

Protection of Neuroblastoma Neuro2A Cells from Hypoxia-Induced Apoptosis by Cyclic Phosphatidic Acid (cPA)

Mari Gotoh[‡], Katsura Sano-Maeda[‡], Hiromu Murofushi, Kimiko Murakami-Murofushi*

The Division of Life Sciences, Ochanomizu University, Tokyo, Japan

Abstract

Cyclic phosphatidic acid (cPA) is a naturally occurring phospholipid mediator with a unique cyclic phosphate ring at the *sn*-2 and *sn*-3 positions of its glycerol backbone. We have previously shown that cPA significantly suppresses ischemia-induced delayed neuronal death and the accumulation of glial fibrillary acidic protein in the CA1 region of the rat hippocampus. These results indicated that the systemic administration of cPA can protect hippocampal neurons against ischemia-induced delayed neuronal cell death. In the current study, we investigated the effects of cPA on neuronal cell death caused by hypoxia *in vitro* and the molecular mechanisms underlying these effects. We used cobalt chloride (CoCl₂) to expose cells to hypoxic conditions *in vitro*. Treating mouse neuroblastoma (Neuro2A) cells with CoCl₂ induced nuclear DNA condensation and phosphatidylserine exposure. However, adding cPA led to the suppression of CoCl₂-induced apoptosis in a cPA dose-dependent manner and attenuated the increase in the Bax/Bcl-2 ratio caused by CoCl₂. Quantitative PCR analysis showed that Neuro2A cells strongly express the LPA₁, LPA₂, and LPA₆, which are G-protein coupled receptors that can be activated by cPA. To date, LPA₁ and LPA₂ have been reported to exhibit antiapoptotic activity. Therefore, to assess the roles of LPA₁ and LPA₂ on cPA-induced neuroprotective functions, Ki16425, a selective LPA₁ and LPA₃ antagonist, was adopted to know the LPA₁ function and siRNA was used to knockdown the expression of LPA₂. On the basis of our results, we propose that cPA-induced protection of Neuro2A cells from CoCl₂-induced hypoxia damage is mediated via LPA₂.

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* E-mail: murofushi.kimiko@ocha.ac.jp

‡ These authors contributed equally to this work.

Introduction

In 1992, cyclic phosphatidic acid (cPA) was originally isolated from the myxamoebae of a true slime mold, *Physarum polycephalum* [1]. Later, the presence of cPA 16:0 and 18:1 has been found in mammalian fluids such as serum and brain tissue [2,3]. And these are major forms of naturally occurring cPA species. Although the chemical formula of cPA is similar to that of lysophosphatidic acid (LPA), cPA has a unique structure consisting of a cyclic phosphate ring at the *sn*-2 and *sn*-3 positions of its glycerol backbone [1]. These features provide cPA with biological functions that are distinct from or oppose the functions of LPA. For example, LPA stimulates cell proliferation, cancer cell invasion, and generates pain, whereas cPA inhibits these activities [4–10].

Previous *in vitro* studies have reported that cPA 16:0, 18:1 and LPA 18:1 elicit neurotrophin-like actions in embryonic hippocampal neurons [11]. We have examined the effects of cPA 18:1 on ischemia-induced delayed neuronal death in the hippocampal CA1 region and found that the systemic administration of cPA 18:1 has *in vivo* neuroprotective effects [12]. However, the mechanisms underlying these effects of cPA 18:1 on hypoxic-ischemic brain injury have not yet been completely understood.

Here, we aimed to investigate the effects of cPA 18:1 on hypoxia-induced apoptosis and the molecular mechanism underlying these effects.

To induce hypoxic/ischemic conditions *in vitro*, we induced apoptosis with cobalt chloride (CoCl₂), which is used as a chemical hypoxia-inducing agent for several types of neural cells [13–16]. Then, we investigated the possible mechanisms of LPA receptor involvement. To date, cPA has been reported to stimulate several LPA G protein-coupled receptors (GPCRs) such as LPA_{1–5} [6,17]. LPA₁, LPA₂, and GPR87 have been shown to exhibit antiapoptotic activity [18]. cPA activates the LPA_{1–5} at significantly higher EC₅₀ concentrations than LPA [6,17]. Although cPA is a weak agonist against the LPA_{1, 3, 4, 5}, it has a stronger efficacy against the LPA₂ (140% maximal efficacy compared with LPA) [6]. Each LPA receptor exhibits a different affinity and efficacy toward cPA and LPA. In addition, each LPA receptor has a individual signaling pathway and function. Thus, cells expressing several LPA receptors might undergo a different cellular phenomenon when stimulated by cPA or LPA.

