A ceramide analog inhibits cPLA2 activity and consequent PGE2 formation in LPS-stimulated macrophages

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1. Introduction

Prostaglandin E2 (PGE2) is one of the most well-studied members of the prostanoids family, which is derived from the unsaturated 20-carbon arachidonic acid (AA). PGE2 synthesis from phospholipids is carried out in three steps: first is the mobilization of AA from membranous phospholipids, through the action of phospholipase A2 (PLA2). There are three distinct groups of PLA2: cytosolic (cPLA2), secreted (sPLA2) and calcium-independent (iPLA2). Each group, subdivided to multiple subtypes, has specific function and localization, but in some cases they can overlap in their activities [1]. Second is the formation of PGH2 from AA by one of the cyclooxygenase (COX) isozymes; either the homeostatic COX-1 or the inducible COX-2. The latter is responsible for elevated production of PGE2 during inflammation [2]. The third step is the conversion of PGH2 to PGE2 by the action of PGE synthases [3].

PGE2 is produced, either constitutively or in response to an external stimulus, by almost all cell types, including immune cells. It exerts its actions by binding to any of its four receptors EP1-4 [4]. PGE2 is best known for its pro-inflammatory effects including plasma leakage, dilatation of smooth muscle, pain, and fever [5], but this lipid can also affect long-term processes, including the initiation and progression of cancer [6,7], Alzheimer [8] and cardiovascular diseases [9]. Additionally, PGE2 can exert homeostatic or in some cases anti-inflammatory effects [10].

Like other inflammatory modulators, PGE2 is essential for mounting a successful inflammatory response against invading pathogens and cancer cells [11], but unregulated or overproduction of PGE2 can lead to human pathology including arthritis [12], migraines [13] and general discomfort associated with inflammation. Therefore, inhibiting PGE2 production via the use of glucocorticosteroids or non-steroidal anti-inflammatory drugs is the common way of reducing pain and fever associated with inflammation.

Abbreviations: CERA-1, ceramide analogue-1; PCERA-1, phospho-ceramide analogue-1; AA, arachidonic acid; cPLA2, cytosolic phospholipase A2; PG, prostaglandin; COX, cyclooxygenase; NO, nitric oxide; C1P, ceramide-1-phosphate; S1P, sphingosine-1-phosphate; PC, phosphatidylcholine; PAPC, 1-palmitoyl-2- arachidonoylphosphatidylcholine; CalB, calcium-dependent lipid-binding.

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The sphingolipid ceramide-1-phosphate (C1P) and the synthetic phospho-ceramide analogue-1 (PCERA-1) have distinct non-overlapping receptor-mediated effects on RAW264.7 macrophages [14]. PCERA-1 is a potent modulator of macrophages activity and inflammation in vivo [15,16] and in vitro [17]. We have previously shown that PCERA-1 down regulates pro-inflammatory cytokine (TNFα, IL-12 and IL-23 p40) production and simultaneously up-regulates production of an anti-inflammatory cytokine (IL-10) [15,17,18]. While these receptor-mediated effects of PCERA-1 on cytokine production have been well documented, the effect of PCERA-1 on non-protein pro-inflammatory modulators has not been investigated. In this work we set out to determine the effect of PCERA-1 on production of the pro-inflammatory mediator PGE2 and nitric oxide (NO). We found that PCERA-1 suppressed LPS-induced PGE2 production by inhibiting the enzymatic activity of cPLA2. Our data further suggest that the inhibitory activity of PCERA-1 is attributed to its dephosphorylated derivative, ceramide analogue-1 (CERA-1), which directly inhibits cPLA2α in a mixed-micelle assay, mimicking the effect of ceramide itself.

