Lysophosphatidic acid in medicinal herbs enhances prostaglandin E_2 and protects against indomethacin-induced gastric cell damage *in vivo* and *in vitro*

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Abstract

Lysophosphatidic acid (LPA) is a bioactive phospholipid that induces diverse biological responses. Recently, we found that LPA ameliorates NSAIDs-induced gastric ulcer in mice. Here, we quantified LPA in 21 medicinal herbs used for treatment of gastrointestinal (GI) disorders. We found that half of them contained LPA at relatively high levels (40–240 μg/g) compared to soybean seed powder (4.6 μg/g), which we previously identified as an LPA-rich food. The LPA in peony (*Paeonia lactiflora*) root powder is highly concentrated in the lipid fraction that ameliorates indomethacin-induced gastric ulcer in mice. Synthetic 18:1 LPA, peony root LPA and peony root lipid enhanced prostaglandin E_2 production in a gastric cancer cell line, MKN74 cells that express LPA_2 abundantly. These materials also prevented indomethacin-induced cell death and stimulated the proliferation of MKN74 cells. We found that LPA was present in stomach fluids at 2.4 μM, which is an effective LPA concentration for inducing a cellular response *in vitro*. These results indicated that LPA is one of the active components of medicinal herbs for the treatment of GI disorder and that orally administered LPA-rich herbs may augment the protective actions of endogenous LPA on gastric mucosa.

Keywords: Lysophosphatidic acid; Medicinal herbs; Indomethacin; Prostaglandin E_2; Cell death; Cell proliferation

Abbreviations: GI, gastrointestinal; LPA, lysophosphatidic acid; PA, phosphatidic acid; TLC, thin-layer chromatography; PL, phospholipid; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; LC/MS/MS, liquid chromatography-tandem mass

spectrometry; CMC, carboxymethylcellulose; PLA_2, phospholipase A_2; PGE_2, prostaglandin E_2; NSAIDs, non-steroidal anti-inflammatory drugs; COX, cyclooxygenase.

1. Introduction

Lysophosphatidic acid (LPA) is a bioactive phospholipid that induces diverse cellular responses including proliferation, protection of cells from apoptosis, and migration of cells [1]. These cellular responses are mediated through six LPA-specific G-protein coupled receptors, $LPA_{1–6}$ [2]. Recent studies have revealed important actions of LPA in the mammalian gastrointestinal (GI) tract [3, 4]. These include inhibition of diarrhea, regulation of intestinal electrolyte transport, protection of intestinal cells from apoptosis, and wound healing [5-9].

Peptic ulcer is a major GI disorder that occurs due to an imbalance in mucosal offensive (gastric acid secretion) and defensive (gastric mucosal integrity) factors [10, 11]. Infection with *Helicobacter pylori*, smoking, drinking alcohol, and chronic ingestion of drugs are major causes of peptic ulcer. Recently, the number of patients with non-steroidal anti-inflammatory drug (NSAID)-induced gastric ulcer is increasing due to increased use of NSAIDs for pain treatment and prevention of thrombosis [12]. NSAIDs cause gastric ulcer by inhibition of cyclooxygenase (COX)-1 and COX-2, which produce a predominant mucosal defense factor, prostaglandin E_2 (PGE_2) [13]. Our recent study showed that orally administered synthetic LPA ameliorates aspirin- and indomethacin-induced gastric ulcer in mice [14, 15]. We also showed that LPA up-regulates COX-2 and enhances production of PGE_2 via activation of LPA_2 receptors, which are located on the apical side of gastric mucosal cells [16]. However, further uncharacterized mechanisms other than COX-2 induction are considered to function in the protective action of LPA, because LPA protects the gastric mucosa from the acute toxicity of NSAIDs.

Medicinal herbs have been traditionally used for the treatment of many diseases, including gastric ulcer [17]. Considering that some medicinal herbs and vegetables contain LPA abundantly [18, 19], it is rational to postulate the existence of anti-ulcer medicinal herbs that contains LPA as an active component. To examine this possibility, we determined the LPA content of 21 herbs that are traditionally used for the treatment of GI disorders. We also aimed to examine the effects of LPA and herbal lipids on NSAID-induced gastric ulcer. The results showed that peony root lipid, which contain highly concentrated LPA, had an ameliorative effect on NSAID-induced gastric ulcer and enhanced PGE_2 production in gastric cells. We also showed evidence that LPA/LPA_2 signaling protects against acute cytotoxicity of NSAIDs in cultured gastric cells.

2. Materials and Methods

2.1 Materials

Herbs used for the treatment of various digestive disorders were selected based on the descriptions in the oldest Chinese traditional herbal medicine book, the Shennong Ben Cao Jing. Coptis rhizome (*Coptis japonica*), moutan cortex (*Paeonia suffruticosa*), atractylodes rhizome (*Atractylodes japonica*), atractylodes lancea rhizome (*Atractylodes lancea*), amomum seed (*Amomum xanthioides*), peony root (*Paeonia lactiflora*), poria sclerotium (*Poria cocos*), and phellodendron bark (*Phellodendron amurense*) were obtained from Yoshimi Seiyaku Co. Ltd. (Osaka, Japan). Licorice root (*Glycyrrhiza glabra*), platycodon root (*Platycodon grandiflorum*), bupleurum root (*Bupleurum falcatum*), zedoary rhizome (*Curcuma zedoaria*), fennel fruit (*Foeniculum vulgare*), dried ginger rhizome (*Zingiber officinale*), and stripped, steamed, and dried ginger rhizome (*Zingiber officinale*) were purchased from Nakaya Hikojuro Co. Ltd.

(Ishikawa, Japan). Sophora root (*Sophora flavescens*) and schisandra fruit (*Schisandra chinensis*) were obtained from Kojima Kampo Co. Ltd. (Osaka, Japan). Pinellia tuber (*Pinellia ternata*), cimicifuga rhizome (*Cimicifuga simplex*), panax rhizome (*Panax japonicus*), and corydalis tuber (*Corydalis turtschaninovii*) were purchased from a local drug store.

2.2 Reagents

One-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (18:1 LPA), 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphate (17:0 LPA), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (16:0 LPC), and arachidonic acid were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Peony root LPA was freshly prepared from peony root powder as described below. Pertussis toxin (PTX) and calcium ionophore A23187 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phos-tag was obtained from Wako Pure Chemical Industries (Osaka, Japan). Carboxymethylcellulose (CMC), aspirin, and indomethacin were obtained from Kanto Chemical Co. (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan), and Nacalai Tesque Inc. (Kyoto, Japan), respectively. A prostaglandin E_2 EIA kit was obtained from Cayman Chemical Co. (Ann Arbor, MI). A lactate dehydrogenase (LDH) assay kit was obtained from Dojindo Laboratories Co. Ltd. (Kumamoto, Japan). A bromo-2-deoxy-uridine (BrdU) cell proliferation ELISA kit was purchased from Roche (Mannheim, Germany).

2.3 Animals

Five-week-old male ICR mice (35g body weight) were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The animals were adapted to an animal room maintained at 24 ± 2 °C and housed in a 12 h light/dark cycle. The care and handling of mice

were in accordance with the National Institute of Health guidelines. All experimental procedures were approved by the Tokushima University Animal Care and Use Committee.

2.4 Extraction of lipid and isolation of LPA from herbs

Lipids were extracted from the medicinal herbs by the Bligh and Dyer method [20] with acidification of the water/methanol phase, as described previously [19]. In brief, 1 g of herb powder was mixed with 15.2 ml of a solvent consisting of chloroform/methanol/water in the ratio of 1:2:0.8 (v/v/v) and centrifuged to collect the supernatant. The pellet was added to the same amount of the mixed solvent consisting of chloroform/methanol/water and centrifuged. The combined supernatant fraction was mixed with an appropriate volume of chloroform and water to make solvent system consisting of chloroform/methanol/water in the ratio of 1:1:0.9 (v/v/v). The resulting two-layer solution was mixed with 0.15 ml of 5 N HCl and centrifuged. Lipids were obtained from the lower phase (chloroform phase). The LPA in the lipid extract was isolated by TLC. The solvent system of the chromatography was chloroform/methanol/28% aqueous ammonia (60:35:8, v/v/v). After development, the plate was dried for a few minutes with blowing air and sprayed with primulin for visualization under UV light. LPA was identified, extracted from the silica gel by the Bligh and Dyer method [20], and quantified by the colorimetric method based on phospho-molybdenum-malachite green formation [21]. The weight of phospholipids (μg/g) was determined from a weight of lipid phosphorus (μg inorganic phosphorus/g) in a way recommended by American Oil Chemists' Society [22].

