Effects of ceramide-1-phosphate on cultured cells: dependence on dodecane in the vehicle

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Abstract Ceramide-1-phosphate (C1P), the product of ceramide kinase, is a sphingophospholipid with recently recognized signaling properties. In particular, it was reported to be mitogenic and capable of direct stimulation of cytosolic phospholipase A2a. Much of the present knowledge has relied on the use of C1P of various acyl chain lengths, together with diverse protocols to deliver it to cultured cells. A mixture of ethanol (or methanol) with dodecane, as the vehicle, has become popular. However, the contribution of this solvent to the observed effects of C1P has not been documented. Here, we show that addition of C1P in ethanol-dodecane to culture medium leads to irreversible cytotoxic effects. These culminate in mitochondrial swelling, vacuole formation, and cell death. Not only the toxicity of C1P, but also its ability to trigger prostaglandin E2 release, is fully dependent upon addition of a premade C1P-dodecane mixture. Furthermore, we show that these effects are not restricted to C1P. They result from the capacity of dodecane to interact with phospholipids; hence, they go undetected with a vehicle control. This study should raise awareness about the use of dodecane for phospholipid delivery and, in turn, help unravel C1P signaling, which is still poorly understood.—Tauzin, L., C. Graf, M. Sun, P. Rovina, N. Bouveyron, M. Jaritz, A. Winiski, N. Hartmann, F. Staedtler, A. Billich, T. Baumruker, M. Zhang, and F. Bornancin. Effects of ceramide-1-phosphate on cultured cells: dependence on dodecane in the vehicle. J. Lipid Res. 2007. 48: 66–76.

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Ceramide-1-phosphate (C1P) was first described in brain synaptic vesicles, HL60 cells, and neutrophils (1–3). Recent insight since the cloning of ceramide kinase (4), the only known enzyme capable of generating C1P from ceramide, has provided evidence that C1P is endowed with signaling functions. C1P was reported to participate in eicosanoid synthesis (5), in which it synergizes with sphingosine-1-phosphate (6). This led to proposal of the cytosolic phospholipase A2a (cPLA2a) as the first molecular target of C1P (7). Other studies have suggested a role of ceramide kinase/C1P in mast cell degranulation (8), regulation of acidic sphingomyelinase and control of apoptosis in bone marrow-derived macrophages (9), calcium mobilization in GH4C1 pituitary and Jurkat T-cells (10, 11), and dopamine release in PC12 cells (12). A role for C1P in fibroblast proliferation was reported a decade ago (13, 14). More recently, C1P was shown to activate phosphatidylinositol 3-kinase and proposed to have a role in macrophage survival (15).

Whether C1P is present in the bloodstream or other extracellular fluids and can be taken up by cells under physiological conditions is currently unknown. Whether C1P interacts with cell surface receptors or needs to be transported across the plasma membrane is equally unknown. Nevertheless, many laboratories, including ours, have tried to elucidate ceramide kinase signaling pathways by adding exogenous C1P to cells in culture. Although reports are still not numerous, there is already complexity attributable to variability in the choice of the acyl chain length of the C1P used, or in the choice of the organic solvent used, such as DMSO (10, 11), methanol/chloroform (16), or ethanol (or methanol)/dodecane (5–7, 14); others have prepared fine dispersions of C1P by sonication of others have prepared fine dispersions of C1P by sonication of the solvent used, such as DMSO (10, 11), methanol/chloroform (16), or ethanol (or methanol)/dodecane (5–7, 14); others have prepared fine dispersions of C1P by sonication of C1P in aqueous medium (13, 15). The ethanol (or methanol)/dodecane mixture, in particular, used at 98:2, was first reported 10 years ago (14) and proposed to be useful to elucidate the biological functions and mechanisms of

Abbreviations: ATCC, American Type Culture Collection; C1P, ceramide-1-phosphate; cPLA2a, cytosolic phospholipase A2a; ER, endoplasmic reticulum; LAMP2, lysosome-associated membrane protein 2; NBD-Cer, nitrobenzo-2-oxa-1,3-diazole-labeled C6-ceramide; PA, phosphatidic acid; PG, phosphatidylglycerol; PGE2, prostaglandin E2; YFP, yellow fluorescent protein.