2. Materials and methods

2.1. Reagents and cell culture

Lipopolysaccharide (LPS: Escherichia coli serotype 055:BS), PMSF, DMSO, [5,6,8,9,11,12,14,15-3H] radiolabelled AA and radiolabelled 3H-PGE2, and the antibodies against PGE2, COX-2 and α-tubulin were purchased from Sigma-Aldrich (St. Louis, MO). Macrophage colony stimulating factor (M-CSF) was purchased from PeproTech (Rocky Hill, NJ). Trypsin, l-glutamine, penicillin and streptomycin were purchased from Biological Industries (Beit Haemek, Israel). DMEM and FBS were purchased from Gibco (Carlsbad, CA). BSA was purchased from Amresco (Solon, OH). Palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (PAPC), D-e-C8-ceramide and D-e-C8-ceramide-1-phosphate (C1P) were purchased from Avanti Polar Lipids (Alabaster, AL). [14C]-PAPC was purchased from American Radiolabeled Chemicals (St. Louis, MO). Triton X-100 was purchased from Pierce (Rockford, IL). Recombinant mouse TNFα (rTNFα) was purchased from R&D Systems (Minneapolis, MN). Griess reagent was purchased from Promega (Madison, WI). Infrared dye-labelled secondary antibodies and blocking buffer were from LI-COR Biosciences (Lincoln, NE). Immobile-FL polyvinylidene fluoride (PVDF) membranes were from Millipore (Billerica, MA). Complete protease inhibitors mixture was purchased from Roche (Mannheim, Germany). PCERA-1 and its non-phosphorylated analog, CERA-1, were synthesized according to published procedures [19,20]. The sodium salt of PCERA-1 was dissolved in PBS, while the free acid PCERA-1 and CERA-1 were dissolved in ethanol. Recombinant human cPLA2α with a His6 tag was expressed and purified as previously described [21,22]. Mouse RAW264.7 macrophage cells, obtained from ATCC (Rockville, MD), were grown to 80–90% confluence in DMEM supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 12.5 U/ml Nystatin (hereafter culture medium) and 10% FBS, at 37 °C in a humidified incubator with 5% CO2.

2.2. Animal care

Male BALB/c mice (12 weeks old), obtained from the animal breeding center of Tel-Aviv University (TAU) were housed in a pathogen-free room under controlled temperature (22–23 °C), humidity, and lighting (12 h light–dark cycles), and were given access to food and water ad libitum. Animal care and experimentation was carried out in accordance with TAU guidelines.

2.3. PGE2 and NO assays

RAW264.7 macrophages were maintained for 48 h prior to the experiment in 96-well plates, at 2 × 10⁵ cells per well, in culture medium supplemented with 5% FBS, at 37 °C in a humidified incubator with 5% CO2. The culture medium was replaced 2 h before treatment in order to avoid the artifact of medium replacement on signaling [23]. The cells were stimulated with LPS (1 μg/ml) at 37 °C for 24 h and/or PCERA-1 (10 μM, unless otherwise indicated), or CERA-1 (50 μM), PGE2 secretion to the medium was measured by radioimmunoassay, as described by Peretz et al. [24]. NO was measured using the gries method according to the manufacturers instructions.

2.4. AA release assay in RAW264.7 macrophages

RAW264.7 cells were grown in 24-well plates, at 2 × 10⁵ cells per well, in culture medium supplemented with 10% FBS. After 24 h the medium was replaced and 0.25 μCi of [3H]-AA was added for an incubation period of 12–16 h. The cells were then thoroughly washed with PBS containing 0.1% BSA, and incubated in a fresh culture medium supplemented with 10% FBS with various treatments as indicated. At the time points indicated, the media were collected and centrifuged, and the radioactivity was determined in the supernatants. The data were presented as the mean ± S.D. (n = 3) of fold-stimulation compared with the control (vehicle-treated cells).

2.5. Isolation and AA release assay of mouse bone marrow-derived macrophages (BMDM)

BALB/c mice were sacrificed and the femoral and tibial marrow were flushed with sterile PBS using a 27-gage needle. Red blood cells were removed by osmotic shock. The cells were re-suspended in culture medium supplemented with 15% FBS and 10 ng/ml M-CSF (hereafter BMDM medium), seeded in Petri dishes at a density of 2.0 × 10⁵ cells per cm², and incubated at 37 °C in a humidified incubator with 5% CO2. Following 1 day, adherent cells were discarded, while non-adherent cells were centrifuged at 1000 × g for 5 min. Cell pellet was re-suspended in fresh BMDM medium (7 ml), and allowed to further differentiate. On day 3, fresh BMDM medium (6 ml) was added to the culture dish. On day 6, the non-adherent cells were discarded and a fresh BMDM medium (7 ml) was added. On day 7, the adherent cells (differentiated BMDM, ~98% homogenous by appearance), re-suspended in BMDM medium, were transferred to 24-well culture plates (0.3 ml/well), at 3 × 10⁵ cells per well. After 24 h, 0.25 μCi of [3H]-AA was added (in fresh medium with 0.1% FBS and 10 ng/ml M-CSF) for an incubation period of 16 h. The cells were then thoroughly washed with medium containing 0.1% BSA, re-suspended in BMDM medium, and treated with LPS (1 μg/ml) and/or CERA-1 (0–50 μM) for 10 h at 37 °C.