2.5 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)

Molecular species of LPA in herbs were determined by MALDI-TOF MS as described previously [15]. An aliquot of LPA was dissolved in 100 µl of methanol containing 0.1% aqueous ammonia. This solution (10 µl) was mixed with 5 µl 0.1 mM ^{68}Zn Phos-tag solution. A small portion (0.5 µl) of this mixture was spotted on a sample plate. Immediately, 0.5 µl of 2, 4, 6-trihydroxyacetophenone (THAP) solution (10 mg/ml in acetonitrile) was layered onto the mixture as a matrix solution. The sample plate was dried for a few minutes, and the matrix/analyte co-crystal that formed was subjected to MALDI-TOF MS. MALDI-TOF mass spectra were acquired using a Bruker Microflex mass spectrometer (Bruker Daltonics, Bremen, Germany) in positive ion detection mode. The wavelength of the nitrogen-emitting laser and the accelerating voltage were 337 nm and 20 kV, respectively. To enhance the reproducibility, 300 laser shots were averaged for each mass spectrum.

2.6 NSAIDs-induced gastric ulcer

Mouse models of aspirin- and indomethacin-induced gastric ulcer were developed as described previously [14, 15]. In brief, aspirin (300 mg/kg body weight), indomethacin (22.9 mg/kg body weight), or peony root lipid was suspended in 3% (w/v) CMC and sonicated for 1 min. Peony root powder was suspended in water. Fasted mice were intragastrically administered peony root lipid or powder suspension in a volume of 0.2 ml. After 0.5 h, 0.2 ml of the aspirin or indomethacin suspension was administered intragastrically. The mice were anesthetized with diethyl ether and sacrificed 3 or 5 h after the aspirin or indomethacin administration, respectively. The isolated stomach was ligated at both ends, filled with 1.5 ml of 2% formalin, and immersed in 2% formalin for 15 min. Then, the stomach was cut along the greater curvature, and the lengths of lesions on the stomach wall were measured using a millimeter scale with a magnifying glass. The total length of lesions was used as a lesion index.

2.7 Cell culture and reverse transcription-PCR (RT-PCR)

MKN74 cells, a human gastric cancer cell line, were obtained from the RIKEN Cell Bank (Tsukuba, Japan). The MKN74 cells were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 ºC. Total RNA from MKN74 cells was prepared by using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The first-strand complementary DNA (cDNA) was synthesized from the purified total cellular RNA with random hexamer primers using the SuperScript™ III synthesis system for RT-PCR kit (Invitrogen, Carlsbad, CA). The cDNA was then subjected to PCR amplification with primer sets and condition as described in supplementary Table 1. The PCR products were examined by electrophoresis on 2% agarose gel, stained with Gelred (Biotium, Hayward, CA) and visualized with UV light. Images of the fluorescent band on the gel were captured by a Fuji LAS-4000 imaging system (FujiFilm, Tokyo, Japan), and the digitized image data were analyzed by NIH image.

2.8 PGE_2 production assay

PGE_2 production in MKN74 cells was performed as described previously [16]. In brief, MKN74 cells were seeded in 35-mm dishes and added 10 μM arachidonic acid (AA) for AA-enrichment in the cells. After 24 h, the medium was changed to serum-free medium, and further incubated for 24 h. Then, the cells were treated with 10 μM acetylsalicylic acid for 30 min to minimize the effect of the preexisting COX activity. After replacement of the medium, cells were incubated with 18:1 LPA, peony root lipid extract or purified peony root LPA in the presence or absence of pertussis toxin (PTX). After 5 h, the cells were stimulated with 1 μM A23187 for 10

178 min. The supernatant were collected and PGE_2 was evaluated by EIA kit according to the
179 manufacturer's instructions.

180 **2.9 Indomethacin-induced cell damage of MKN74 cells**

181 MKN74 cells were seeded at a density of $1x10^5$ in 35-mm polystyrene dishes and serum starved
182 for 24 h. LPA dissolved in 0.3% BSA in PBS were added 2 h before addition of indomethacin
183 solution. The final concentration of indomethacin was fixed at 0.8 mM. After 30 h, the cells were
184 gently washed with PBS and stained with propidium iodide (PI) for 30 min. The extent of cell
185 death was determined by observation with a fluorescent microscope Axiovert 200 M (Zeiss,
186 Oberkochen, Germany). For the LDH assay, MKN74 cells were treated in the same manner as
187 described above. At the end of incubation, 100 μl of the culture medium supernatant was added
188 per well of a 96-well microplate. The LDH activity was measured according to the instructions
189 of the LDH assay kit. The activity was expressed as % of maximum release of LDH that can be
190 obtained in a sample of the lysis buffer-treated cells. The extent of cell death was also
191 determined by flow cytometric analysis. The PI-stained cells were subjected to a flow cytometer
192 (Becton Dickinson) that was operated using Cell Quest software, and at least 10,000 cells were
193 analyzed for each sample.

194 **2.10 Cell proliferation assay**

195 MKN74 cells seeded in 35-mm polystyrene dishes were serum-starved for 24 h. Then they
196 were cultured with or without synthetic or peony root-derived LPA in the presence or absence of
197 PTX. After 24 h, cells were harvested by trypsinization, mixed with trypan blue dye, and the
198 number of living cells was counted using a hemocytometer. The proliferation of MKN74 cells
199 was also confirmed by a BrdU cell proliferation assay. In brief, $2x10^3$ cells were seeded in 96-

well microplates in 100 μl/well culture medium and kept in serum-starved condition for 48 h. The synthetic or peony root-derived LPA was added to the cells and further incubated for 24 h. BrdU was added to the cell culture 4 h before termination of incubation. The incorporated BrdU was determined as described by the manufacturer's protocol.

2.11 Determination of LPA in a mouse stomach fluid

Stomachs of overnight-fasted mice were isolated and gently washed with PBS. The stomach was cut along the greater curvature. The stomach inner surface was carefully washed with a small amount of PBS. After addition of 17:0 LPA (0.5 nmol) as an internal standard, lipids were extracted from the stomach washing solution by using an acidified Bligh and Dyer method as described above. Extracted lipids were dissolved in 0.8 ml of methanol and filtered through 0.2 μm nylon filter. After filtration, methanol was evaporated and reconstituted in 0.1 ml of methanol/water mixture (95:5, v/v) containing 5 mM ammonium formate for LC/MS/MS. LC/MS/MS was performed as described previously [23] using a quadrupole-linear iontrap hybrid mass spectrometry system, 4000 Q TRAP™ (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) with an Agilent 1100 liquid chromatography system (Agilent Technologies, Wilmington, DE, USA). In the negative ion mode of operation with multiple reactions monitoring, Q1 was set to the deprotonated molecular ion of each class of LPA as the precursor ion. The fragment ions, $[\text{deprotonated cyclic glycerophosphate}]^-$ at m/z 153 were selected for Q3. The ratios of the negative ion peak areas of the endogenous LPA to that of the corresponding internal standard were calculated.

2.12 Statistical analysis

Statistical analyses of the difference between two means were performed by Student's *t*-test.

3. Results

3.1 Abundance of LPA in medicinal herbs

Our previous study [19] revealed that LPA is abundant in cruciferous plants, such as cabbage leaves and radish roots (9.2 and 2.3 μg/g wet weight, respectively). Soybean seed powder was also found to be rich in LPA (4.6 μg/g). In this study, we determined LPA content in 21 dried medicinal herbs used for the treatment of GI disorders (Fig. 1A). Compared to LPA-rich foods, about half of the medicinal herbs contained LPA at high levels (40–240 μg/g). Among them, peony root powder (240 μg/g) contained the highest level of LPA. It was 52 times that of soybean seed powder. We previously revealed that phosphatidic acid (PA), a diacyl derivative of LPA, serves as a source of LPA in the digestive tract [14]. The amounts of PA in these medicinal herbs were comparable (Fig. 1B) to those in cabbage and soybean, which were characterized as PA-rich foods previously [24]. Surprisingly, the percentage of LPA in total phospholipids (PLs) in peony root was 11%, which is 30 and 400 times of those in cabbage leaves and soybean seed powder, respectively (Table 1). These results indicated that peony root powder contains abundant LPA with a high concentration in its lipid fraction. This is evident from the relative intensity of TLC bands of its lipid extract (Supplementary Fig. 1).

MALDI-TOF MS of PA and LPA in medicinal herbs showed that the predominant PA species were 16:0/18:2 and 18:2/18:2 (or 18:1/18:3) PA (Supplementary Table 2) and the predominant LPA species were 16:0, 18:2 LPA (Supplementary Table 3). This is also the case in peony root as shown in Fig. 2A, B.