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action of CIP. Since then, it has become increasingly popular. In particular, two described processes, the mitogenicity of C1P (14) and its ability to stimulate cPLA2 (5–7), have relied on the use of dodecane.

When addressing the capacity of CIP to act as a mitogenic signal in fibroblasts, we observed, unexpectedly, that this phospholipid was toxic in the conditions used. The study reported here was undertaken to understand the molecular and cellular basis of these toxic effects.

**EXPERIMENTAL PROCEDURES**

**Materials**

Absolute ethanol and methanol were from Merck, and dodecane was from Aldrich. C8-ceramide and C8-CIP were from Cayman, C18-ceramide was from Avanti, and natural C1P (a mixture of C18-CIP and C24-CIP isolated from bovine brain) as well as C8-phosphatidic acid (C8-PA) and phosphatidic glycerol were from Sigma. Nitrobenzo-2-oxa-1,3-diazole-labeled C6-ceramide (NBD-Cer) was from Molecular Probes. HP-TLC plates were from Merck. Lipoprotein-free FBS and LDL were from Sigma.

**Plasmids**

Human lysosome-associated membrane protein 2 (LAMP2) cDNA was generated by PCR from a human multiple tissue cDNA library (Clontech) using primer A (5’TCCGCTGAGGTCGCGGCCTGCTCTGCGGGGTC) and primer B (5’GCCGCGATCCTGTGGTTGTTGTTGTTGCGAGGTCTGATATCCAGCA- TAACCTTT). For mammalian expression, a plasmid containing LAMP2 fused to yellow fluorescent protein (YFP), was constructed by in-frame insertion of the generated LAMP2 cDNA into the pEF-P-N1 vector, between XhoI and BamHI sites. LAMP2 was fused to the N terminus of YFP with a 6-histidine linker in between. The plasmid was verified by DNA sequencing on the entire fusion insert. Golgi-YFP plasmid was obtained from Clontech.

**Cell culture and treatments**

COS-1 cells (used in Figs. 2 and 5–7 below), obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, were cultured in DMEM with 10% FBS at 37°C and 5% CO2 in a humidified atmosphere. Cells were seeded on 24-well plates at 0.25 × 104 cells/cm² (0.06 × 104 cells/cm² for Fig. 2). Treatments were performed in 1% FBS-containing DMEM for 24 h, unless stated otherwise, and residual viability was measured using Alamar Blue (Serotec). A549 cells (used in Figs. 1 and 9 below), obtained from the American Type Culture Collection (ATCC), were grown in low-glucose DMEM with 10% FBS. For experiments, cells were seeded at 0.15 × 105 cells/cm². The FBS content was reduced to 2% at 2 h before treatments. After 2–5 h of treatment, prostaglandin E2 (PGE2) release was assayed in culture supernatants (see below). Primary fibroblasts (used in Figs. 2–4 below) were isolated from Balb/c mouse kidneys, grown in DMEM plus 10% FBS, and used within 7–10 days of isolation under the same conditions as for A549 cells, with seeding densities between 0.08 and 0.15 × 105 cells/cm². Wild-type CHO cells (used in Fig. 8 below and supplementary Fig. I), obtained from the ATCC, were maintained in Ham’s F-12 medium supplemented with 10% FBS (HyClone, Logan, UT). CV-1, a monkey kidney fibroblast cell line (used in Fig. 8 below), was obtained from the ATCC and grown in medium containing 90% Eagle’s MEM and 10% FBS. For cell treatment, FBS content was reduced to 2%. C8-CIP was used throughout the study.

**Fluorescence microscopy**

COS-1 cells were seeded into four chambered coverslips (Lab-TEK II; Nalgé Nunc) at 2 × 10⁶ cells per chamber. Twenty-four hours after seeding, cells were switched to 1% FBS-containing medium and treated. Live-cell fluorescence microscopy was done on an Axiovert 200 M inverted microscope equipped with an AxioCam MRc high-resolution microscopy camera (Zeiss) and Plan-NeoFluar 40×/1.30 oil differential interference contrast and Plan-Apochromat 63×/1.40 oil differential interference contrast objectives.

