Published in final edited form as:

Immunol Lett. 2011 March 30; 135(1-2): 136–143. doi:10.1016/j.imlet.2010.10.014.

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

Meir Goldsmith^a, Ala Daka^a, Nadia F. Lamour^b, Roi Mashiach^c, Yifat Glucksam^a, Michael M. Meijler^c, Charles E. Chalfant^b, and Tsaffir Zor^{a,*}

^a Department of Biochemistry and Molecular Biology, Life Sciences Institute, Tel-Aviv University, Tel-Aviv 69978, Israel

^b Department of Biochemistry and Molecular Biology, Virginia Commonwealth University-School of Medicine, Richmond, VA 23298-0614; Hunter Holmes McGuire Veterans Administration Medical Center, Richmond, VA 23249; and The Massey Cancer Center, Richmond, VA 23298

^c Department of Chemistry, Ben-Gurion University of the Negev, Be'er-Sheva 84105, Israel

Abstract

Prostaglandin E₂ (PGE₂) is an important mediator of the inflammatory response. Phospho-ceramide analogue-1 (PCERA-1), a synthetic phospholipid-like molecule, was previously reported to modulate pro- and anti-inflammatory cytokine production. We show here that PCERA-1 inhibited LPS-stimulated PGE₂ production in RAW264.7 macrophages, without affecting COX-2 expression. Furthermore, PCERA-1 efficiently suppressed arachidonic acid (AA) release in response to LPS. The dephosphorylated derivative of PCERA-1, ceramide analogue-1 (CERA-1), mimicked the inhibitory effect of PCERA-1 on AA release and PGE₂ production in macrophages. Inhibition of PGE₂ production by CERA-1 was completely rescued by addition of exogenous AA. Importantly, PCERA-1 and ceramide-1-phosphate (C1P) stimulated the enzymatic activity of cPLA₂α in an *in-vitro* assay, whereas CERA-1 and ceramide inhibited both basal and C1P-stimulated cPLA₂α activity. Collectively, these results indicate that CERA-1 suppresses AA release and subsequent PGE₂ production in LPS-stimulated macrophages by direct interaction with cPLA₂, and suggest that ceramide may similarly counteract C1P effect on cPLA₂ activity in cells. The suppression of PGE₂ production is suggested to contribute to the anti-inflammatory action of PCERA-1.

Keywords

phospholipase A₂; arachidonic acid; prostaglandin E₂; LPS; ceramide; ceramide-1-phosphate

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

*Corresponding author. Tel/Fax: 972-3-640-7192; tsaffyz@tauex.tau.ac.il.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 activates [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.

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 (TNF α , 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 mediators 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 cPLA₂ α 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:B5), PMSF, DMSO, [5,6,8,9,11,12,14,15-³H] radiolabeled AA and radiolabeled 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). 1-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 TNF α (rTNF α) was purchased from R&D Systems (Minneapolis, MN). Griess reagent was purchased from Promega (Madison, WI).

Infrared dye-labeled secondary antibodies and blocking buffer were from Li-COR Biosciences (Lincoln, NE). Immobilon-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 MEM medium 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 hours 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 hours prior to the experiment in 96-well plates, at $2 \cdot 10^5$ 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 hours 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 hours 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 griess 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 $2 \cdot 10^5$ cells per well, in culture medium supplemented with 10% FBS. After 24 hours the medium was replaced and 0.25 µCi of [³H]-AA was added for an incubation period of 12–16 hr. 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 is 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 was 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 $2.0 \cdot 10^5$ 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 1000g for 5 min. cells 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 $3 \cdot 10^5$ cells per

well. After 24 hours, 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 hours. The cells were then thoroughly washed with medium containing 0.1% BSA, re-suspended in BMDM medium, and treated with LPS (1 $\mu\text{g/ml}$) and/or CERA-1 (0–50 μM) for 10 hr at 37°C.