2.6. Mixed-micelle assay for cPLA2α

In vitro activity of cPLA2α was measured in a phosphatidylcholine (PC) mixed-micelle assay in a standard buffer composed of 80 mM HEPES (pH 7.5), 150 mM NaCl, 10 μM free Ca2+, and 1 mM dithiothreitol. The assay also contained 0.3 mM PAPC with 85,000 dpm [14C]-PAPC, 2 mM Triton X-100, 26% glycerol, and 1 μg of purified cPLA2α protein in a total volume of 200 μl.

To prepare the substrate, an appropriate volume of cold PAPC in chloroform, [14C]-PAPC in toluene–ethanol (1:1) solution, and the indicated lipids (C8–C1P, C8–ceramide, PCERA-1 free acid, or CERA-1) were evaporated under nitrogen. Triton X-100 was added to the dried lipid to give a 4-fold concentrated substrate solution (1.2 mM PAPC). The solution was probe-sonicated on ice (3 cycles of 1 min
on and 1 min off) and glycerol was added. The reaction was initiated by addition of the enzyme for 45 min at 37 °C, and was stopped by the addition of 2.5 ml Dole reagent (2-propanol/heptane/H2SO4 (0.5 M), in a 20:5:1 v/v/v ratio). Determination of the [14C]-AA product was performed using the Dole procedure as previously described [25,26].

2.7. Protein determination

Protein was determined by a modification of the Bradford procedure, which yields linear results, increased sensitivity, and reduced detergent interference, as previously described by Zor and Selinger [27]. BSA served as standard.

2.8. Statistical analysis

Data were analyzed using Student’s t-test wherever applicable. In all cases, differences of p < 0.05 were considered to be significant. EC50 values were calculated by non linear regression curve fitting, using the DeltaGraph software.

3. Results

3.1. PCERA-1 inhibits PGE2 production in LPS-stimulated macrophages

Treatment of RAW264.7 macrophage with LPS for a duration of 24 h increased PGE2 production by 22-fold (Fig. 1A). Co-treatment with PCERA-1 inhibited up to 66% of LPS-stimulated PGE2 production in a dose-dependent manner with an EC50 of 1 μM (Fig. 1A). To determine whether the residual PGE2 production is attributed to LPS or to PCERA-1 itself, the cells were treated with LPS and/or PCERA-1. While PCERA-1 inhibited 70% of the LPS-stimulated PGE2 production, PCERA-1 alone did not affect basal PGE2 production in unstimulated cells (Fig. 2A). As anti-inflammatory agents often suppress both PGE2 and NO [28,29], and since NO suppression may lead to PGE2 suppression [30], we decided to measure NO production in the LPS-stimulated RAW264.7 macrophages. Interestingly, PCERA-1 did not inhibit LPS-induced NO production (Fig. 1B). PGE2 production in LPS-stimulated macrophages can also be positively regulated by TNFα [31], which is released from these cells considerably earlier than PGE2, and thus accumulates in the culture medium. We have previously shown that PCERA-1 suppresses the production and secretion of TNFα in response to LPS-stimulation of macrophages [17]. Therefore, we examined now whether the inhibitory effect of PCERA-1 on PGE2 production is secondary to its inhibitory effect on TNFα secretion and subsequent autocrine or paracrine activity. To this end, exogenous TNFα was added to the cell culture (of all treatments) in excess, and LPS-induced PGE2 production was measured again in the presence and absence of PCERA-1. Fig. 2B shows that the effect of PCERA-1 on PGE2 production is independent of extra-cellular TNFα level.

The committed step of PG biosynthesis is performed by a cyclooxygenase that converts AA to PGH2, a precursor of PGE2 [2]. Expression of COX-2 is highly induced [32], and is essential for PGE2 production [33], in LPS-stimulated macrophages. We therefore investigated whether the effect of PCERA-1 on PGE2 production was correlated with modulation of COX-2 expression. As shown in
metabolites. Each data point represents the mean ± S.D. (n = 3).