LAMP2-YFP, Golgi-YFP, or MitoTracker Green fluorescence in living CHO and CV1 cells was captured using charge-coupled device imaging systems that include standard filters for YFP or FITC, objectives of 100× magnification, a Nikon TE2000 microscope, and a CoolSnap HQ Monochrome charge-coupled device camera (Photometrics). The CoolSnap HQ camera was controlled with MetaMorph software (Universal Imaging Corp.). To visualize mitochondria with phase-contrast time-lapse imaging, cells were cultured in Lab-TEK chambered coverslips and imaged with a Hamamatsu electron microscopy camera and a Nikon TE300 microscope with 100× objective. Cells were kept in the LiveCell incubation system mounted on the Hamamatsu/Nikon TE300 imaging system, with 5% CO2 supply at 37°C, during the imaging intermission to keep them growing normally.

**Preparation of NBD-C1P**

NBD-C1P was prepared from NBD-Cer, using recombinant *Escherichia coli* diacylglycerol kinase (Calbiochem) and a cardio-lipin/acylglucosyl-based micellar assay, according to published procedures (18). NBD-C1P was separated from NBD-Cer by preparative HP-TLC using butanol-acetic acid-water (3:1:1) as the developing solvent. NBD-C1P was extracted two times with CHCl3/methanol/HCl (100:100:1) with vortexing followed by sonication, concentrated in a SpeedVac, and washed twice with ethanol. The obtained NBD-C1P, pure to 97%, was quantified with a spectrophotometer, based on the spectral characteristics of the NBD moiety, and dried down until use.

**DNA microarray analysis**

A549 cells were grown in 75 cm² flasks (1.1 × 10⁶/flask; day 1) in low-glucose DMEM with 10% FBS. On day 3, FBS was reduced to 2%. After a 2 h preincubation, cells were stimulated in quin-
duplicate with medium only (1 h), vehicle only [0.1% ethanol-
dodecane (98:2)] (1 and 3 h), or 10 μM C1P in ethanol-
dodecane (1 and 3 h). Cells were harvested with trypsin and spun.
Cells were lysed for total RNA extraction using Stratagene’s
Absolutely RNA Miniprep (400800), and the RNA was stored at
−20°C until target labeling. RNA integrity was confirmed using
denaturing agarose gel electrophoresis with 1% SeaKem Agarose
LE (Cambrex) prepared in 1% formaldehyde/MOPS buffer
(Ambion) as well as with the RNA 6000 Nano LabChip system
on Bioanalyzer 2100 (Agilent Technologies). The experiment
was conducted on a GeneChip Human Genome 133 2.0 Plus
Expression Array (Affymetrix). The oligonucleotide probes were
25-mers, and 11 probe pairs per sequence were used. Double-
stranded cDNA synthesis was carried out with 5 μg of total RNA
(SuperScript double-stranded cDNA synthesis kit; Invitrogen)
according to the manufacturer’s instructions. After purification
(QIAquick; Qiagen), the cDNA was transcribed in vitro in the
presence of biotinylated ribonucleotides (Bioarray high-yield
T7 DNA transcription kit; ENZO Life Sciences, Inc.). The la-
beled copy RNA was purified on an affinity resin (RNeasy;
Qiagen), quantified, and fragmented into strands of ~50–200
nucleotides in length. Hybridization was carried out at 45°C for
~18 h. The arrays were stained on a GeneChip Fluidics Work-
station 450 and scanned on GeneArray Scanner 3000 accord-
ing to the manufacturer’s technical manual (Affymetrix). The scanned images were converted into numerical values of signal
intensity and into categorical expression-level measurements using
Micro Array Analysis Suite version 5.0 software (MAS5; Affy-
metrix). The signal intensities were uploaded into GeneSpring
(Agilent Technologies) for filtering and statistical analysis. First,
the signal intensities of each sample were centered on 1 through
division by the individual sample mean. Then, the treatment
samples (C1P) were normalized toward the control samples
(vehicle). The probe was subsequently filtered for present or
marginal flags, MAS5 norm values > 50, and GeneSpring Error
Model-derived control values > 30.34. On average, a fraction of
~38% of all probe sets passed these filters. Finally, we filtered for
Benjamini and Hochberg false-discovery rate-adjusted t-test
P values < 0.01, which should reflect significant changes from 1.