2.6. Mixed-Micelle Assay for cPLA $_2\alpha$

In-vitro activity of cPLA $_2\alpha$ 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 Ca^{2+} , and 1 mM dithiothreitol. The assay also contained 0.3 mM PAPC with 85,000 dpm [^{14}C]-PAPC, 2 mM Triton X-100, 26% glycerol, and 1 μg of purified cPLA $_2$ protein in a total volume of 200 μl .

To prepare the substrate, an appropriate volume of cold PAPC in chloroform, [^{14}C]-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/ H_2SO_4 (0.5 M), in a 20:5:1 v/v/v ratio). Determination of the [^{14}C]-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. EC_{50} values were calculated by non linear regression curve fitting, using the DeltaGraph software.

3. Results

3.1. PCERA-1 inhibits PGE $_2$ production in LPS-stimulated macrophages

Treatment of RAW264.7 macrophages with LPS for a duration of 24 hours increased PGE $_2$ production by 22-fold (Fig. 1A). Co-treatment with PCERA-1 inhibited up to 66% of LPS-stimulated PGE $_2$ production in a dose-dependent manner with an EC_{50} of 1 μM (Fig. 1A). To determine whether the residual PGE $_2$ 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 PGE $_2$ production, PCERA-1 alone did not affect basal PGE $_2$ production in unstimulated cells (Fig. 2A). As anti-inflammatory agents often suppress both PGE $_2$ and NO [28,29], and since NO suppression may lead to PGE $_2$ 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). PGE $_2$ production in LPS-stimulated macrophages can also be positively regulated by TNF α [31], which is released from these cells considerably earlier than PGE $_2$, and thus accumulates in the culture medium. We have previously shown that PCERA-1 suppresses the production and secretion of TNF α in response to LPS-stimulation of macrophages [17]. Therefore, we examined now whether the inhibitory effect of PCERA-1 on PGE $_2$ production is secondary to its inhibitory effect on TNF α secretion and subsequent autocrine or paracrine activity. To this end, exogenous TNF α 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. Figure 2B shows that the effect of PCERA-1 on PGE₂ production is independent of extra-cellular TNF α 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 figure 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 hr, 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 hours (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 α 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.

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 cPLA₂ α in a mixed-micelle assay, using radioactive PAPC as a substrate. Interestingly, cPLA₂ α activity was modestly elevated, rather than inhibited, by PCERA-1 (Fig. 5B). The endogenous sphingolipid C1P is a direct activator of cPLA₂ α [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 cPLA₂ α 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 cPLA₂ α (Fig. 5C). Taken together, these findings suggest that PCERA-1 indirectly inhibits AA production in macrophages, via dephosphorylation and direct inhibition of cPLA₂ α by CERA-1.

Next, we measured the effect of the phosphate-free CERA-1 on LPS-stimulated production of PGE₂ in the RAW264.7 macrophages. As expected, we found that CERA-1 efficiently inhibited PGE₂ production in LPS-stimulated cells (Fig. 6A), mimicking its parent compound, PCERA-1 (Fig. 1A and 2A–B). The apparent EC₅₀ was similar to that obtained

for inhibition of AA release (compare to Fig. 5A). CERA-1 itself had no measurable activity in resting macrophages (Fig. 6B).