In this study, using neuroblastoma Neuro2A cells, we examined the effects of the neuroprotective functions that are specific to cPA 18:1 and LPA 18:1 and involve LPA receptors and also

investigated the molecular mechanisms underlying these effects. Our results show that although both lipid mediators exhibit neuroprotective functions for Neuro2A, the signal pathway initiated by cPA and LPA may potentially differ.

Materials and Methods

1. Pharmacologic agents

cPA 18:1 was chemically synthesized as previously described [19]. Bovine serum albumin (BSA; fraction V, fatty acid free) and 1-oleoyl-*sn*-lysophosphatidic acid (LPA 18:1) were purchased from Sigma-Aldrich (St. Louis, MO). LPA and cPA were dissolved in PBS containing 0.1% (w/v) BSA. The inhibitor of the LPA₁, ₃, that is, Ki16425 (Cayman Chemicals, MI), was dissolved in DMSO and added to Neuro2A cells at a working concentration of 10 μ M at 20 min prior to adding cPA or LPA.

2. Cell culture and treatment

Mouse Neuro2A cells were obtained from the Department of Biochemistry, Faculty of Medicine, the University of Tokyo. The cells were originally obtained from the American Type Culture Collection (ATCC cat. no. CCL131). The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Biowest, FL), 0.2% NaHCO₃, and 0.06% glutamine. Cells were grown at 37°C in a humidified incubator containing 5% CO₂. For all the experiments, 1.0 \times 10⁶ Neuro2A cells were seeded into a 100-mm dish and incubated for 4 hours with culture media containing 10% FBS. The cells were then subjected to serum starvation for 16 hours with serum-free DMEM.

3. Cell viability

To determine cell viability, cells were seeded (4 \times 10⁴ cells/well) in a 96-well plate. Serum-starved Neuro2A cells were exposed to various concentrations of CoCl₂ for 24 hours. The cells were then stained with 10 μ M calcein-AM (Dojindo, Kumamoto, Japan) in the dark for 30 min at 37°C and washed with phosphate-buffered saline (PBS). The fluorescence intensity (em/ex, 485/530 nm) of each well was measured using a CytoFluor series 4000 fluorescence microplate reader (Applied Biosystems, Tokyo, Japan). Data were calculated as the percent cell viability compared to that of controls without CoCl₂ treatment and have been presented as the mean and SE values for triplicate wells.

4. DAPI staining

Neuro2A cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Then, the nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10 min at room temperature. The chromatin structures of the cells were observed under a fluorescence microscope equipped with a UV combination filter (Nikon Corp., Tokyo, Japan).

5. Measurement of reactive oxygen species generation

Intracellular reactive oxygen species (ROS) generation was measured using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA; Molecular Probes, Inc., OR). Serum-starved Neuro2A cells in 96-well plates were exposed to 300 μ M CoCl₂ and stained with 10 μ M CM-H₂DCFDA in the dark for 30 min. Subsequently, the cells were washed with PBS, and the fluorescence intensity (em/ex, 485/530 nm) of each well was measured using the CytoFluor series 4000 microplate reader. The data were

calculated as the relative ROS induction compared to that of controls (without CoCl₂ treatment, time 0) and have been presented as the mean and SE values of triplicate wells.

6. Flow cytometric analysis

Serum-starved Neuro2A cells were exposed to 300 μ M CoCl₂ in the presence of 10 μ M cPA or LPA. After 24 hours, the extent of apoptosis in Neuro2A cells was quantified by flow cytometric analysis by using the Cell Lab Quanta™ SC MPL (Beckman Coulter, CA). Cell staining was performed with FITC-conjugated Annexin V (Trevigen, MD) or DEVD-FMK (a caspase-3 inhibitor that irreversibly binds to activated caspase-3, BioVision, CA) in accordance with the manufacturer's instructions.

7. Cell adhesion assay

Serum-starved Neuro2A cells were exposed to 300 μ M CoCl₂ in the presence of 10 μ M cPA or LPA. After 24 hours, the non-adherent cells were removed by washing 3 times with PBS. The number of adherent cells (cells/cm²) was determined using an inverted phase-contrast microscope.