RESULTS

Dodecane is needed for C1P-induced PGE2 release

The effects of exogenously added C1P in cells that have
been reported in the literature are based on the use of C1P
either dispersed in aqueous medium (13, 15) or dissolved
in DMSO (10, 11), in chloroform-methanol (16), or in a
mixture of ethanol (or methanol) and dodecane (5–7,
14). To evaluate and compare these vehicles for C1P deliv-
er, we used an already described functional readout, such
as the capacity of C1P to activate cPLA2α and trigger PGE2
production (5). We treated A549 cells with 10 μM C8-C1P,
dissolved in DMSO, ethanol, ethanol-dodecane (98:2), or
chloroform-methanol (50:50), or dispersed in medium after
sonication. As seen in Fig. 1, only when solubilized in
ethanol-dodecane could C1P stimulate PGE2 release after 2 h of incubation. Ethanol alone did not enable C1P to trigger PGE2 release. Of note, when viability was assayed subsequently, a significant decrease was observed, but only with cells that had been treated with C1P in ethanol-dodecane. Neither on PGE2 release nor on viability did the vehicle itself (ethanol-dodecane) exhibit any effect of its own.

PGE2 release and decreased viability as a function of dodecane concentration

In the experiment described above, we used a 2% dodecane-containing ethanolic solution to vehicle C1P, as described in the original study (14). We then examined the proportion of dodecane needed to support C1P-mediated effects. The lowest concentration of dodecane tested [0.1% (v/v) in ethanol] was already effective, resulting in some PGE2 release (Fig. 2). PGE2 release increased as a function of dodecane concentration, reaching a plateau between 0.5% and 1% dodecane. Strikingly, induction of PGE2 release and cell death occurred in parallel with the concentrations of dodecane in ethanol used to solubilize C1P (Fig. 2).
count to correct the potential of C1P to stimulate PGE\textsubscript{2} release (5), this would imply that the higher the molar ratio of dodecane to C1P, the more effective C1P appears to be. This experiment suggests that dodecane in the vehicle interacts with C1P in a way that cannot be recapitulated by an independent injection of vehicle at the time of cell treatment. Therefore, how the stock solution is prepared affects the results and the interpretations thereof. In the remainder of this study, except when stated otherwise, we used 10 μM C1P arising from 10 mM stock solutions made in ethanol-dodecane (98:2) (i.e., dodecane/C1P \(\gg\) 10).

Other phospholipids in ethanol-dodecane can induce PGE\textsubscript{2} release: dependence on cPLA\textsubscript{2}

Evidence was provided both in vitro and in cell-based assays that cPLA\textsubscript{2a} is a direct target of C1P (5–7). Furthermore, the selectivity toward C1P was established using lipid vesicles of various composition (7). Given the evidence reported above for a combination of C1P and dodecane as a basis for the induction of PGE\textsubscript{2} release, we wanted to examine the case of other phospholipids. As shown in Fig. 4A, PA was also able to promote PGE\textsubscript{2} release in fibroblasts in a concentration-dependent manner. That this is dependent upon cPLA\textsubscript{2} activity was established using the selective cPLA\textsubscript{2a} inhibitor, efipladib (19) (Fig. 4B). Therefore, it appears that the capacity of phospholipids to stimulate a cPLA\textsubscript{2}-dependent PGE\textsubscript{2} release when presented to cells in ethanol-dodecane is not restricted to C1P. Phosphatidylglycerol (PG) was also tested and showed enhanced PGE\textsubscript{2} release as well (see supplementary Fig. I). As noted above for Fig. 3, the final amplitude of the measured PGE\textsubscript{2} release seemed to inversely correlate with the induced toxicity of the respective phospholipid (Fig. 4 and supplementary Fig. I).