3.2 Anti-ulcer effect of peony root lipid and powder

As shown previously, orally administered aspirin (300 mg/kg body weight) produces 15–20 mucosal lesions in the gastric corpus of mice [14]. The lesions were linear and extended from the fundic area to the pyloric area as erosion. Similar morphological lesions were observed in the experiments with indomethacin (22.9 mg/kg body weight) (Fig. 3A). We used the total length of lesions as the lesion index.

Orally administered peony root lipid reduced indomethacin-induced lesion formation in a dose-dependent fashion (Fig. 3A, B). The maximum reduction was observed when mice were administered 1 mM of peony root lipid (Fig. 3A, B), which corresponds 4.4 mg PLs/kg (animal body weight). We confirmed that synthetic LPA at 1 mM has protective effect against indomethacin-induced lesion formation (Fig. 3B). We also examined the gastro protective effect of peony root powder on an aspirin-induced acute gastric ulcer mouse model. The peony root powder at 2 g/kg body weight effectively reduced gastric mucosal lesion formation (Fig. 3C). This dose of the powder corresponds to the administration of 1 mM peony root lipid.

3.3 Enhancement of PGE_2 production by LPA-rich herbal lipids in gastric cells

According to the Human Protein Atlas database (http://www.proteinatlas.org/), LPA_2, LPA_5, and LPA_6 are expressed abundantly in human stomach epithelia. On the other hand, expression levels of LPA_1, LPA_3, and LPA_4 are very low. Firstly, we examined $LPAR_{1–6}$ mRNA expression in human gastric cancer cell line, MKN74 cell. Results showed that order of abundance of LPAR mRNA was LPA_2=LPA_5>LPA_6. Levels of mRNA of LPA_1, LPA_3, and LPA_4 were under detectable (Fig. 4A). The relative abundance of mRNA of LPARs in MKN74 cells is good agreement with the expression profile of LPARs in human stomach tissue. We used this cell line as a representative mucosal cell model of the human stomach. We confirmed that synthetic 18:1

LPA can enhance PGE_2 production in MKN74 cells (Fig. 4B). This is consistent with our previous observation that LPA enhances PGE_2 production via up-regulation of COX-2 [16]. This effect was also observed with LPA prepared from peony root. Peony root lipid, which has concentrated LPA, enhanced PGE_2 production at high efficacy. Enhancements of PGE_2 production induced by LPA and peony root lipid were completely abolished by PTX (Fig. 4C), suggesting the involvement of Gi-coupled receptor in their actions. It should be mentioned that high concentration of the peony root lipid (10 μM) did not induce PGE_2 production (Fig. 4B). This is due to cytotoxicity of the lipid extract which contain various components other than LPA. In fact, we observed that most of the cells were floating at the end of the incubation with peony root lipid at 10 μM.

3.4 LPA protects indomethacin-induced cell injury of MKN74 cells

Effect of LPA on indomethacin-induced cell injury was examined in MKN74 cells. As shown in Fig 5A, non-treated MKN74 cells were tightly attached each other. We found that treatment of the cells with 0.8 mM indomethacin results in loss of attachment and rounding of their cell shape (Fig. 5A), a typical morphological change in apoptotic cells [25, 26]. When the cells were treated with 0.8 mM indomethacin plus 10 μM LPA, the indomethacin-induced morphological change was not observed. In fact, LPA caused cell spreading with an extended edge, indicating the disappearance of apoptotic symptoms (Fig. 5A). The protective action of LPA on indomethacin-induced cellular damage was quantified by directly counting PI-positive cells (dead cells). We found that LPA reduced the number of PI-positive cells in a dose-dependent fashion (Fig. 5B). LPA from peony root also significantly reduced cell death at 10 μM (Fig. 5B). The protective action of LPA against indomethacin-induced cellular damage was also confirmed by LDH leakage (Fig. 5C). To determine the involvement of G-protein-coupled receptors in these

observations, the effect of pretreatment of PTX, a specific inhibitor of Gi-protein, was examined. Flow cytometric analysis was applied for this experiment to quantify the population of dead cells in the total cells. Results showed that the effect of LPA against indomethacin-induced cell death was completely abolished by pre-treatment of PTX, indicating the involvement of Gi-coupled receptors in the LPA action (Fig. 5D). This is also confirmed by the morphological change of the cells (data not shown). We found that LPC did not show a protective effect on indomethacin-induced cell death (Fig. 5D).

3.5 LPA stimulated proliferation of MKN74 cells

Treatment of MKN74 cells with LPA induced proliferation in a dose-dependent manner (Fig. 6A). LPA-induced cell proliferation was also confirmed by measuring incorporation of the thymidine analog BrdU into the cells (Fig. 6B). Peony root LPA also stimulated the proliferation of MKN74 cells (Fig. 6A, B). LPA-induced cell proliferation was partially and significantly inhibited by PTX (Fig. 6C). Again, LPC had no proliferative effect.

3.6 LPA concentration in a mouse stomach fluid

In order to better understand the role of LPA in the physiology of the stomach, it is necessary to know the concentration and molecular species of LPA in stomach fluid. The volume of stomach fluid was assumed to be 0.08 ml. This is based on the fact that the area of the mucus layer is 400 mm^2 and the thickness of mucus gel layer is 0.2 mm [27]. We found that the total LPA concentration in the stomach fluid was 2.4 μM (Fig. 7). The major species of LPA in the stomach fluid were found to be 16:0, 18:0, 18:1, and 18:2 LPAs (Fig. 7). The concentration of these LPA species was found to be 0.3–0.7 μM. Other LPA species, such as 16:1, 18:3, 18:4,

310 20:0, 20:1, 20:2, 20:4, 22:0, 22:4, and 22:6, were present in low (0.01–0.1 μM) level
311 (Supplementary Fig. 2).

312 **4. Discussion**

313 Research in medicinal herbs has identified many active components that exert anti-ulcer effects
314 with diverse mechanisms of action. These include compounds belonging to flavonoids, alkaloids,
315 tannins, and saponins [28, 29]. Dietary phospholipids, such as soy-derived PC, are also shown as
316 reducing agents for NSAIDs-induced gastric ulcer [30]. In this study, we found that LPA in
317 medicinal herbs is a potential component for prevention of gastric mucosal injury. This notion is
318 based on several observations. Firstly, synthetic LPA and herbal LPA showed ameliorative
319 activity against cytotoxic effect of indomethacin. Secondly, LPAs and LPA-rich lipid enhanced
320 PGE_2 production, an important cytoprotective factor in GI mucosa. Thirdly, peony root lipid, an
321 LPA-rich herbal lipid identified here, significantly ameliorated indomethacin-induced gastric
322 lesions in mice. Lastly, there were many LPA-rich herbs in Chinese traditional medicines used
323 for the treatment of GI disorders. We discuss on mechanisms of these effects in detail.

324 A well-known mechanism of NSAID-induced gastric mucosal lesions is the inhibition of
325 COX-1 and COX-2 enzymes and a resulting decrease in gastroprotective PGE_2. The importance
326 of PGE_2 in the integrity of stomach mucosa is evident from the fact that common anti-ulcer drugs,
327 such as rebamipide and geranylgeranylacetone, up-regulate COX-2, leading to the enhancement
328 of PGE_2 production [31, 32]. Consistent with our previous study [16], LPA from medicinal herb
329 was found to enhance PGE_2 production in human gastric cancer cell line, MKN74 cells. We also
330 showed that peony root lipid, which has concentrated LPA, enhanced PGE_2 production with Gi-
331 mediated manner. Surprisingly, the efficacy of PGE_2 production of peony root lipid is higher

than that expected from the LPA content in the lipid extract. At present, we do not know other components in peony root lipid that enhance PGE_2 production along with LPA. Considering that PGE_2-enhancement was completely abolished by PTX, there may be components that increase LPA action in the lipid. Further study is needed for clarification of this point.

NSAIDs have been reported to induce apoptosis in gastric mucous cells [25, 26]. The mechanism of the cytotoxicity is uncoupling of mitochondrial oxidative phosphorylation and inhibition of the electron transport chain, leading to depletion of intracellular ATP, cellular Ca^{2+} toxicity, and generation of reactive oxygen species [13]. It is also reported that NSAIDs chemically interact with cell membrane phospholipids, disrupt membrane permeability, and form membrane pores [13]. In this study, we found that pretreatment of MKN74 cells with LPA prevents indomethacin-induced cell shape change, LDH leakage and cell death. The protective effect of LPAs against indomethacin-induced cell death was completely abolished by PTX, indicating the involvement of Gi-coupled receptor.