8. RNA interference

Neuro2A cells were transfected with Accell SMARTpool siRNA specific for the mouse LPA₂ or Accell non-targeting siRNA (Dharmacon, Inc., CO) with Accell siRNA delivery media, according to the manufacturer's protocol. The cells were used 3 d after transfection.

9. RNA isolation and real-time PCR

Total RNA was extracted from Neuro2A cells by using the Isogen reagent (Nippongene, Toyama, Japan). It was used as a template for subsequent cDNA synthesis with oligo dT primers by using the Omniscript RT Kit (Qiagen, CA). mRNA levels were quantified using an ABI 7300 real-time PCR machine (Foster City, CA) and SYBR Premix Ex Taq II (Takara Bio Inc., Siga, Japan). Gene-specific primer sets were used as previously reported [12,20]. To quantify the knockdown levels of LPA₂ mRNA, the following 2 primer pair sets were used: primer set I, 5'-GTTGAGGT-CACTCCCACGTT-3' (F) and 5'-CGTGCCCTTCCCTAACCTT-3' (R); and primer set II, 5'-AGGCTGGATATGGT-CATTGC-3' (F) and 5'-GCAGCTCCAGCAGAAATGTA-3' (R). The data were analyzed using the delta Ct method. The expression level of each LPA receptor was normalized to β -actin expression as previously described [12,20].

10. Western blot analysis

Neuro2A cells were collected and subjected to western blot analysis to detect Bax and Bcl-2 protein expression. Proteins were separated by SDS-PAGE by using a 15% polyacrylamide gel and then transferred to an Immobilon-P Transfer Membrane (Millipore). Using anti-Bax or anti-Bcl-2 antibodies (1:1000 dilution, Cell Signaling Technology, Inc., MA) and horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000 dilution; Kirkegaard & Perry Laboratories Inc., MD), immunodetection was performed using an enhanced chemiluminescence (ECL) system (GE Healthcare UK Ltd, Amersham Place, Little Chalfont, England).

11. Statistical analysis

All the values have been reported in terms of mean \pm SE values. The data were analyzed using one-way analysis of variance (ANOVA) and subsequently with Dunnett's test. A *P* value less than 0.05 was considered to be statistically significant.

Results and Discussion

1. CoCl₂-induced apoptosis in Neuro2A cells

Neuro2A cells were treated with various concentrations of CoCl₂. After 24 hours, exposure of Neuro2A cells to CoCl₂ significantly decreased cell viability in a CoCl₂ dose-dependent manner (Fig. 1A). Exposure to 300 μM CoCl₂ for 24 hours resulted in 61% viable cells compared to control cells (100%). The mode of cell death, necrosis, or apoptosis was determined by DAPI staining. After exposure to CoCl₂, the cells displayed apoptotic morphology characterized by the condensation of chromatin, as shown in Fig. 1B. Moreover, to assess intracellular ROS generation, we measured the oxidation of CM-H₂DCFDA [13]. CoCl₂ treatment has been reported to significantly increase ROS levels within 1 h of incubation [21]. We also observed that treatment of Neuro2A cells with CoCl₂ for 15 min induced oxidative stress by enhancing ROS levels (Fig. 1C). Our data show that exposure of Neuro2A cells to CoCl₂ rapidly increased ROS levels and might initiate apoptosis signaling. Meanwhile it was revealed that Neuro2A did not generate superoxide by treatment of CoCl₂ for 0–30 min (data not shown).

Flow cytometric analysis with FITC-Annexin V was used to analyze the rate of apoptosis induced by CoCl₂ (Fig. 1D). Representative data show that exposure to 300 μM CoCl₂ for 24 hours resulted in 54.5% FITC-Annexin V-positive Neuro2A cells in the entire cell population. On the other hand, no exposure to CoCl₂ for 24 hours resulted in only 8.9% FITC-Annexin V-positive Neuro2A cells in the entire cell population. These results

suggest that stimulation by 300 μM CoCl₂ for 24 hours induced apoptosis in Neuro2A cells. Therefore, these conditions were used to induce apoptosis in Neuro2A cells in all subsequent experiments.