To further confirm the mechanism of suppression of PGE₂ production by CERA-1, we performed an experiment in which the production of PGE₂ in RAW264.7 macrophages was measured at two distinct phases. Initially, the cells were treated with LPS and/or CERA-1 for 24 hours, and the medium was collected for PGE₂ analysis. This first phase examines the effect of CERA-1 on the whole process of PGE₂ production. Then, the cells were washed, incubated with exogenous AA for a short period of 20 min, and the medium was collected again for PGE₂ analysis. The exogenous AA is expected to rescue PGE₂ production in CERA-1-treated cells, if the only defect in these cells is AA release. Thus, this second phase is designed to be distinct between inhibition of AA release and any inhibitory effect on the downstream enzymes, COX and PGE synthase. As shown in Fig. 6B, CERA-1 blocked 90% of PGE₂ production during 24 hours 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 cPLA₂ 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 TNF α and IL-12p40, and to up-regulate production of the anti-inflammatory cytokine 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 evaluate the mechanism by which PCERA-1 suppresses PGE₂ production, we initially examined whether the inhibitory effect of PCERA-1 on TNF α production was involved, as TNF α by itself is capable of elevating PGE₂ levels. We found that addition of exogenous TNF α 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 TNF α 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 RAW264.7 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 recombinant purified cPLA₂ α , is consistent with previous reports showing that cPLA₂ is accountable 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 potentiates cPLA₂ activity in an intra-cellular receptor-independent manner ([39] and Fig. 5B–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-1 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-1 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 phosphatase 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 μ M and 1 μ M, 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 cPLA₂ α (Fig. 5B–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 solubility.

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], cPLA₂ 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 length 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).

Kitatani *et al.* found that exogenous ceramide, as well as ceramide formed *in-situ* following the addition of SMase, accelerated calcium-dependent cPLA₂ translocation and AA release [44]. The later discovery of the stimulatory role of C1P in cPLA₂ translocation 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 downstream. Importantly, basal AA release in resting (not stimulated by LPS) primary macrophages was also inhibited by CERA-1. This finding 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 can not 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 mechanism. 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 rises 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 physiological 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 cPLA₂α, 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 TNF α , 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], provide 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.

Acknowledgments

This work was supported by a grant from the European Commission (IRG #021862 to T.Z.) and by grants from Veteran's Administration (VA Merit Review I and a Research Career Scientist Award to C.E.C.) and from the National Institutes of Health (HL072925 and CA117950 to C.E.C.). We are grateful to Mrs. Nava Silberstein for superb technical assistance. We thank Dr. Hugh Rosen and Dr. Nathanael S. Gray for supply of reagents, and specifically Mr. Peter Ding and Dr. Mark Parnell for chemical synthesis of PCERA-1. We are grateful to Orna Ernst and to Dr. Amiram Raz for helpful advises and for critical reading of the manuscript.

Abbreviations

CERA-1	ceramide analogue-1
PCERA-1	phospho-ceramide analogue-1
AA	arachidonic acid
cPLA₂	cytosolic phospholipase A ₂
PG	prostaglandin
COX	cyclooxygenase
NO	nitric oxide
C1P	ceramide-1-phosphate
S1P	sphingosine-1-phosphate
PC	phosphatidylcholine
PAPC	1-palmitoyl-2-arachidonoylphosphotidylcholine
CaLB	calcium-dependent lipid-binding

References

1. Kudo I, Murakami M. Phospholipase A₂ enzymes. Prostaglandins Other Lipid Mediat. 2002; 68–69:3–58.
2. Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol. 1998; 38:97–120. [PubMed: 9597150]
3. Noguchi K, Ishikawa I. The roles of cyclooxygenase-2 and prostaglandin E₂ in periodontal disease. Periodontol 2000. 2007; 43:85–101. [PubMed: 17214837]