Fig. 3. PCERA-1 does not affect expression of COX-2. RAW264.7 macrophages were incubated at 37 °C for 24 h with LPS (1 µg/ml) and/or PCERA-1 (10 µM). DMEM served as vehicle. Cell extracts (30 µg protein) were subjected to SDS-PAGE and, after transfer to immobilon-FL PVDF, the membranes were co-exposed to antibodies against COX-2 and tubulin (for normalization).

Fig. 3. LPS strikingly up-regulated the expression of COX-2, whereas PCERA-1 had no measurable effect by itself or in combination with LPS.

3.2. PCERA-1 inhibits LPS-stimulated AA formation

Production of the COX-2 substrate, AA, is considered to be the rate limiting step in PG formation. As PCERA-1 did not inhibit COX-2 expression, we next examined whether it acts by preventing the formation of AA in LPS-stimulated RAW264.7 macrophages. To this end we incubated the cells with radiolabelled AA for a period of 24 h, during which it was incorporated into triacylglycerols and phospholipids. The effect of LPS and PCERA-1 on the activity of PLA2 was then determined by measurement of AA release into the medium. Our data reveal that LPS-stimulated cells released radiolabelled AA and its metabolites into the medium with a 5-fold maximal increase at 12 h (Fig. 4A). The released radioactivity accounted for 5.0% of the AA that was incorporated into the cells. Strikingly, PCERA-1 completely abolished LPS stimulation of AA release (Fig. 4A). PCERA-1 itself had no effect on AA release in the absence of LPS (data not shown).

3.3. Non-phosphorylated CERA-1 inhibits LPS-stimulated AA release and PGE2 production, and in vitro cPLA2 activity

CERA-1, the non-phosphorylated derivative of PCERA-1, was initially used as a negative control in the AA release assay. Surprisingly, CERA-1 dose-dependently inhibited AA release in LPS-stimulated RAW264.7 macrophages (Fig. 5A). The apparent EC50 was an order of magnitude higher than that of PCERA-1 (compare Fig. 5A to Fig. 1A). The released radioactivity accounted for 12.2% of the AA that was incorporated into the cells.

Fig. 4. PCERA-1 blocks AA release from LPS-stimulated RAW264.7 cells. RAW264.7 macrophages were labelled with 3H-AA as described under Section 2. The cells were stimulated with LPS (1 µg/ml) in the presence (black) or absence (dashed line and diamonds) of PCERA-1 (10 µM), or treated with vehicle (gray). At the indicated time points the medium was examined for the presence of radiolabelled AA and its metabolites. Each data point represents the mean ± S.D. (n = 3).

Fig. 5. AA release and cPLA2 activity are inhibited by the phosphate-free CERA-1. RAW264.7 macrophages were labelled with 3H-AA as described under Section 2. The cells were incubated at 37 °C for 12 h with the indicated concentrations of CERA-1 (0–100 µM, represented by increasing gray intensity) and with LPS (1 µg/ml), and the medium was examined for the presence of radiolabelled AA and its metabolites. Each data point represents the mean ± S.D. (n = 3), relative to control cells that were not stimulated with LPS (also represented by the dashed line). (B and C) Recombinant cPLA2 activity was measured in a mixed-micelle assay for 45 min at 37 °C in the absence or presence of C8-C1P (2.5 mol %), and with either PCERA-1 (2.5 or 10 mol %), CERA-1 (10 mol %), C8-ceramide (10 mol %), or vehicle, as described under Section 2. Each data point represents the mean ± S.D. (n = 3).