It has been reported that LPA stimulates proliferation of diverse types of cells, including gastric cancer cells [33]. In this study, we found that LPA stimulated proliferation of MKN74 cells. The proliferative effect of LPA was partially but significantly abolished by PTX. The partial inhibition of PTX in LPA-induced proliferation of MKN74 indicates the involvement of Gi as well as G12/13 and Gq/11/14 in the LPA response. Our result is consistent with previous reports showing that LPA stimulates proliferation of NIH3T3 in both PTX- sensitive and -insensitive manners [34].

We observed membrane budding-like structure in LPA-treated MKN74 cells (Supplementary Fig. 3). They form almost vesicles, and their size is considerably smaller than those of LPA-

induced membrane blebs reported by Valentine et al. [35]. At present, we do not know the biological significance of this phenomenon induced by LPA. Miyake et al. reported that MKN28 cells secrete mucin when the plasma membrane of the cells is injured in the presence of calcium [36]. They also found microvilli on the plasma membrane of MKN28 cells. If the phenomenon observed here is one of the steps of mucin secretion from gastric cells, the physiological function of the LPA-induced vesicle secretion is to strengthen the mucus gel layer, a protective barrier of the stomach wall.

We found that LPA_2 and LPA_5 are predominant LPAR in MKN74 cells. This is good agreement with the expression profile of LPARs in human stomach tissue [http://www.proteinatlas.org/]. Here, we showed that LPA-induced PGE_2 production, anti-apoptosis, and proliferation in MKN74 cells were all Gi-mediated responses. LPA_2 has been known to couple with Gi in many cells [8, 37, 38]. On the other hand, LPA_5 seems to be coupled with Gq rather than Gi in many cells [39, 40]. Considering these facts, it is reasonable to assume that LPA_2 is involved in these observations and that LPA_2 expressed on the apical membrane of gastric mucous cells [16] plays important role in gastric mucosal integrity.

In this study, for the first time, we determined the LPA concentration in stomach fluid. We found that LPA concentration in stomach fluid was 2.4 μM. The LPA concentration in stomach fluid is three times higher than that in saliva (0.9 μM) [41] and similar or relatively higher level to that in human plasma or serum LPA (0.1–2.4 μM) [42-44]. The presence of LPA in stomach fluid is reasonable because LPA receptors are expressed in the apical side of gastric mucosal cells [14, 16]. We found that the level of LPA in the stomach fluid is in a range that induces a diverse response in gastric cells *in vitro* (Fig. 4, 5, 6). We also found that the abundant LPA species in the stomach fluid were 16:0, 18:0, 18:1, and 18:2 LPA. These LPA species are potent

agonists for LPA receptors [45] and showed potent gastro protective effects *in vivo* and *in vitro* (Fig. 3, 5). These results suggested that endogenous LPAs have a possibility to play vital roles in gastric epithelial cells, and that ingested LPAs contribute to mucosal integrity by augmentation of LPA in stomach fluid.

5. Conclusion

The present study revealed the abundant existence of LPA in medicinal herbs that are used for treatment of GI disorders. An LPA-rich herb, peony root had a significant gastro protective effect on NSAID-induced gastric ulcer. We also revealed that in addition of PGE_2 enhancement, LPA protects against NSAID-induced acute cell toxicity and stimulates the proliferation of gastric cells. LPA_2 in gastric mucosal cells are considered to be involved in these LPA actions.

Conflicts of interest

No conflicts of interest

Author contributions

S. A. is primary author of manuscript, conducted most of experiments and data analysis. S. W., A. T., K. K. and T. T. designed this study. A. Y. contributed to analysis of medicinal herbs. K. F., M. M. R., T. F., T. S. and T. I. contributed to cultured cell experiments. K. M. conducted mass spectrometric analysis of LPA in stomach fluid and receptor expression analysis. E. K. and K. T. conducted microscopic observation including electron microscope. All authors of the manuscript have approved this manuscript.

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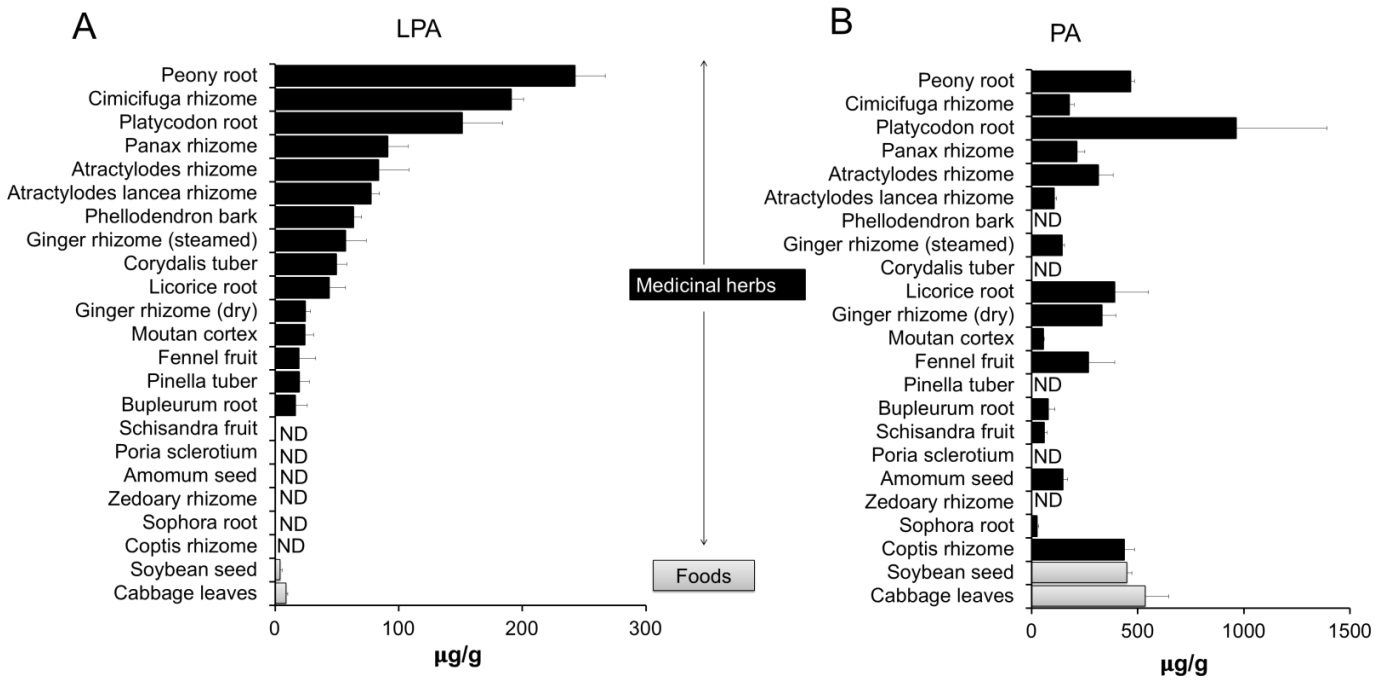
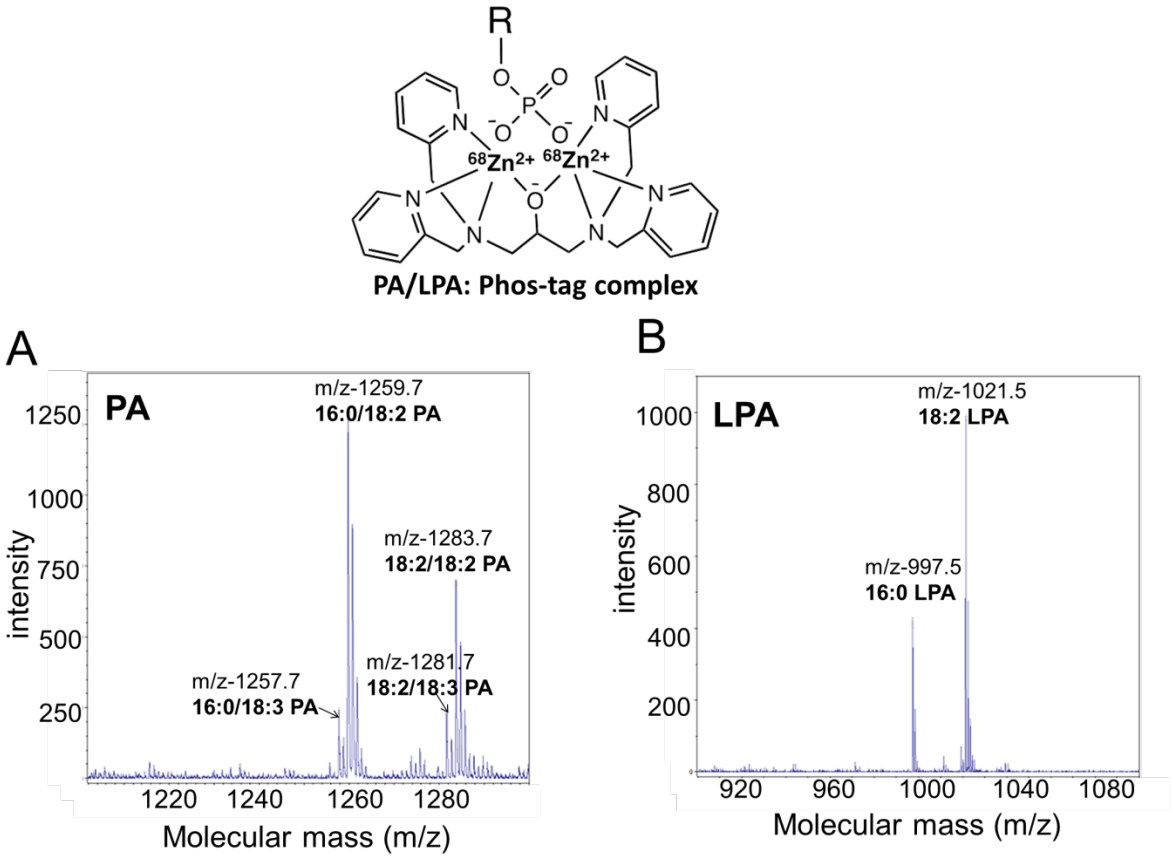