4. Negishi M, Sugimoto Y, Ichikawa A. Prostaglandin E receptors. *J Lipid Mediat Cell Signal*. 1995; 12:379–391. [PubMed: 8777580]
5. Harris SG, Padilla J, Koumas L, Ray D, Phipps RP. Prostaglandins as modulators of immunity. *Trends Immunol*. 2002; 23:144–150. [PubMed: 11864843]
6. Pereg D, Lishner M. Non-steroidal anti-inflammatory drugs for the prevention and treatment of cancer. *J Intern Med*. 2005; 258:115–123. [PubMed: 16018788]
7. Wang D, Dubois RN. Prostaglandins and cancer. *Gut*. 2006; 55:115–122. [PubMed: 16118353]
8. Hoshino T, Nakaya T, Homan T, Tanaka K, Sugimoto Y, Araki W, Narita M, Narumiya S, Suzuki T, Mizushima T. Involvement of prostaglandin E2 in production of amyloid-beta peptides both in vitro and in vivo. *J Biol Chem*. 2007; 282:32676–32688. [PubMed: 17767011]
9. Cheng Y, Wang M, Yu Y, Lawson J, Funk CD, Fitzgerald GA. Cyclooxygenases, microsomal prostaglandin E synthase-1, and cardiovascular function. *J Clin Invest*. 2006; 116:1391–1399. [PubMed: 16614756]
10. Park JY, Pillinger MH, Abramson SB. Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases. *Clin Immunol*. 2006; 119:229–240. [PubMed: 16540375]
11. Rocca B, FitzGerald GA. Cyclooxygenases and prostaglandins: shaping up the immune response. *Int Immunopharmacol*. 2002; 2:603–630. [PubMed: 12013502]
12. Egan KM, Wang M, Fries S, Lucitt MB, Zukas AM, Pure E, Lawson JA, FitzGerald GA. Cyclooxygenases, thromboxane, and atherosclerosis: plaque destabilization by cyclooxygenase-2 inhibition combined with thromboxane receptor antagonism. *Circulation*. 2005; 111:334–342. [PubMed: 15655126]
13. Davis RJ, Murdoch CE, Ali M, Purbrick S, Ravid R, Baxter GS, Tilford N, Sheldrick RL, Clark KL, Coleman RA. EP4 prostanoid receptor-mediated vasodilatation of human middle cerebral arteries. *Br J Pharmacol*. 2004; 141:580–585. [PubMed: 14744815]
14. Levi M, Meijler MM, Gomez-Munoz A, Zor T. Distinct receptor-mediated activities in macrophages for natural ceramide-1-phosphate (C1P) and for phospho-ceramide analogue-1 (PCERA-1). *Mol Cell Endocrin*. 2010; 314:248–255.
15. Avni D, Goldsmith M, Ernst O, Mashiach R, Tuntland T, Meijler MM, Gray NS, Rosen H, Zor T. Modulation of TNF α , IL-10 and IL-12p40 levels by a ceramide-1-phosphate analog, PCERA-1, in vivo and ex-vivo in primary macrophages. *Immunol Lett*. 2009; 123:1–8. [PubMed: 19185589]
16. Matsui T, Kondo T, Nishita Y, Itadani S, Nakatani S, Omawari N, Sakai M, Nakazawa S, Ogata A, Ohno H, Obata T, Nakai H, Toda M. Highly potent inhibitors of TNF- α production. Part 1: Discovery of chemical leads. *Bioorg Med Chem Lett*. 2002; 12:903–905. [PubMed: 11958990]
17. Goldsmith M, Avni D, Levy-Rimler G, Mashiach R, Ernst O, Levi M, Webb B, Meijler MM, Gray NS, Rosen H, Zor T. A ceramide-1-phosphate analogue, PCERA-1, simultaneously suppresses tumour necrosis factor (TNF)- α and induces interleukin (IL)-10 production in activated macrophages. *Immunology*. 2008; 127:103–115. [PubMed: 18793216]
18. Goldsmith M, Avni D, Ernst O, Glucksam Y, Levy-Rimler G, Meijler MM, Zor T. Synergistic IL-10 induction by LPS and the ceramide-1-phosphate analog PCERA-1 is mediated by the cAMP and p38 MAP kinase pathways. *Mol Immunol*. 2009; 46:1979–1987. [PubMed: 19362373]
19. Matsui T, Kondo T, Nakatani S, Omawari N, Sakai M, Mori H, Ogata A, Kato J, Ohno H, Obata T, Nakai H, Toda M. Synthesis, further biological evaluation and pharmacodynamics of newly discovered inhibitors of TNF- α production. *Bioorg Med Chem*. 2003; 11:3937–3943. [PubMed: 12927854]
20. Matsui T, Kondo T, Nishita Y, Itadani S, Tsuruta H, Fujita S, Omawari N, Sakai M, Nakazawa S, Ogata A, Mori H, Kamoshima W, Terai K, Ohno H, Obata T, Nakai H, Toda M. Highly potent inhibitors of TNF- α production. Part II: metabolic stabilization of a newly found chemical lead and conformational analysis of an active diastereoisomer. *Bioorg Med Chem*. 2002; 10:3787–3805. [PubMed: 12413833]
21. Stahelin RV, Subramanian P, Vora M, Cho W, Chalfant CE. Ceramide-1-phosphate binds group IVA cytosolic phospholipase α 2 via a novel site in the C2 domain. *J Biol Chem*. 2007; 282:20467–20474. [PubMed: 17472963]
22. Subramanian P, Stahelin RV, Szulc Z, Bielawska A, Cho W, Chalfant CE. Ceramide 1-phosphate acts as a positive allosteric activator of group IVA cytosolic phospholipase A2 α and enhances

