3\) was determined by a competitive ligand-binding assay and quantified by a standard curve. Results are expressed as mean ± S.E. of duplicate determinations from three similar experiments.

**Fig. 7. Enhancement of tyrosine phosphorylation of phospholipase C-\(\gamma\)1 by bradykinin stimulation in glycosphingolipid depleted human ECV304 cells.**

A. Controls and treated cells (100 nM D-\(t\)-EtDO-P4) were stimulated with 2 µM bradykinin for 2 min. Following stimulation, cells were lysed, and the cell lysates (600 µg protein) were immunoprecipitated with anti-phosphotyrosine antibody. The immune precipitates (*upper panel*) and 10 µg protein of cell lysates (*lower panel*) were subjected to a 4-13% gradient SDS-PAGE. After transfer the membrane was probed with a monoclonal antibody to phospholipase C-\(\gamma\)1. B. Control cells were stimulated with 2 µM bradykinin for different time intervals. C. Treated cells were exposed to 100 nM D-\(t\)-EtDO-P4 for 48 hours, and were then stimulated with 2 µM bradykinin for various times. A blot representative of three independent experiments is shown.
Table I. Lipid composition of ECV304 cells and sphingolipid enriched fractions isolated by density gradient centrifugation.

| Lipid                           | Whole cell lipids (nmol/2.0 x 10^6 cells) | Mole fraction of whole cell lipids | Microdomain lipids (nmol/2.0 x 10^6 cells) | Mole fraction of microdomain lipids | Fraction of whole cell lipids recovered in microdomain fractions |
|---------------------------------|------------------------------------------|-----------------------------------|-------------------------------------------|------------------------------------|---------------------------------------------------------------|
| Phosphatidylcholine             | 405 ± 15                                 | 0.27                              | 20 ± 3                                    | 0.22                               | 0.05                                                          |
| Phosphatidylethanolamine        | 256 ± 21                                 | 0.17                              | 14 ± 2                                    | 0.15                               | 0.055                                                         |
| Phosphatidylinositol and phosphatidylserine | 134 ± 8                              | 0.09                              | 3 ± 1                                     | 0.03                               | 0.02                                                          |
| Triglyceride                    | 225 ± 17                                 | 0.15                              | 1 ± 0.5                                   | 0.01                               | 0.004                                                         |
| Cholesterol                     | 355 ± 45                                 | 0.24                              | 35 ± 6                                    | 0.38                               | 0.10                                                          |
| Sphingomyelin                   | 98 ± 5                                   | 0.06                              | 13 ± 3                                    | 0.14                               | 0.13                                                          |
| Glucosylceramide                | 22 ± 4                                   | 0.015                             | 5 ± 1                                     | 0.05                               | 0.23                                                          |
| Lactosylceramide                | 2 ± 1                                    | 0.001                             | 0.5 ± 0.1                                 | 0.005                              | 0.25                                                          |

Whole cell and sphingolipid enriched domain lipids were measured as detailed under methods. The data represent the mean ± SD of three separate experiments. The recovered microdomain lipid phosphate was compared to the total cellular lipid phosphate in 2.0 x 10^7 cells. The microdomain lipid phosphate was 50 ± 2 nmol versus 1500 ± 100 nmol for intact cells (n=3).
by guest on March 17, 2020

D-\(\alpha\)-EtDO-P4 (100 nM)

GlcCer
LacCer
Gb\(_3\)
GM\(_3\)
IP: anti-Phosphotyrosine
WB: anti-PLCγ1

A.

D-\text{t}-\text{EtDO-P4} \quad 0 \quad 50 \quad 100 \quad 150 \quad 200 \quad 300 \quad \text{(nM)}

\rightarrow \text{PLC-γ1 (phosphorylated)}

B.

D-\text{t}-\text{EtDO-P4} \quad 0 \quad 50 \quad 100 \quad 150 \quad 200 \quad 300 \quad \text{(nM)}

\text{GlcCer} \quad + \quad + \quad + \quad + \quad + \quad + \quad \text{(1 \muM)}

\rightarrow \text{PLC-γ1 (phosphorylated)}

C.

D-\text{t}-\text{EtDO-P4} \quad 0 \quad 50 \quad 100 \quad 150 \quad 200 \quad 300 \quad \text{(nM)}

\text{LacCer} \quad + \quad + \quad + \quad + \quad + \quad + \quad \text{(1 \muM)}

\rightarrow \text{PLC-γ1 (phosphorylated)}

D.

D-\text{t}-\text{EtDO-P4} \quad 0 \quad 50 \quad 100 \quad 150 \quad 200 \quad 300 \quad \text{(nM)}

\text{liposomes (only)} \quad + \quad + \quad + \quad + \quad + \quad + \quad \text{(1 \muM)}

\rightarrow \text{PLC-γ1 (phosphorylated)}
A.  
IP: anti-Phosphotyrosine  
WB: anti-PLCγ1  

PLCγ1 (phosphorylated)

| D-t-O2P4-4 (nM) | 0  | 50 | 100 | 150 | 200 |
|-----------------|----|----|-----|-----|-----|
| BK (2 μM)       | +  | +  | +   | +   | +   |

B.  
IP: anti-Phosphotyrosine  
WB: anti-PLCγ1  

PLCγ1 (phosphorylated)

| BK (2 μM)       | 0  | 0.5 | 1   | 2   | 3   |
|-----------------|----|-----|-----|-----|-----|

C.  
IP: anti-Phosphotyrosine  
WB: anti-PLCγ1  

PLCγ1 (phosphorylated)

| D-t-O2P4-4 (nM) | 100 | 100 | 100 | 100 | 100 | 100 |
|-----------------|-----|-----|-----|-----|-----|-----|
| BK (2 μM)       | 0   | 0.5 | 1   | 2   | 3   | 4   |