The inhibitory activity of CERA-1 in the AA release assay could be either direct or possibly indirect, due to phosphorylation by a kinase resulting in the in situ formation of an active PCERA-1. To distinguish between these two scenarios, we tested the ability of PCERA-1 and CERA-1 to directly inhibit the in vitro activity of purified cPLA2α in a mixed-micelle assay, using radioactive PAPC as a substrate. Interestingly, cPLA2α activity was modestly elevated, rather than inhibited, by PCERA-1 (Fig. 5B). The endogenous sphingolipid C1P is a direct activator of cPLA2. To distinguish between these two scenarios, we tested the ability of PCERA-1 and CERA-1 to directly inhibit the in vitro activity of purified cPLA2α in a mixed-micelle assay, using radioactive PAPC as a substrate. Interestingly, cPLA2α activity was modestly elevated, rather than inhibited, by PCERA-1 (Fig. 5B). The endogenous sphingolipid C1P is a direct activator of cPLA2α [21,22,34], and therefore we hypothesized that PCERA-1, a synthetic analog of C1P, would be a competitive inhibitor of C1P. However, as shown in Fig. 5B, PCERA-1 did not affect C8-C1P-stimulated cPLA2α activity. In contrast, the phosphate-free compounds, CERA-1 and C8-ceramide, effectively inhibited the basal as well as the C8-C1P-stimulated in vitro activity of cPLA2α (Fig. 5C). Taken together, these findings suggest that PCERA-1 indirectly inhibits AA production in macrophages, via
Fig. 6A shows that PCERA-1 blocked 90% of PGE2 production during 24 h of LPS stimulation. However, exogenous AA fully rescued PGE2 production in these cells during the following 20 min (Fig. 6B), indicating that the effect of CERA-1 was limited to inhibiting the release of the COX substrate, AA. Collectively, these data point at CERA-1 as a modulator of PGE2 production in LPS-stimulated cells via direct cPLA2α inhibition.

3.4. CERA-1 inhibits AA release in bone marrow-derived macrophages (BMDM)

As the findings described above were observed in a macrophage cell line (RAW264.7), the effect of CERA-1 on AA release was also studied in BMDM, which are primary macrophages. In these cells, basal AA release accounted for 10.2% of the AA that was incorporated into the cells, and LPS treatment modestly elevated AA release to 14.0% of the total. Interestingly, CERA-1 dose-dependently inhibited both basal and LPS-stimulated AA release in these primary macrophages, with potency similar to that observed in RAW264.7 macrophages (Fig. 7). Thus, the observed activity of CERA-1 in an in vitro assay using a purified recombinant enzyme, as well as in primary macrophages, confirms that cPLA2 inhibition by CERA-1 is not influenced by the identity of the chosen cell line, and highlights the potential anti-inflammatory benefit of CERA-1 in an in vivo setting.

4. Discussion

Recent work carried out in our laboratory identified PCERA-1 as a potent immune modulator with the ability to down-regulate production of pro-inflammatory cytokines such as TNFα and IL-12p40, and to up-regulate production of the anti-inflammatory cytokines IL-10 [15,17,18]. In order to further elucidate the anti-inflammatory activity of PCERA-1 we examined whether PCERA-1 could affect the production and release of PGE2 and NO from LPS-stimulated macrophages. Production of these two inflammatory mediators is frequently coupled in LPS-stimulated macrophages, and NO positively regulates PGE2 production [30,35]. The results presented here reveal that while PCERA-1 was incapable of inhibiting NO production, it efficiently inhibited the formation of PGE2 in LPS-stimulated RAW264.7 macrophages. Protein expression levels of COX-2 were sharply induced by LPS treatment and this elevation was not affected by PCERA-1. Taken together, our findings regarding the lack of effect of PCERA-1 on COX-2 induction and on NO production, insinuated that PCERA-1 inhibits PGE2 production by down-regulating the enzymatic activity of COX-2, either directly or indirectly, rather than affecting its expression level. To evalu-
ate the mechanism by which PCERA-1 suppresses PGE2 production, we initially examined whether the inhibitory effect of PCERA-1 on TNFα production was involved, as TNFα by itself is capable of elevating PGE2 levels. We found that addition of exogenous TNFα did not abolish the effect of PCERA-1 on PGE2 production, indicating that the inhibitory effect of PCERA-1 on PGE2 production was not secondary to prior TNFα inhibition by PCERA-1.

PGE2 production depends on PLA2-mediated liberation of the COX substrate, AA, from phospholipids in cellular membranes. We found that PCERA-1 completely abolished LPS-stimulated release of AA in RW264.7 macrophages. The release of AA may be mediated by the products of several PLA2 genes (cPLA2, sPLA2, iPLA2), possessing the same enzymatic activity, but differing in cellular location and in mechanism of activation and regulation [1]. Our observation that PCERA-1 and its non-phosphorylated derivative CERA-1 directly affect the enzymatic activity of recombiant purified cPLA2α, is consistent with previous reports showing that cPLA2 is account-able for at least the majority, if not all, of LPS-induced AA release in RAW264.7 macrophages [36]. It is therefore inferred that PCERA-1 inhibited the activity of cPLA2 in LPS-stimulated macrophages.