2. cPA protected Neuro2A cells against CoCl₂-induced apoptosis

To examine the effects of cPA on CoCl₂-induced apoptosis, Neuro2A cells were treated with CoCl₂ in the presence or absence of cPA. Twenty-four hours later, the number of adherent cells (cells/cm²) was counted (Fig. 2A). At a concentration of 10 μM, cPA was observed to inhibit CoCl₂-induced cell detachment. Although LPA is less potent than cPA, it also inhibited cell detachment. These results suggest that cPA and LPA could potentially attenuate CoCl₂-induced Neuro2A cytotoxicity.

We then investigated the effects of cPA and LPA on CoCl₂-induced apoptosis by measuring exposure of phosphatidylserine (PS) and activation of caspase-3. Exposure of PS on the surface of the cell membrane is related to the occurrence of early stages of apoptotic cell death and can be detected using Annexin V (PS-binding protein). Flow cytometric analysis with FITC-Annexin V showed that cPA-treatment significantly decreased the number of FITC-Annexin V-positive Neuro2A cells in a bell-shaped dose-dependent manner after exposure to CoCl₂. At the most effective cPA-concentration (10 μM), the number of FITC-Annexin V-positive cells decreased to 30% of those in the vehicle control. LPA (10 μM) also exhibited neuroprotective effects on Neuro2A cells,

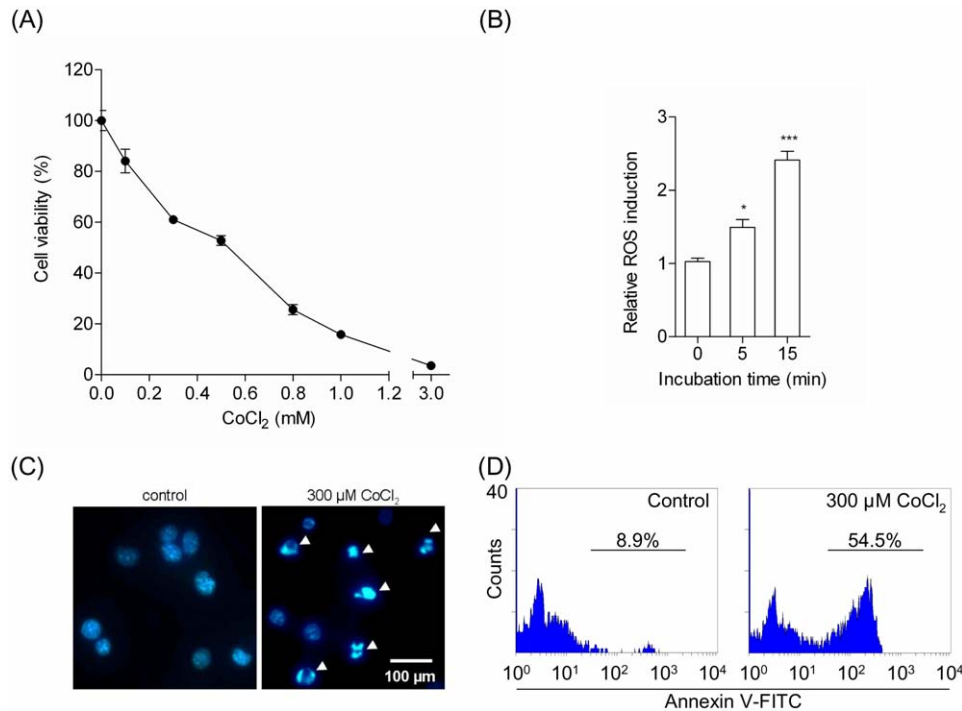


Figure 1. Treatment with CoCl₂ induces apoptosis in Neuro2A cells. (A) Effects of CoCl₂ on the viability of Neuro2A cells. Neuro2A cells were incubated with various concentrations of CoCl₂ for 24 hours. Cell viability was estimated as described in the materials and methods section. The data represent the mean \pm SE values from triplicate independent experiments. (B) Generation of reactive oxygen species (ROS) induced by CoCl₂. Neuro2A cells were incubated with 300 μM CoCl₂, and ROS generation was measured after 5 and 15 min. The data represent the mean \pm SE values from triplicate independent experiments (* P <0.05, *** P <0.001 vs. the CoCl₂-treated group). (C) Morphologic changes in the nuclei of CoCl₂-treated Neuro2A cells. Neuro2A cells incubated in the absence (left) or presence (right) of 300 μM CoCl₂ for 24 hours were fixed and stained with DAPI and examined by fluorescence microscopy. (D) Analysis of apoptosis-associated PS exposure on CoCl₂-treated Neuro2A cells by using FITC-Annexin V. Neuro2A cells were treated with (right) or without (left) 300 μM CoCl₂ for 24 hours and stained with FITC-Annexin V. The population of FITC-Annexin V-positive Neuro2A cells was quantified by flow cytometry. doi:10.1371/journal.pone.0051093.g001