Fig. 1. Abundance of LPA in medicinal herbs

Amounts of (A) LPA and (B) PA isolated from herbs were determined by measurement of their lipid phosphorus. Data represent means ± SD of three independent experiments. Herbs and foods except for cabbage leaves were dry weight. ND: Not detectable (less than 2 µg/g).



548

549 **Fig. 2. MALDI TOF mass spectra of PA and LPA isolated from peony root**

550 (A) PA and (B) LPA from peony root were analyzed by MALDI-TOF MS as their Phos-tag
551 complexes. Chemical structure shown is a complex of a phosphate monoester compound with
552 Phos-tag.

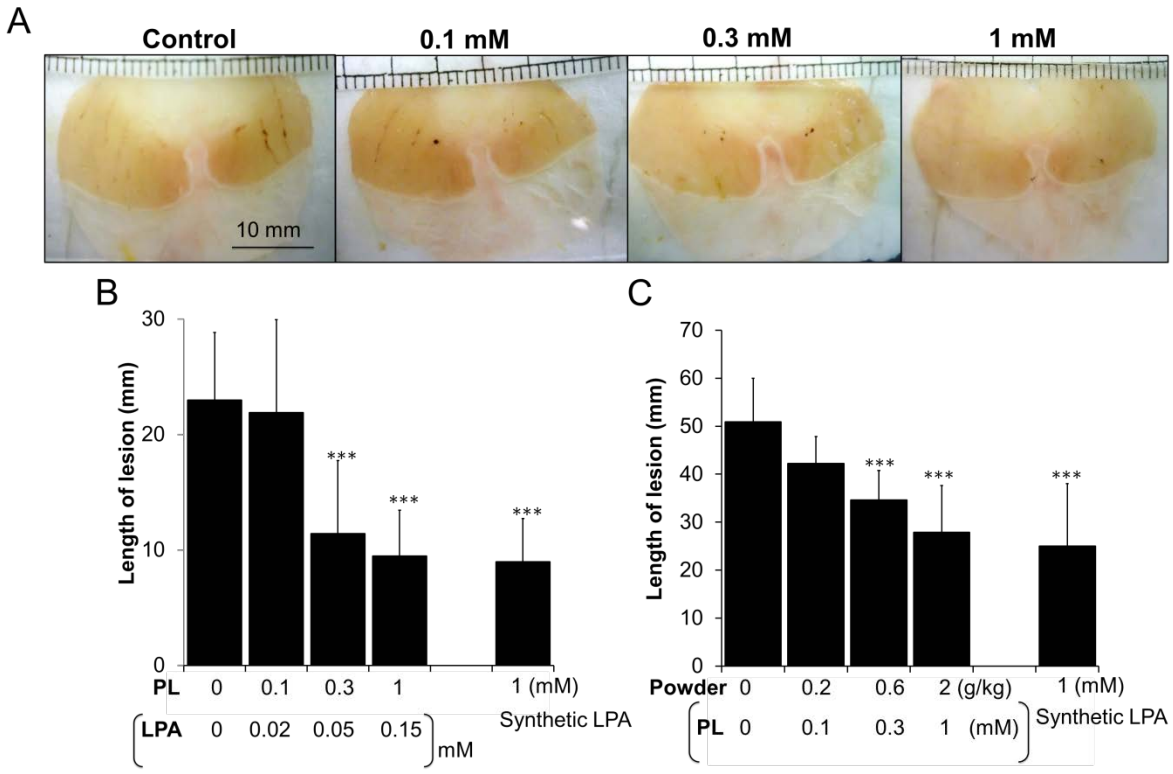


Fig. 3. Anti-ulcer effect of peony root lipid and powder

(A, B) Peony root lipid was suspended in 3% CMC. Aliquots of the suspension (0.2 ml) were orally administered to mice. After 30 min, 0.2 ml of indomethacin (22.9 mg/kg) in 3% CMC was intragastrically administered. The total lengths of lesions on the stomach wall were measured at 5 h after injection of indomethacin. (B) Indicated concentrations are based on the amounts of total phospholipid. For example, "1 mM PL" indicates administration of peony lipid containing 0.2 μmol of phospholipid in 0.2 ml of the suspension. Values in the parenthesis indicate concentrations of LPA in peony root lipid suspensions. This is deduced from Table1. The numbers of mice of each group were 15 for control (0 mM) and 5–15 for others. ***P<0.005 versus control. (C) Mice were intragastrically administered 0.2 ml water or 0.2 ml peony root powder suspended in water. Peony root powder in a dose of 2 g/kg body weight contains 1 mM phospholipids. Mice were sacrificed 3 h after aspirin administration (300 mg/kg). The numbers of mice of each group were 5–10. ***P<0.005 versus control. Synthetic LPA (16:0 LPA) at 1 mM (5.7 μmol/kg body weight) was used as positive control. Error bar represents SD.