- the interaction of the enzyme with phosphatidylcholine. *J Biol Chem.* 2005; 280:17601–17607. [PubMed: 15743759]
23. Smith ER, Jones PL, Boss JM, Merrill AH Jr. Changing J774A.1 cells to new medium perturbs multiple signaling pathways, including the modulation of protein kinase C by endogenous sphingoid bases. *J Biol Chem.* 1997; 272:5640–5646. [PubMed: 9038174]
 24. Peretz A, Degani-Katzav N, Talmon M, Danieli E, Gopin A, Malka E, Nachman R, Raz A, Shabat D, Attali B. A tale of switched functions: from cyclooxygenase inhibition to M-channel modulation in new diphenylamine derivatives. *PLoS ONE.* 2007; 2:e1332. [PubMed: 18159230]
 25. Lamour NF, Subramanian P, Wijesinghe DS, Stahelin RV, Bonventre JV, Chalfant CE. Ceramide 1-phosphate is required for the translocation of group IVA cytosolic phospholipase A2 and prostaglandin synthesis. *J Biol Chem.* 2009; 284:26897–26907. [PubMed: 19632995]
 26. Subramanian P, Vora M, Gentile LB, Stahelin RV, Chalfant CE. Anionic lipids activate group IVA cytosolic phospholipase A2 via distinct and separate mechanisms. *J Lipid Res.* 2007; 48:2701–2708. [PubMed: 17890681]
 27. Zor T, Selinger Z. Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Anal Biochem.* 1996; 236:302–308. [PubMed: 8660509]
 28. Ianaro A, Ialenti A, Maffia P, Sautebin L, Rombola L, Carnuccio R, Iuvone T, D'Acquisto F, Di Rosa M. Anti-inflammatory activity of macrolide antibiotics. *J Pharmacol Exp Ther.* 2000; 292:156–163. [PubMed: 10604943]
 29. Amin AR, Vyas P, Attur M, Leszczynska-Piziak J, Patel IR, Weissmann G, Abramson SB. The mode of action of aspirin-like drugs: effect on inducible nitric oxide synthase. *Proc Natl Acad Sci USA.* 1995; 92:7926–7930. [PubMed: 7544010]
 30. Perkins DJ, Kniss DA. Blockade of nitric oxide formation down-regulates cyclooxygenase-2 and decreases PGE2 biosynthesis in macrophages. *J Leukoc Biol.* 1999; 65:792–799. [PubMed: 10380901]
 31. Jupp OJ, Vandenabeele P, MacEwan DJ. Distinct regulation of cytosolic phospholipase A2 phosphorylation, translocation, proteolysis and activation by tumour necrosis factor-receptor subtypes. *Biochem J.* 2003; 374:453–461. [PubMed: 12786601]
 32. Harper KA, Tyson-Capper AJ. Complexity of COX-2 gene regulation. *Biochem Soc Trans.* 2008; 36:543–545. [PubMed: 18482003]
 33. Graf BA, Nazarenko DA, Borrello MA, Roberts LJ, Morrow JD, Phipps RP. Proinflammatory signals upregulate COX-2 and increase PGE2 production in biphenotypic B/macrophage cells. *Ann N Y Acad Sci.* 2000; 905:294–296. [PubMed: 10818468]
 34. Pettus BJ, Bielawska A, Subramanian P, Wijesinghe DS, Maceyka M, Leslie CC, Evans JH, Freiberg J, Roddy P, Hannun YA, Chalfant CE. Ceramide 1-phosphate is a direct activator of cytosolic phospholipase A2. *J Biol Chem.* 2004; 279:11320–11326. [PubMed: 14676210]
 35. Perez-Sala D, Lamas S. Regulation of cyclooxygenase-2 expression by nitric oxide in cells. *Antioxid Redox Signal.* 2001; 3:231–248. [PubMed: 11396478]
 36. Qi HY, Shelhamer JH. Toll-like receptor 4 signaling regulates cytosolic phospholipase A2 activation and lipid generation in lipopolysaccharide-stimulated macrophages. *J Biol Chem.* 2005; 280:38969–38975. [PubMed: 16176925]
 37. Van Brocklyn JR, Lee MJ, Menzeleev R, Olivera A, Edsall L, Cuvillier O, Thomas DM, Coopman PJ, Thangada S, Liu CH, Hla T, Spiegel S. Dual actions of sphingosine-1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. *J Cell Biol.* 1998; 142:229–240. [PubMed: 9660876]
 38. Granado MH, Gangoiti P, Ouro A, Arana L, Gonzalez M, Trueba M, Gomez-Munoz A. Ceramide 1-phosphate (C1P) promotes cell migration Involvement of a specific C1P receptor. *Cell Signal.* 2009; 21:405–412. [PubMed: 19041940]
 39. Wijesinghe DS, Subramanian P, Lamour NF, Gentile LB, Granado MH, Szulc Z, Bielawska A, Gomez-Munoz A, Chalfant CE. The chain length specificity for the activation of group IV cytosolic phospholipase A2 by ceramide-1-phosphate. Use of the dodecane delivery system for determining lipid-specific effects. *J Lipid Res.* 2009; 50:1986–1995. [PubMed: 19075030]