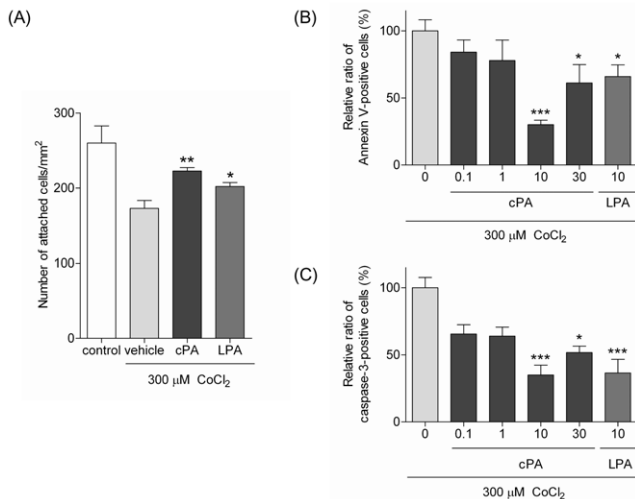


Figure 2. cPA protects against CoCl₂-induced apoptosis in Neuro2A cells. (A) The effects of cPA and LPA on a number of adhesive Neuro2A cells treated with CoCl₂. Neuro2A cells were incubated with 300 μM CoCl₂ in the presence of 10 μM cPA or LPA for 24 hours, and the number of cells attached to the surface of dishes was determined (*P<0.05, ***P<0.01 vs. the CoCl₂-treated group). (B & C) Analysis of the percent of FITC-Annexin V- and FITC-DEVD-FMK-positive Neuro2A cells after CoCl₂ treatment by flow cytometry. Neuro2A cells were incubated with 300 μM CoCl₂ and various concentrations of either cPA or LPA for 24 hours. Cells were subsequently stained with either FITC-Annexin V (B) or FITC-DEVD-FMK, an activated caspase-3 inhibitor (C) and subjected to flow cytometric analysis. The data represent the mean ± SE values from triplicate independent experiments (*P<0.05, **P<0.01, ***P<0.001 vs. the CoCl₂-treated group). doi:10.1371/journal.pone.0051093.g002

as shown in Fig. 2B. However, the neuroprotective effects were not exhibited at lower concentrations (0.1 and 1 μM) of LPA (data not shown).

The cleavage of caspase-3 has been shown to be an important trigger for the execution of apoptosis [22,23]. Treatment of Neuro2A cells with 300 μM CoCl₂ significantly stimulated caspase-3 activity, which was attenuated by cPA in a bell-shaped dose-dependent manner, as shown in Fig. 2C. To confirm flow cytometric analysis, we performed western blot analysis to measure protein level of pro-caspase-3 using anti-caspase-3 antibody. It was revealed that level of pro-caspase-3 was significantly decreased by treatment of Neuro2A cells with 300 μM CoCl₂ for 24 hours (data not shown), indicating pro-caspase-3 was activated. The decrease of pro-caspase-3 was attenuated by the addition of cPA 18:1 or LPA 18:1. These results are consistent with the results from flow cytometric analysis as shown in Fig. 2C.

In the case of measuring caspase-3 activity, 10 μM LPA but not lower concentration of LPA was observed to attenuate the caspase-3 activity. These results strongly indicate that cPA decreases the exposure of PS and caspase-3 activity and attenuates CoCl₂-induced apoptosis.

3. Effects of CoCl₂ and cPA on the expression of the Bcl-2 protein family in Neuro2A cells

The Bcl-2 family is an important regulator of various apoptotic pathways [22]. Because hypoxia induces an increase in the expression of proapoptotic Bcl-2 family members, such as Bax, and decrease in the expression of antiapoptotic Bcl-2 family members, such as Bcl-2, we examined the effects of CoCl₂-induced apoptotic signaling on Bax and Bcl-2. Bax and Bcl-2 protein levels

were determined by western blot analysis, and a time-course analysis of Bax and Bcl-2 protein expression ratios was performed (Fig. 3). The Bax/Bcl-2 ratio increased over time with CoCl₂ treatment. However, in the presence of either cPA or LPA (10 μM), the ratio did not increase and remained at the ratio seen in control cells for up to 12 hours. Thus, cPA and LPA suppressed the CoCl₂-induced increase in the Bax/Bcl-2 ratio and prevented CoCl₂-induced apoptosis.