It is well established that the sphingolipid sphingosine-1-phosphate (S1P) has both extra-cellular primary messenger and intra-cellular second messenger roles [37]. Likewise, recent reports have shown such a duality also for another endogenous sphingolipid, C1P, which stimulates macrophage migration in an extra-cellular receptor-mediated manner [38], whereas it poten-tially acts as an agonist in an intra-cellular receptor-independent manner [39] and Fig. 5B and C). C1P directly binds cPLA2, stabilizes the interaction of the enzyme with membranes where its substrate is found, and subsequently activates it [21,34,39]. C1P is endogenously formed by ceramide kinase upon stimulation of A549 lung adenocarcinoma cells with IL-β or with a Ca2+ ionophore [40]. Importantly, down-regulation of ceramide kinase in these cells by siRNA completely abolished AA release in response to IL-β or a Ca2+ ionophore, indicating that C1P is required for cPLA2 activation by these two agents [40]. When exogenous C1P was incubated with cells in culture, it underwent uptake and accumulation in the intra-cellular membranes, where activated cPLA2 can be found [39]. A mechanism of uptake into the cells was previously suggested by Boudker and Futerman to consist on the sequential activities of an extra-cellular-phoshatase and an intra-cellular kinase, which act on C1P and ceramide (following flip-flop), respectively [41]. We have previously shown that PCERA-1, a synthetic C1P analog, modulates cytokine production in macrophages in an extra-cellular manner, presumably via a GPCR [17,42]. While this as-of-yet unidentified PCERA-1 receptor may also be upstream of cPLA2 suppression, the different EC50 values for the effects of PCERA-1 on cytokine modulation and cPLA2 inhibition (0.1 μM and 1 μM, respectively), argues against receptor-dependency. Moreover, both PCERA-1 and CERA-1 inhibited LPS-induced AA release (Fig. 5A); CERA-1, but not PCERA-1, directly inhibited the in vitro enzymatic activity of cPLA2α (Fig. 5B and C); and in contrast PCERA-1, but not CERA-1, modulates cytokine production via a cell-surface receptor [17,42]. Taken together, these findings are consistent with a mechanism by which exogenously added PCERA-1, as suggested for C1P [41], is dephosphorylated in situ by a cell surface phosphatase, and the resulting hydrophobic CERA-1 penetrates the cell and directly inhibits cPLA2. The difference in potency between PCERA-1 and CERA-1 is likely to be related to their different solubilities. C1P and PCERA-1 have distinct non-overlapping receptor-mediated effects on RAW264.7 macrophages [14]. Interestingly, both C1P and PCERA-1 stimulated the enzymatic activity of cPLA2 in the in vitro assay (Fig. 5B). Therefore, in contrast to the distinct cell-surface receptors [14], C1P appears to be a common intra-cellular target for C1P and PCERA-1. Of note, the effect of PCERA-1 was considerably smaller than that of C1P, and PCERA-1 had no additive or inhibitory effect on C1P-stimulated cPLA2 activity (Fig. 5B), implying that PCERA-1 weakly interacts with the C1P-binding site on cPLA2.

 Whereas C1P and PCERA-1 stimulated cPLA2 activity, ceramide and CERA-1, their respective dephosphorylated derivatives, inhibited both basal and C1P-stimulated cPLA2 activity (Fig. 5C). Ceramide was more potent than CERA-1, in accordance with the relative potency of the phosphorylated compounds. Thus, C1P/PCERA-1 and ceramide/CERA-1 possibly act as agonists and inverse agonists, respectively, of cPLA2. In support of this suggestion, Huwiler et al. showed by photo-affinity labeling that ceramide specifically binds to the calcium-dependent lipid-binding (CaLB) domain of cPLA2 [43], while we have previously shown that the CaLB domain specifically binds C1P [21] and mediates its effect on cPLA2 activity [34]. In contrast to our finding, Huwiler et al. found that ceramide stimulated, rather than inhibited, cPLA2 activity [43]. The contrasting activity may possibly be explained by the different fatty acid chain lengths present in the ceramide species employed in the two studies. Alternatively, it is likely that the non-physiologic conditions (absence of salt and pH 8.5) employed by Huwiler et al. resulted in cPLA2 stimulation by ceramide, whereas the physiologic salt and pH conditions employed in our study led to cPLA2 inhibition by ceramide (Fig. 5C).