40. Pettus BJ, Bielawska A, Spiegel S, Roddy P, Hannun YA, Chalfant CE. Ceramide kinase mediates cytokine- and calcium ionophore-induced arachidonic acid release. *J Biol Chem.* 2003; 278:38206–38213. [PubMed: 12855693]
41. Boudker O, Futerman AH. Detection and characterization of ceramide-1-phosphate phosphatase activity in rat liver plasma membrane. *J Biol Chem.* 1993; 268:22150–22155. [PubMed: 8408075]
42. Avni D, Philosoph A, Meijler MM, Zor T. The ceramide-1-phosphate analogue PCERA-1 modulates tumour necrosis factor-alpha and interleukin-10 production in macrophages via the cAMP-PKA-CREB pathway in a GTP-dependent manner. *Immunology.* 2010; 129:375–385. [PubMed: 19922425]
43. Huwiler A, Johansen B, Skarstad A, Pfeilschifter J. Ceramide binds to the CaLB domain of cytosolic phospholipase A2 and facilitates its membrane docking and arachidonic acid release. *Faseb J.* 2001; 15:7–9. [PubMed: 11099485]
44. Kitatani K, Oka T, Murata T, Hayama M, Akiba S, Sato T. Acceleration by ceramide of calcium-dependent translocation of phospholipase A2 from cytosol to membranes in platelets. *Arch Biochem Biophys.* 2000; 382:296–302. [PubMed: 11068881]
45. Ambs P, Baccarini M, Fitzke E, Dieter P. Role of cytosolic phospholipase A2 in arachidonic acid release of rat-liver macrophages: regulation by Ca²⁺ and phosphorylation. *Biochem J.* 1995; 311 (Pt 1):189–195. [PubMed: 7575453]

Fig. 1.