4. The antiapoptotic function of cPA is mediated by LPA₂

To gain insight into the molecular mechanism underlying the protective effects of cPA on CoCl₂-induced apoptosis in Neuro2A cells, we focused on the signaling pathways initiated by LPA receptors. cPA has been shown to activate LPA₁₋₅ [6,17]. Recently, we examined whether cPA could activate LPA₆ by measuring the LPA₆-mediated neurite retraction of B103 cells as described previously [24,25]. We observed that cPA could activate LPA₆, although its efficacy and affinity were less potent than those of LPA (unpublished data). Quantitative PCR analysis showed that Neuro2A cells highly expressed LPA_{1, 2, 6} and p2y10 (Fig. 4A). Although LPA₁, LPA₂, and GPR87 have been speculated to have antiapoptotic activity [18], Neuro2A cells did not express GPR87. Therefore, we focused on the functions of the LPA₁ and LPA₂ in cPA-mediated antiapoptotic activity. In order to inhibit LPA₁, we used Ki16425 (10 μM), which is an LPA receptor antagonist with selectivity for the LPA₁ and LPA₃ [26]. As shown in Fig. 4B, Ki16425 pretreatment attenuated LPA-mediated antiapoptotic activity for Neuro2A cells. However, the antiapoptotic activity of cPA was not affected by Ki16425. These results indicate that the antiapoptotic activity for Neuro2A of LPA may be mediated by LPA₁, whereas that of cPA was not mediated by LPA₁. We then used siRNA to knockdown LPA₂ and assess the effects of reduced LPA₂ expression on the antiapoptotic activity for Neuro2A cells of cPA and LPA. Compared to the mRNA expression level of LPA₂ in non-targeting siRNA-transfected cells (control), the expression level in LPA₂-targeted siRNA-transfected cells decreased by 14.4% (Fig. 4C). In addition, the antiapoptotic activity of cPA on CoCl₂-induced Neuro2A cell apoptosis was largely abolished after knockdown of LPA₂ (Fig. 4D). In the case of LPA, the effect of the suppression of its antiapoptotic activity on CoCl₂-induced Neuro2A cell apoptosis was less apparent. Therefore, we propose that cPA-induced protection of Neuro2A cells from CoCl₂-induced apoptosis might potentially be mediated by LPA₂ and that LPA-induced protection of Neuro2A cells might be mediated mainly by LPA₁ but not LPA₂.

The LPA₁ and LPA₂ differ in their dose response to cPA and LPA. LPA₁ can be activated by lower concentrations of cPA and LPA than LPA₂ [6]. This difference in dose response has been speculated to have important biological implications [27]. These receptors can cooperate and respond over a wide range of LPA concentrations. For example, LPA₁ is activated by LPA with 100% efficacy (EC₅₀, 130 nM) and cPA with 66% efficacy (EC₅₀, 1.7 μM). LPA₂ is activated by LPA with 100% efficacy (EC₅₀, 3 nM) and cPA with 140% efficacy (EC₅₀, 180 nM) [6]. We speculate that the affinity for the ligand, efficacy of the ligand, and expression level of receptors in individual cells are important factors for LPA receptor signaling. Most cells express multiple LPA receptors and work cooperatively. Therefore, determining the signal response of individual LPA receptors is not easy. LPA₁ and LPA₂ are coexpressed in Neuro2A cells. The neuroprotective functions for Neuro2A of cPA could possibly be mediated by LPA₂. Furthermore, the neuroprotective function of LPA might be mediated mainly by LPA₁ but not LPA₂ in Neuro2A cells. On the basis of these results, we suggest that 10 μM LPA could activate