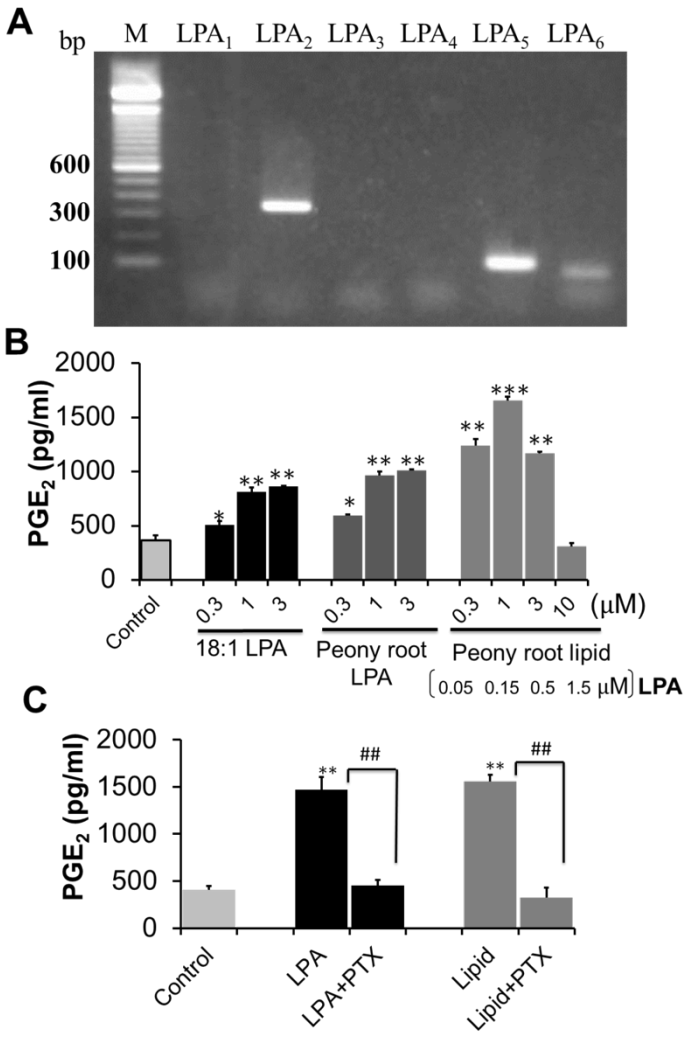
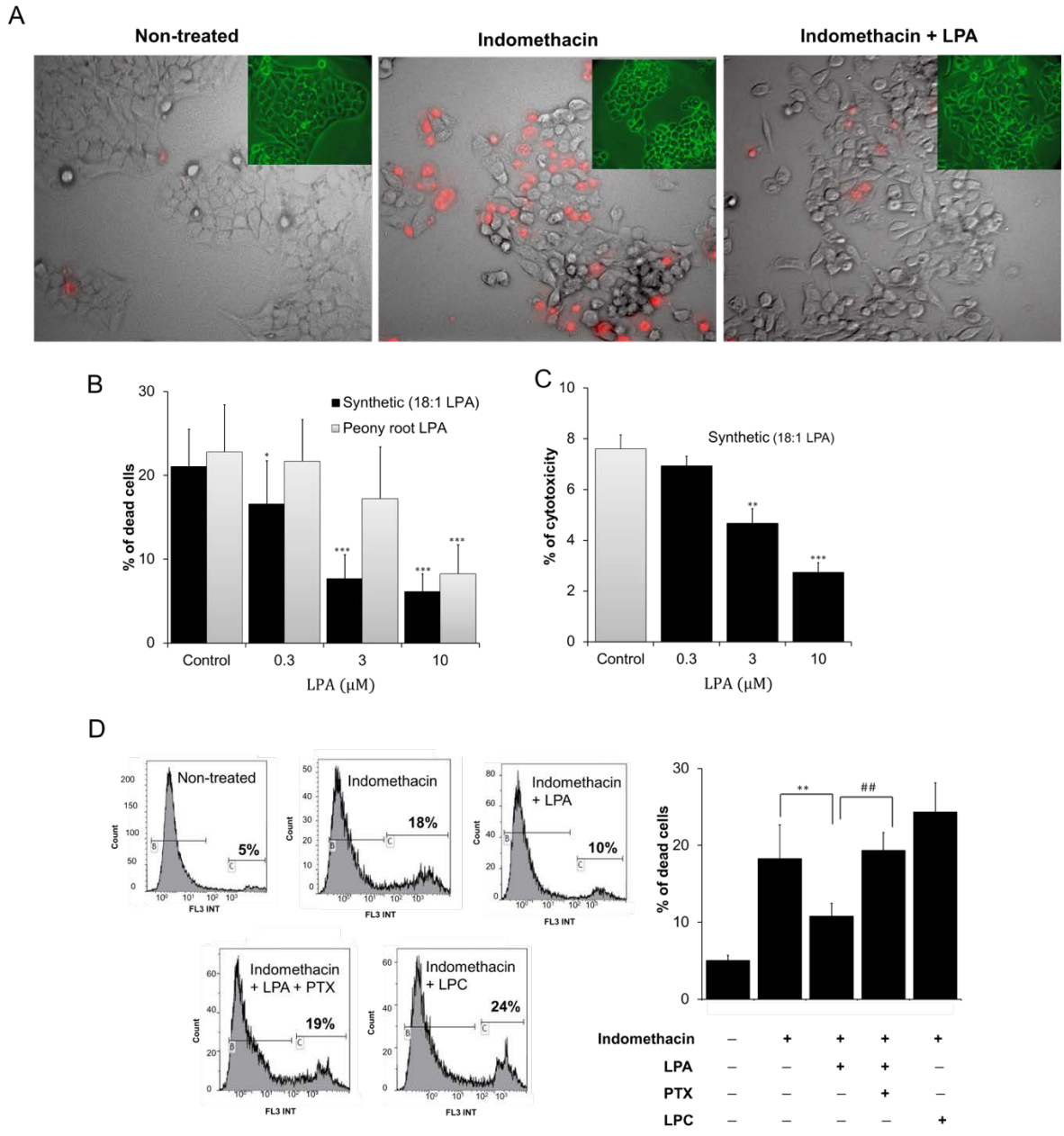


Fig. 4. Enhancement of PGE_2 production by LPA and peony root lipid.

(A) mRNA profile of LPARs (LPA_{1-6}) of MKN74 cells. (B) MKN74 cells repleted with arachidonic acid were preincubated with the indicated concentration of LPA or peony root lipid for 5 h before stimulation with 1 μM A23187 for 10 min. PGE_2 released into the culture media was measured using an ELISA kit. Indicated values in parenthesis are the concentration of LPA in the peony root lipid. Each value shown is mean ± SD. (C) MKN74 cells were incubated with 3 μM synthetic LPA (18:1 LPA) or 3 μM peony root lipid in the absence or presence of 100 ng/ml of PTX for 5 h before stimulation with 1 μM A23187 for 10 min. PGE_2 released into the culture media was measured using an ELISA kit. Each value shown is the mean ± SD. *P<0.05, **P<0.01, ***P<0.005 versus control and $^{\#\#}$ P<0.01.



591

592 **Fig. 5. LPA protect indomethacin-induced cell injury in MKN74 cells.**

593 (A) Serum-starved MKN74 cells were incubated with 0.8 mM indomethacin with or without 10
594 μM 18:1 LPA. After 30 h, dead cells are stained with propidium iodide (PI). The image shown is
595 phase-contrast microscopic photo merged with a fluorescent microscopic photo for indication of
596 PI-positive cells. (B, C) Serum-starved MKN74 cells were incubated with 0.8 mM indomethacin
597 in the absence or presence of increasing concentration of LPAs. (B) The percentage of dead cells

was calculated by counting PI-positive cells in several randomized subfields in each dish from three different experiments. (C) LDH leakage in the culture media was determined to know the extent of the cellular damage. (D) MKN74 cells were incubated with 0.8 mM indomethacin in the absence or presence of 10 μM 18:1 LPA or 16:0 LPC with or without PTX (100 ng/ml). Flow cytometry was performed 30 h after indomethacin treatment. Values in the flow cytometry chart indicate the % of dead cells. Data represent means ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.005 versus control and $^{\#\#}$ P<0.01.

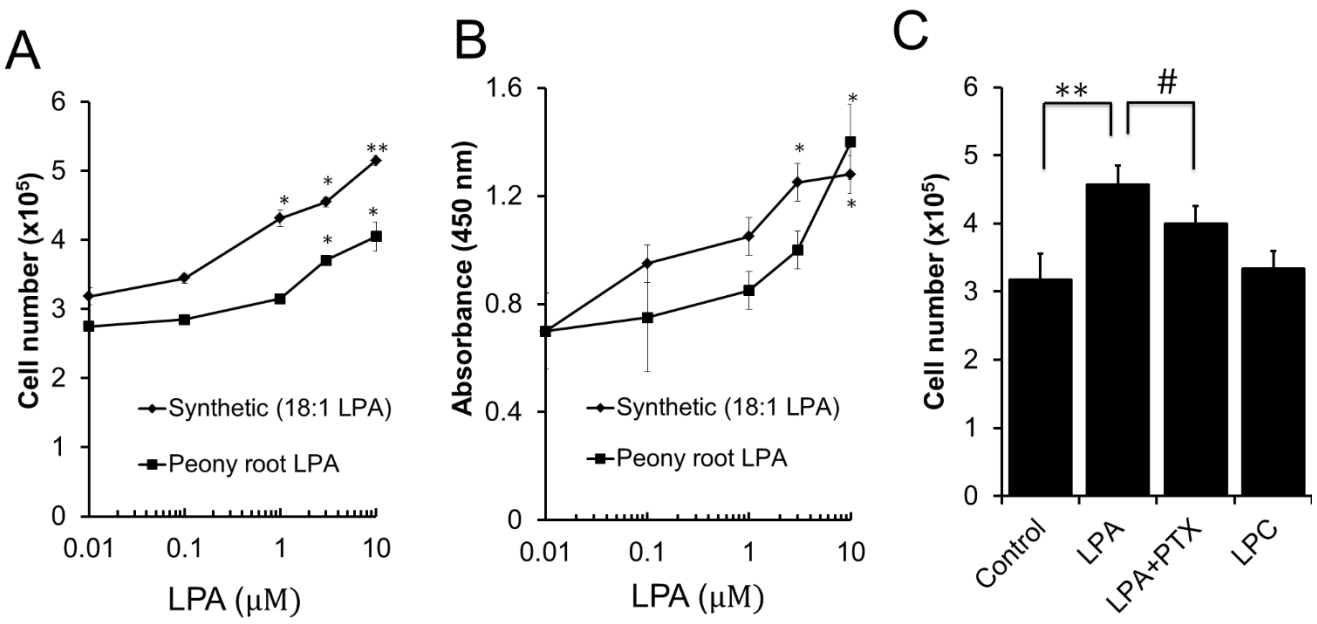


Fig. 6. LPA stimulated proliferation of MKN74 cells

(A, B) MKN74 cells were serum starved for 24 h, and then, treated with different concentrations of LPA. After 24 h, the extent of proliferation was determined by direct counting or BrdU incorporation. (C) The proliferation assay was conducted in the presence or absence of 100 ng/ml of PTX or with 16:0 LPC (10 μM) instead of 18:1 LPA. Data represent means ± SD of three independent experiments. *P<0.05, **P<0.01 versus control and $^{\#}$P<0.05.