Dodecane does not improve the uptake of fluorescent C1P

The first study reporting the use of dodecane with C1P (14) mentioned its benefit to enhance C1P penetration into cells. We synthesized NBD-C1P from NBD-Cer and studied the cellular uptake of both lipids in the presence and absence of dodecane. As seen in Fig. 5A, NBD-Cer itself was readily absorbed and metabolized into glucosylceramide and sphingomyelin, regardless of the presence of dodecane. In sharp contrast, very little fluorescence was associated with cells treated with NBD-C1P (Fig. 5A). Most of it likely arose from the dephosphorylation of NBD-C1P and subsequent conversion to glucosylceramide and sphingomyelin. Only minute amounts of NBD-C1P were found associated with the cells after 5 min of incubation, which dodecane did not significantly increase (Fig. 5B, C). Therefore, the effects of dodecane may not result from increased delivery of C1P to cells.

C8-C1P with dodecane, but not C8-Cer with dodecane, is toxic to cells: protection by serum LDL

We compared the influence of dodecane on cell viability when used as a vehicle for Cer and C1P. Figure 6A shows that concentrations of either C8-C1P or C8-Cer < 0.5 μM are well tolerated and might even slightly increase viability compared with vehicle alone. However, at >0.5 μM, C8-C1P was found to be very toxic, with an EC\textsubscript{50} of \(\sim 3\) μM. This EC\textsubscript{50} value matches the concentrations that have been used to date to characterize C1P effects and reported

![Image](http://www.jlr.org/content/suppl/2007/01/10/M600399-JLR200700399-supplemental.html)
in the literature. Remarkably, C8-Cer, even at concentrations as high as 10 μM, was barely toxic. Similar observations were made when using natural C1P (a mixture of C18-C1P and C24-C1P; EC50 ≈ 3 μM) and C18-Cer (EC50 ≈ 10 μM) (data not shown). The toxicity of C1P in ethanol-dodecane was most obvious in the presence of low serum amounts but also occurred in the absence of serum and with increased serum levels (Fig. 6B). The partial protection afforded by increased concentrations of serum was lost when the serum used had been depleted of lipoproteins beforehand (Fig. 6C). Consistently, the addition of LDL to 1% serum-containing medium was sufficient to protect cells from C1P-mediated cell death (Fig. 6D). Epidermal growth factor could not substitute for LDL to prevent C1P/dodecane toxicity, implying that the protection afforded by LDL was not the result of an effect on cell proliferation (data not shown).

**C1P-dodecane-mediated cell death happens after drastic mitochondria vacuolation**

Addition of C1P in ethanol-dodecane (hereafter referred to as C1P-dodecane) rapidly affected cell viability (Fig. 7, left). Before cells eventually detached from the substratum (usually after a few hours; see below), cell viability had decreased dramatically, to ~30% of untreated levels. Moreover, the toxic effects of C1P-dodecane were found to be irreversible: changing the medium after various incubation times did not rescue the treated cells (Fig. 7, left). After 3 h of incubation in the presence of C1P-dodecane, cells had flattened and an intense production of vacuoles was observed in cells labeled with fluorescent calcein AM (Fig. 7, right). At later time points, cell extensions became visible, and from 5 h onward, cells massively detached from the substratum. Fluorescent calcein AM was also used to probe the viability of cells treated with C1P-dodecane. Combined with the ethidium homodimer-1 fluorescent dye, it failed to reveal any damage of the plasma membrane. This was further probed by measuring the extracellular release of lactate dehydrogenase, which did not increase upon C1P-dodecane treatment (data not shown). Therefore, although a contribution of the plasma membrane could not be ruled out, these observations suggested that the toxicity of C1P-dodecane probably affected intracellular organelles more than the plasma membrane itself.
To identify these organelles, we used fluorescent probes such as the LAMP2-YFP, which localizes to late endosomes and lysosomes, and Golgi-YFP. LAMP2-YFP was expressed as tubulovesicular structures in cells under vehicle control (see supplementary Fig. IIA–C). C1P-dodecane treatment did not significantly alter the distribution and morphology of the late endocytic compartment. Most of the vacuoles generated under C1P-dodecane treatment did not colocalize with the LAMP2 compartment (see supplementary Fig. IID–F). C1P-dodecane-induced vacuoles also did not colocalize with the Golgi-YFP-labeled Golgi apparatus (see supplementary Fig. IIG–I), even in cells rounded up under these conditions (see supplementary Fig. IIJ–L). Therefore, both probes indicate that the late endosomal and lysosomal compartments as well as the Golgi complex probably did not contribute significantly to the formation of vesicles that occurred upon C1P-dodecane treatment. In contrast, by tracking the mitochondria with the MitoTracker Green stain, we observed that C1P-dodecane-induced vacuoles are in fact swollen mitochondria (Fig. 8). Even more, there might be a possibility of fusion of mitochondria with the endoplasmic reticulum (ER), as indicated by the following: i) enlarged mitochondria appeared to be associated with ER fibers; ii) the MitoTracker stain was able to stain both mitochondria and ER in cells treated with C1P but not with vehicle (Fig. 8G, H); and iii) at the end of the swelling, large vacuoles appeared close to the nuclear envelope, a region that is part of the ER system (Fig. 8D–H).