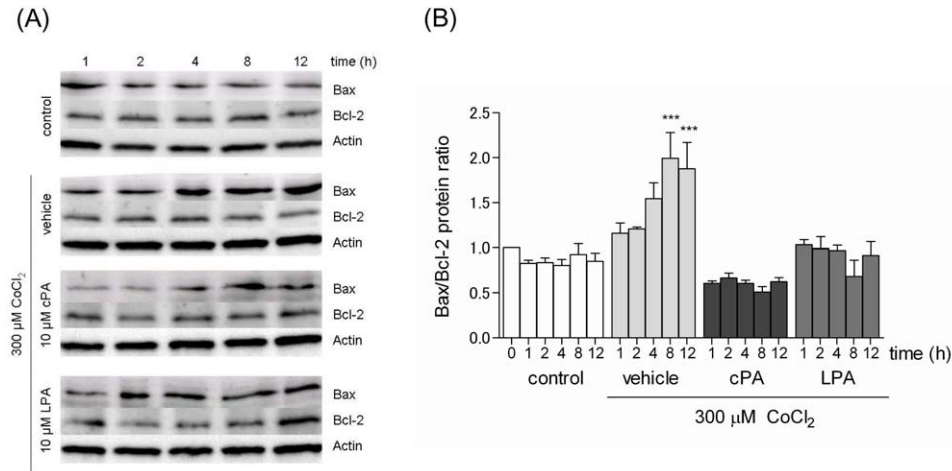


Figure 3. Effects of cPA and LPA on the expression of the Bcl-2 protein family. (A) Neuro2A cells were incubated with 300 μM CoCl₂ in the presence of 10 μM cPA or LPA for the time indicated. Protein levels of Bax, Bcl-2, and β-actin were determined by western blot analysis. (B) The expression of Bax and Bcl-2 was determined using a densitometer, and the Bax/Bcl-2 ratio was calculated and normalized to cells without any treatment. The data represent the mean ± SE values from triplicate independent experiments (**P<0.01 vs. CoCl₂-treated group). doi:10.1371/journal.pone.0051093.g003