PCERA-1 inhibits production of PGE₂, but not of NO, in LPS-stimulated RAW264.7 macrophages. A) Mouse macrophage RAW264.7 cells were incubated at 37°C for 24 hours with the indicated concentrations of PCERA-1 (0–100 μM, represented by increasing gray intensity) and with LPS (1 μg/ml). PGE₂ release to the medium was measured by RIA. Each data point represents the mean ± S.D. (n=6). B) RAW264.7 macrophages cells were incubated with LPS (1 μg/ml) in the presence (black bars) or absence (gray bars) of PCERA-1 (10 μM), or with vehicle (open bars), for the indicated time. NO production was measured using the Griess reagent. Each data point represents the mean ± S.D. (n=6).

Fig. 2.

Inhibition of PGE₂ production by PCERA-1 is independent of TNFα level. A) RAW264.7 macrophages were incubated at 37°C for 24 hours with LPS (1 μg/ml) and/or PCERA-1 (10 μM). PGE₂ release to the medium was measured by RIA. Each data point represents the mean ± S.D. (n=6). B) PGE₂ production was assayed as in panel A, in the presence (solid bars) or absence (open bars) of exogenous recombinant TNFα (7 ng/ml).

Fig. 3.

PCERA-1 does not affect expression of COX-2. RAW264.7 macrophages were incubated at 37°C for 24 hours 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. 4.

PCERA-1 blocks AA release from LPS-stimulated RAW264.7 cells. RAW264.7 macrophages were labeled with ^3H -AA as described under “materials and methods”. The cells were stimulated with LPS (1 $\mu\text{g}/\text{ml}$) 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 $\pm$ S.D. (n=3).

Fig. 5.

AA release and cPLA $_2$ activity are inhibited by the phosphate-free CERA-1. RAW264.7 macrophages were labeled with ^3H -AA as described under “materials and methods”. The cells were incubated at 37°C for 12 hours with the indicated concentrations of CERA-1 (0–100 μM , represented by increasing gray intensity) and with LPS (1 $\mu\text{g}/\text{ml}$), and the medium was examined for the presence of radiolabelled AA and its metabolites. Each data point represents the mean $\pm$ S.D. ($n=3$), relative to control cells that were not stimulated with LPS (also represented by the dashed line). B-C) Recombinant cPLA $_2$ (1.0 μg) 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 “materials and methods”. Each data point represents the mean $\pm$ S.D. ($n=3$).

Fig. 6.

The phosphate-free CERA-1 inhibits PGE₂ production in LPS-stimulated RAW264.7 macrophages by blocking production of the precursor, AA. A) Mouse macrophage RAW264.7 cells were incubated at 37°C for 24 hours with LPS (1 μg/ml) and with the indicated concentration of CERA-1 (0–100 μM, represented by increasing gray intensity). The medium was collected and its PGE₂ content was measured by a radioimmunoassay. Each data point represents the mean ± S.D. (n=5). B) Following a 24 hours incubation with LPS and/or CERA-1 (100 μM), and medium collection, the cells were washed and incubated with exogenous AA (30 μM) for a period of 20 min, after which the COX inhibitor indomethacin (10 μM) was added in order to stop the reaction, the medium was again collected and its PGE₂ content measured as above. The treatments depicted at the X-axis refer to the 24 hours incubation and were not present during the following 20 min incubation with exogenous AA. PGE₂ production during these 20 min was negligible in the absence of exogenous AA (0.15 ± 0.04 ng/mg protein).

Fig. 7.

The phosphate-free CERA-1 inhibits AA release in LPS-stimulated and resting bone marrow-derived macrophages (BMDM). Mouse BMDM were labeled with ³H-AA as described under “materials and methods”. The cells were then incubated at 37°C for 10 hours with LPS (1 μg/ml) and/or with the indicated concentration of CERA-1 (0–50 μM, represented by increasing gray intensity), and the medium was examined for the presence of radiolabelled AA and its metabolites. Each data point represents the mean ± S.D. (n=3).