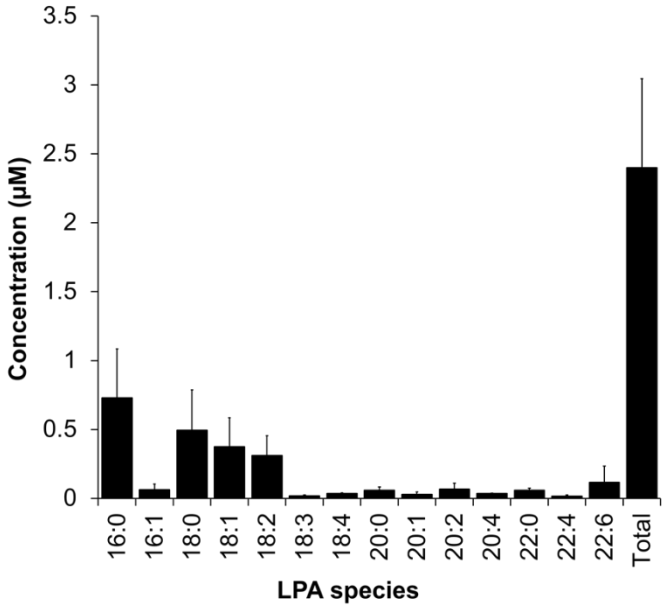


Fig. 7. LPA concentration in a mouse stomach fluid

Stomachs of fasted mice were isolated and cut along the greater curvature. The stomach mucosal surface was washed with a small amount of PBS. Lipids were extracted from the stomach washing solution and subjected to LC/MS/MS by using 17:0 LPA as an internal standard. Data represent means ± SD of three independent experiments.

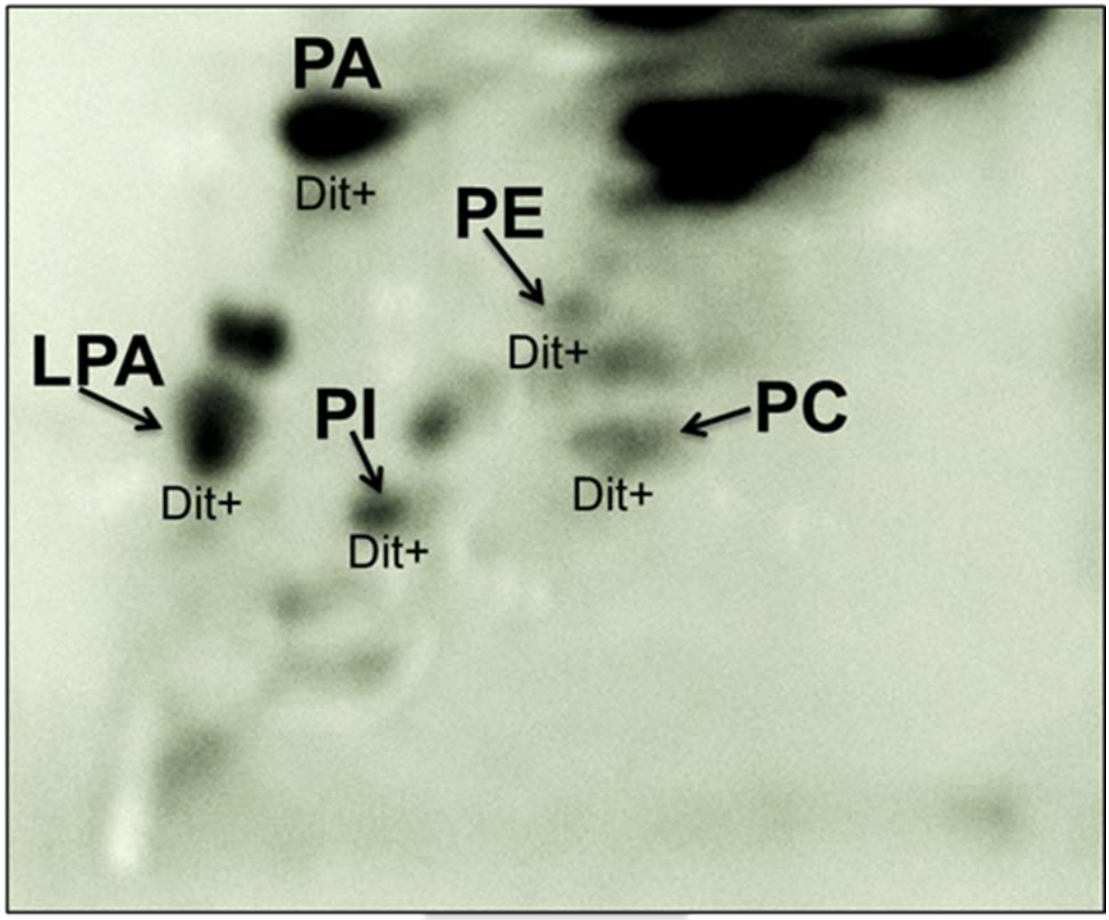
Table 1

LPA is highly concentrated in the lipid fraction of peony root.

	Cabbage		Soybean		Peony root	
	μg/g	(%)	μg/g	(%)	μg/g	(%)
Total phospholipid	2300 ± 160	-	13000 ± 2700	-	2200 ± 140	-
PA	540 ± 110	(24)	450 ± 20	(3.0)	460 ± 20	(21)
LPA	9 ± 1	(0.4)	5 ± 2	(0.03)	240 ± 20	(11)

Value in cabbage is wet weight. Values in soybean and peony root are dry weight. Values in parentheses are percentage in total phospholipid.