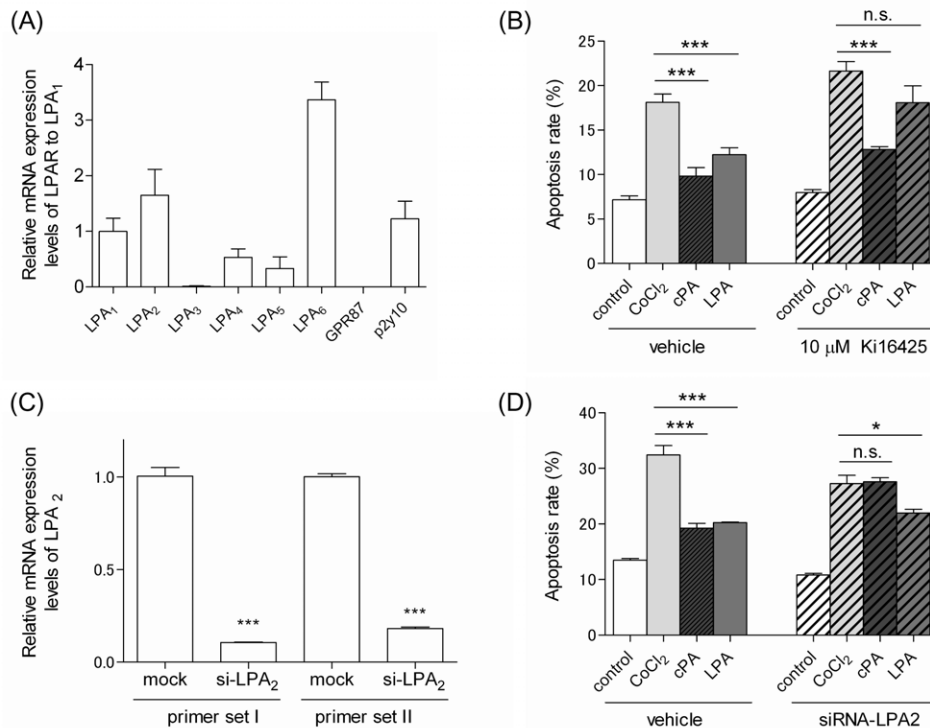


Figure 4. Effects of LPA receptors on the neuroprotective functions of cPA and LPA against CoCl₂-induced apoptosis. (A) Expression of LPA receptors in Neuro2A cells. Total RNA was extracted from Neuro2A cells, and the expression level of each LPA receptor was determined by quantitative real-time PCR. The expression levels were normalized to those of LPA₁ and expressed in terms of the mean ± SE values. (B) The effects of Ki16425 on the neuroprotective functions of cPA and LPA against CoCl₂-induced apoptosis of Neuro2A cells. Neuro2A cells were pretreated with or without 10 μM Ki16425 for 20 min. Subsequently, the cells were incubated with 300 μM CoCl₂ in the presence of 10 μM cPA or LPA for 24 hours. The cells were then stained with FITC-Annexin V and subjected to flow cytometric analysis. The data represent the mean ± SE values from triplicate independent experiments (**P<0.01 vs. the CoCl₂-treated group). (C) Expression of LPA₂ in Neuro2A cells. Neuro2A cells were transfected with siRNA against LPA₂ or non-target siRNA. Total RNA was extracted from each transfected Neuro2A cell, and the expression level of each LPA receptor was determined by quantitative real-time PCR. The expression levels of LPA₂ was normalized to those of Neuro2A cells transfected with non-target siRNA. The resulting data represent the mean ± SE values (**P<0.01 vs. the mock group). (D) The effects of LPA₂ knockdown on the neuroprotective effects of cPA and LPA against CoCl₂-induced apoptosis of Neuro2A cells. Neuro2A cells transfected with either siRNA against LPA₂ or non-target siRNA were incubated with 300 μM CoCl₂ in the presence of 10 μM cPA or LPA for 24 hours. Cells stained with FITC-Annexin V were subjected to flow cytometric analysis. The data represent the mean ± SE values from triplicate independent experiments (*P<0.05, **P<0.01 vs. the CoCl₂-treated group; n.s., not significant). doi:10.1371/journal.pone.0051093.g004

LPA₁ but not LPA₂. An LPA concentration of 10 μ M is too high to activate LPA₂. At high concentrations, LPA₂ can undergo endocytic downregulation and degradation. On the other hand, 10 μ M cPA activates only LPA₂. The affinity of LPA₂ for cPA is lower than that for LPA, which may prevent the receptors from undergoing endocytic downregulation on exposure to 10 μ M of cPA. This may also be because the efficacy of cPA against LPA₂ is higher than that of LPA. More remarkable effects were observed when the LPA₂ was activated by cPA than when it was activated by LPA. Moreover, because LPA₂ has a higher affinity for cPA than LPA₁ does [6], cPA might prefer LPA₂ over LPA₁. Here, we have shown that cPA and LPA exhibit antiapoptotic activity for Neuro2A cells but their originating signaling pathways differ. We speculate that the receptors have differences in efficacy and affinity for cPA and LPA. Although many questions remain to be answered, the current study shows that lipid mediators that exhibit antiapoptotic activity and have overlapping receptors function via different receptors in apoptosis. We hypothesize that these differences in receptor preference and the efficacy of cPA and LPA can explain why cPA exhibits biological functions distinct from those of LPA.

We propose that cPA could be a promising therapeutic agent to treat ischemic brain injury. In order to develop novel compounds with medicinal properties, we have synthesized stable derivatives of cPA [6,10,28]. Moreover, we have also shown that each enantiomer of several cPA derivatives exhibits the same efficiency toward autotaxin inhibition [29–32]. On the basis of these

findings, we are currently investigating effective therapeutic compounds to treat disorders such as cancer and neurodegeneration.

Conclusion

CoCl₂ treatment induced apoptosis in Neuro2A cells, involving an increase in ROS generation and the Bax/Bcl-2 ratio. cPA increased the cell survival rate, while decreasing the number of FITC-Annexin V- and FITC-DEVD-FMK-positive cells among CoCl₂-treated Neuro2A cells in a bell-shaped dose-dependent manner. The protective effect of cPA on CoCl₂-induced apoptosis in Neuro2A cells was not inhibited by the LPA₁ and LPA₃ antagonist Ki16425. However, the effect of LPA was inhibited by Ki16425. Knockdown of LPA₂ in Neuro2A cells largely affected the neuroprotective response of cPA but not that of LPA. Therefore, we propose that the molecular mechanism underlying the protective effect of cPA might differ from that for LPA. In addition, we suggest that cPA-induced protection of Neuro2A cells from CoCl₂-induced hypoxia damage might be mediated via LPA₂.

Author Contributions

Conceived and designed the experiments: HM KMM. Performed the experiments: MG KSM. Analyzed the data: MG KSM. Contributed reagents/materials/analysis tools: MG KSM. Wrote the paper: MG KSM HM KMM.

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