DNA microarray analysis of C1P-dodecane-induced cellular effects

We performed a DNA microarray analysis of A549 cells treated either with or without vehicle or C1P at 10 μM for 1 or 3 h. This study indicated that C1P-dodecane can statistically significantly affect the expression of ~2,200 genes, as measured by 2,823 probe sets, most evident by 3 h (Fig. 9A; see supplementary Table I). Such a large set of regulated genes was unexpected. However, this may be consistent with the cellular stress induced by C1P-dodecane underscored in this study. According to MetaCore (GeneGo, Inc.), >300 gene ontology processes would statistically significantly associate with these 2,200 genes ($P \leq 0.01$). The top gene ontology processes were “transcription,” “regulation of transcription, DNA-dependent,” and “regulation of progression through cell cycle.” MetaCore also listed >80 statistically significantly associated known pathways (maps). Here, “MAPK cascade,” “IL-6,” and “IL-1” maps ranked top. Surprisingly, cyclooxygenase-2 mRNA was one of the most strongly upregulated genes upon C1P-dodecane treatment, whereas cPLA$_{2\alpha}$ mRNA levels were not modified (Fig. 9B).

DISCUSSION

When testing various solvents reportedly used for C1P cell delivery and monitoring PGE$_2$ release as a functional readout, we observed that ethanol, only when it contained dodecane, enabled the capacity of C1P to induce PGE$_2$ release (Fig. 1). The impact of dodecane increased in a concentration-dependent manner, reaching a plateau at 1% (Fig. 2). It is likely, therefore, that colloidal dispersions of C1P-dodecane are being produced via the injection of ethanolic solutions in culture medium (20, 21). To verify whether dodecane might have facilitated the cellular uptake of added C1P, we prepared fluorescent C1P. In
agreement with a recent report (22), the presence of dodecane did not enhance the uptake and metabolism of fluorescent NBD-Cer, which was followed in parallel (Fig. 5). We show now that dodecane did not increase the cellular uptake of NBD-C1P either (Fig. 5). This appears to contrast with the findings of Gomez-Munoz and coworkers (14), who reported an increased uptake of 32P-labeled C1P in the presence of methanol-dodecane. However, in this study, methanol-dodecane, as a vehicle for C1P, was not compared with methanol alone but rather with aqueous sonication of C1P. Collectively, therefore, it can be hypothesized that the permissive effect of dodecane on C1P-induced PGE2 release is not a consequence of an increased cellular uptake of C1P. Moreover, dodecane also supported PA and PG stimulation of PGE2 release (Fig. 4; see supplementary Fig. 1), leading us to consider a possible direct role of dodecane itself in these observed effects.