Kitanai et al. found that exogenous ceramide, as well as ceramide formed in situ following the addition of SMase, accel-erated calcium-dependent cPLA2 translocation and AA release [44]. The later discovery of the stimulatory role of C1P in cPLA2 translo-ca tion and AA release [34,40], rationalized the above effect of ceramide as being indirect, following conversion of ceramide to C1P by ceramide kinase. Accordingly, silencing of ceramide kinase by siRNA blocked the stimulatory effect of ceramide on AA release (D.S. Wijesinghe and C.E. Chalfant, unpublished results), indicating that C1P, rather than ceramide, activates cPLA2 in cells. The results presented here further demonstrate that ceramide and its synthetic analog CERA-1 inhibit cPLA2 activity in cells and in the mixed-micelle assay. Accordingly, exogenous AA fully rescued LPS-stimulated PGE2 production in CERA-1-treated cells, indicating that the defect in these cells is at the level of AA release, and not down-stream. Importantly, basal AA release in resting (not stimulated by LPS) primary macrophages was also inhibited by CERA-1. This find-ing further suggests that CERA-1 blocks the enzymatic activity of cPLA2, rather than interferes with upstream LPS signaling.

While our data are consistent with a mechanism of direct cPLA2 inhibition by CERA-1, it should be noted that this mechanism has not been explicitly demonstrated in cells. Therefore, we cannot rule out the possibility that CERA-1 regulates a biochemical step upstream of cPLA2 activation, in a receptor-independent manner. There are two observations that cast some doubt on a direct mecha-nism. First, we found that while PCERA-1 inhibited LPS-stimulated AA release, it did not inhibit zymosan-stimulated AA release (data not shown). Interestingly, we further observed that PCERA-1 failed to inhibit LPS-induced AA release in the presence of zymosan (data not shown), suggesting that with regards to PCERA-1 activity, the mechanism by which zymosan activates cPLA2 overrides the mechanism by which LPS activates cPLA2. Notably, enzymatic activation by LPS is considerably slower and lower in magnitude than by zymosan, implying distinct mechanisms [45]. The different kinetics of cPLA2 activation by LPS and zymosan, may therefore be consistent with a direct mechanism for CERA-1 that is relevant only for slow LPS-induced or basal cPLA2 activity, due possibly to slow accumulation of CERA-1 in internal membranes.

A second doubt on the direct mechanism arises from the high concentration (10 mol%) required for CERA-1 to significantly and directly inhibit the in vitro activity of cPLA2 in the mixed-micelle assay, a concentration that may be unreachable in a physiologi-
cal setting. However, it is possible that CERA-1 would be more potent in a native membrane than in the artificial mixed micelle that does not reflect the natural lipid composition. Moreover, CERA-1 may be actively transported and concentrated in a subset of internal membranes where activated cPLA2α resides, as has been demonstrated for exogenous C1P [39]. The synthesis of a traceable CERA-1 analog may help to explicitly determine whether CERA-1 indeed co-localizes with C1Pα, as suggested by the data presented here.

In conclusion, in this study we show that PCERA-1 inhibits PGE2 production in LPS-stimulated macrophages. The mechanism of this activity apparently involves PCERA-1 dephosphorylation, resulting in formation of CERA-1 which directly inhibits cPLA2-mediated AA production. Our in vitro study with recombinant cPLA2 suggests that CERA-1 mimics the activity of ceramide, blocking both basal and C1P-stimulated cPLA2 activity.

It was previously demonstrated by Matsui et al. that PCERA-1 dramatically rescues mice from sepsis caused by injection of a lethal dose of LPS [20]. The ability of PCERA-1 (via CERA-1) to inhibit LPS-induced PGE2 production in macrophages, taken together with our previous findings regarding the ability of PCERA-1 to suppress production of the pro-inflammatory cytokines TNFα, IL-12p40 and IL-23p40, and to elevate production of the anti-inflammatory cytokine IL-10, in LPS-stimulated macrophages and in vivo [14,15,17,18], provides a solid mechanistic basis for the potent in vivo activity of PCERA-1 in the animal model of septic shock. PCERA-1 may therefore be considered for the treatment of inflammation-linked pathologic states.

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