A recent study showed that dodecane is able to interact with phospholipid bilayers, leading to vesicle aggregation, perturbation of the gel-fluid transition and lamellar-hexagonal transitions, and promotion of flip-flop movements (23). This study further showed that alkane-to-phospholipid interaction ratios can exceed 1,000. We provide in Fig. 3 the first biological evidence suggesting that C1P-dodecane may cause mitochondrial and ER membrane fusion (G, H). Bar = 10 μm. Middle: CV-1 cells were treated with 10 μM C1P made from 10 mM stock solutions in ethanol-dodecane (98:2). Mitochondria were then labeled with MitoTracker Green FM in live cells as described above for CHO cells. In 50–70% of cells treated with C1P, besides vacuoles, MitoTracker also labels a network of fiber-like structures, including the nuclear envelope, which is reminiscent of endoplasmic reticulum (ER). Both swollen mitochondria and ER structures appear to associate with each other, indicating that C1P-dodecane may cause mitochondrial and ER membrane fusion. Time-lapse images were captured using phase-contrast to visualize mitochondrial morphology under C1P-dodecane treatment. Mitochondrial enlargement and vacuolation can be clearly followed (red arrows). The vacuolation started at ~20 min after treatment in such conditions. Most of the mitochondria became vacuolated in the cells.
of C1P to dodecane in the original preparation that is important. In this respect, the phospholipid (here C1P) acts to emulsify the alkane (here dodecane), the final emulsion being responsible for the observed cellular effects.

This work suggests that a combination of phospholipids, such as C1P or PA, with dodecane leads to an exquisite activation of the cPLA2/PGE2 cascade. Indeed, efipladib, a selective cPLA2 inhibitor, completely abolished stimulation (Fig. 4). Remarkably, microarray experiments (Fig. 9) showed increased cyclooxygenase-2 mRNA expression upon C1P-dodecane treatment. Therefore, if cPLA2 is indeed a target for C1P, working with C1P in ethanolic dodecane adds an unspecific level of stimulation of the arachidonic cascade that may explain the high levels of PGE2 release achievable. This unspecific activation may also explain why PA, which was reported to be incapable of cPLA2 activation (24), was found here to strongly induce PGE2 release. Of note, the concentration-dependence of C1P-dodecane-induced cell death paralleled that required for PGE2 production (Fig. 2). Nevertheless, these two events are not linked, because toxicity of C1P-dodecane also occurred in the presence of efipladib (data not shown).

C8-C1P-dodecane was shown to be more toxic than C8-Cer-dodecane (Figs. 6, 7). These findings may not account for the biological effect of the respective sphingolipids but rather may result from their distinct ability to interact with dodecane (23). C1P-dodecane-mediated toxicity was most evident in low-serum-containing medium, but remarkably, it was detectable at all serum concentrations, and even in its absence (Fig. 6). We showed that the lower toxicity observed at high serum levels results from the presence of increased levels of lipoproteins. Lipoproteins are enriched in phospholipids and therefore may be able to act as a sink. The original report on the use of C1P-dodecane (14) mentioned its positive effect on the proliferation of NIH 3T3 fibroblasts, constitutively over-expressing the epidermal growth factor receptor. In light of the toxicity of C1P-dodecane identified and characterized here, it is difficult to understand how this combination might have enhanced cell proliferation. Because recent...
was also able to stimulate PGE2 release and to give rise to vesicles, such as C1P-dodecane; PG-dodecane was less effective on PGE2 release, probably as a result of higher toxicity (see supplementary Fig. 1). Together, the molar ratio of the anionic phospholipid versus the alkane, rather than the identity of the phospholipid and the alkane, is likely to be key. In this context, the biophysical studies of Urbina et al. (23) showed that shorter alkanes, such as octane and decane, have lipid bilayer-modifying properties as well.

In summary, cellular activity of C1P, as reported here, is seen only when CIP and dodecane are added to the culture medium from a premade mixture in ethanol. The cellular effects, including major mitochondrial toxicity, are difficult to ascribe solely to CIP or dodecane, because they only occur in the presence of the C1P-dodecane combination. A decade ago, ethanol-dodecane was advertised as a delivery vehicle to elucidate the biological function of ceramide (27), and this was applied soon afterward to CIP (14). Since that time, many studies have been performed using this solvent mixture as a vehicle. However, results from this report indicate that interpretation of the data generated in these previous studies is not straightforward. For future work, attempts to modulate endogenous CIP levels, rather than exogenous administration, may prove more beneficial to understand the “true” cellular effects of C1P.

NOTE ADDED IN PROOF

The authors further checked by LC-MS, and found there was no change in C8-C1P cellular uptake after 1 hr, be the vehicle ethanol-dodecane or ethanol alone.

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