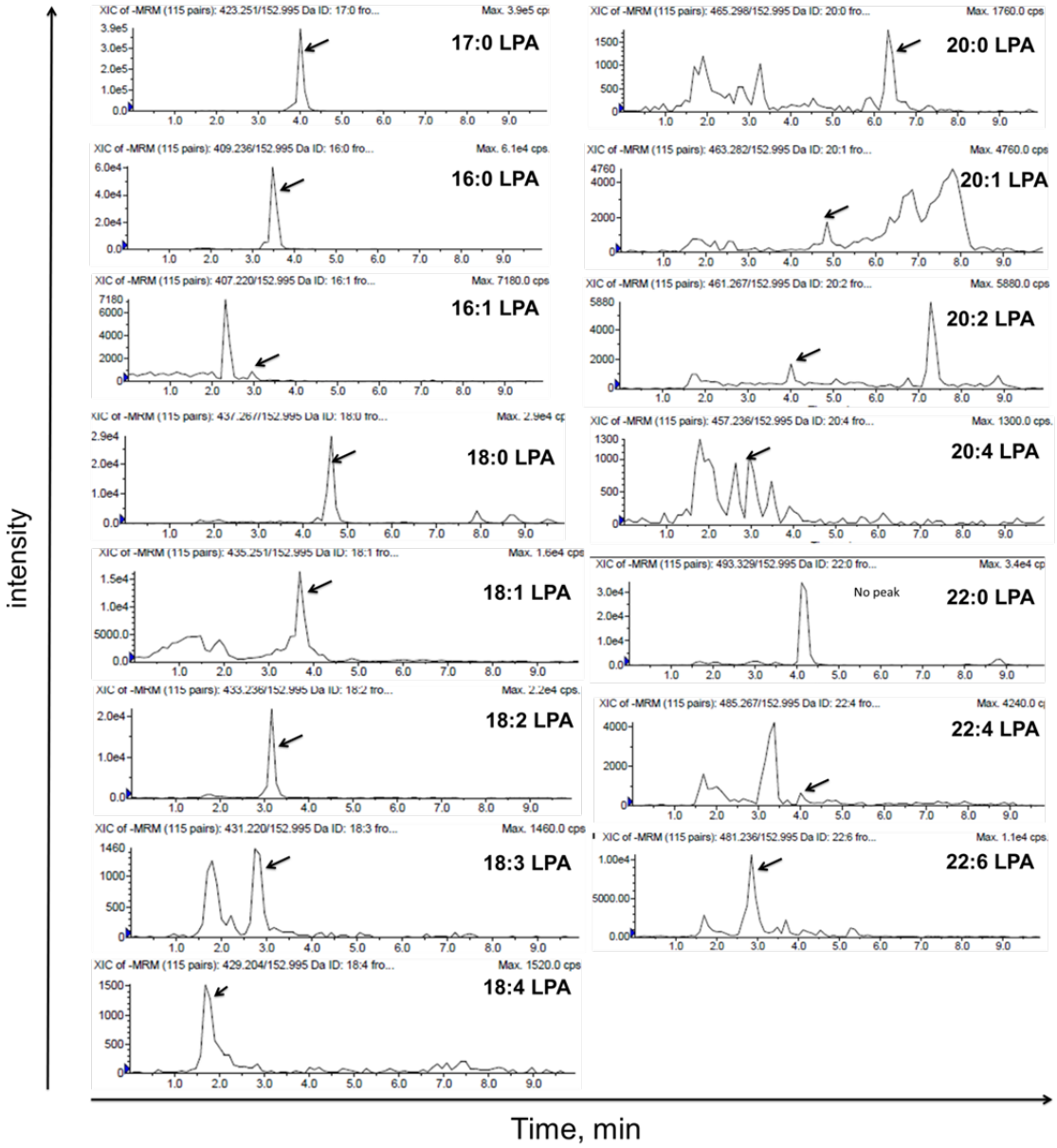
Supplementary Fig. 1



Supplementary Fig. 1. Two-dimensional TLC of peony root lipid

Peony root lipid was separated by two-dimensional TLC. The solvent systems for the first and second chromatography were chloroform/methanol/28% ammonia (60:35:8, v/v/v) and chloroform/acetone/methanol/acetic acid/water (50:20:10:13:5, v/v/v/v/v), respectively. PA, phosphatidic acid; LPA, lysophosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol. Dit, Dittmer-positive spot indicating phospholipid.

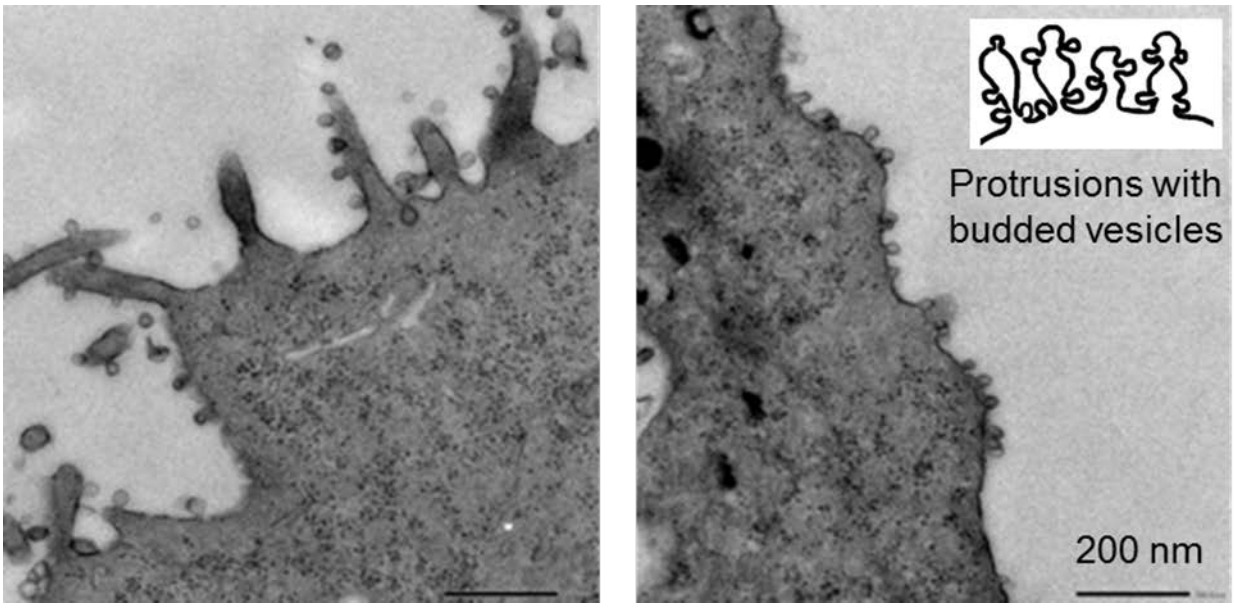
Supplementary Fig. 2



Supplementary Fig. 2. Determination of LPA molecular species in a mouse stomach fluid by LC/MS/MS.

A stomach of fasted mouse was isolated, and cut along the greater curvature. The stomach inner surface was washed with a small amount of PBS. Lipid was extracted from the washing solution, and subjected to LC/MS/MS for determination of LPA using 17:0 LPA as an internal standard.

Supplementary Fig. 3



Supplementary Fig. 3. LPA-induced structural change in plasma membrane of MKN74 cells.

MKN74 cells were treated with 10 μM 16:0 LPA for 3 h and fixed with 3% glutaraldehyde. After treatment with 1% osmium, cells were stained with 2% uranyl acetate. Serial section of the cells in each 70–80 nm in thickness, were cut with an ultramicrotome and examined with an electron microscope. Large protrusions with budded vesicles were observed in plasma membrane of the cells.

Supplementary Table 1.

Primer sequences and PCR conditions used in this study

Name	Sequence	Denaturation	Annealing	Extension
LPA_1	Forward: 5'-GAGGAATCGGGACACCATGAT-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec
	Reverse: 5'-ACATCCAGCAATAACAAGACCAATC-3'			
LPA_2	Forward: 5'-CATCATGCTTCCCGAGAACG-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec
	Reverse: 5'-GGGCTTACCAAGGATACGCAG-3'			
LPA_3	Forward: 5'-GCTCCCATGAAGCTAATGAAGACA-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec
	Reverse: 5'-AGGCCGTCCAGCAGCAGA-3'			
LPA_4	Forward: 5'-CAGTGCCTCCCTGTTTGTCTTC-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec
	Reverse: 5'-GAGAGGGCCAGGTTGGTGAT-3'			
LPA_5	Forward: 5'-AGCAACACGGAGCACAGGTC-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec
	Reverse: 5'-CCAAAACAAGCAGAGGGAGGT-3'			
LPA_6	Forward: 5'-CCGCCGTTTTTGTTCAGTC-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec
	Reverse: 5'-GAGATATGTTTTCCATGTGGCTTC-3'			

Supplementary Table 2. Molecular species composition of PA in medicinal herbs

	*34:3	34:2	34:1	36:5	36:4	36:3	36:2
Peony root	9	53	–	10	28	–	–
Cimicifuga rhizome	–	75	–	–	25	–	–
Panax rhizome	–	60	16	–	24	–	–
Atractylodes rhizome	–	67	–	–	33	–	–
Atractylodes lancea rhizome	–	66	–	–	34	–	–
Phellodendron bark	–	–	–	–	–	–	–
Ginger rhizome (steamed)	–	–	–	–	100	–	–
Corydalis tuber	–	–	–	–	–	–	–
Licorice root	–	62	–	–	38	–	–
Ginger rhizome (dry)	16	75	–	–	9	–	–
Moutan cortex	–	55	–	–	45	–	–
Fennel fruit	–	32	16	–	16	22	14
Pinellia tuber	–	–	–	–	–	–	–
Bupleurum root	–	65	–	–	35	–	–
Schisandra fruit	–	25	–	–	45	28	–
Poria sclerotium	–	–	–	–	–	–	–
Amomum seed	15	40	45	–	–	–	–
Zedoary rhizome	–	–	–	–	–	–	–
Sophora root	–	53	–	–	47	–	–
Coptis rhizome	–	47	–	9	30	14	–

The possible assignable PA species are 16:0/18:3 (34:3), 16:0/18:2 (34:2), 16:0/18:1 (34:1), 18:2/18:3 (36:5), 18:1/18:3 or 18:2/18:2 (36:4), 18:1/18:2 (36:3), and 18:1/18:1 (36: 2). *Total carbon number and number of double bonds in the fatty acid residues. The horizontal dashes lines indicate "not detectable". Our detection limit of PA in MALDI-TOF MS method is around 3 nmol/g herbs.

Supplementary Table 3. Molecular species composition of LPA in medicinal herbs

	16:0	18:1	18:2	18:3
Peony root	29	–	71	–
Cimicifuga rhizome	42	8	41	9
Platycodon root	12	–	83	4
Panax rhizome	42	–	58	–
Atractylodes rhizome	33	–	67	–
Atractylodes lancea rhizome	41	–	58	–
Phellodendron bark	36	–	56	8
Ginger rhizome (steamed)	23	41	36	–
Corydalis tuber	35	–	59	6
Licorice root	17	14	58	11
Ginger rhizome (dry)	44	–	46	–
Moutan cortex	21	14	57	8
Fennel fruit	41	27	32	–
Pinellia tuber	41	–	58	–
Bupleurum root	100	–	–	–
Schisandra fruit	–	–	–	–
Poria sclerotium	–	–	–	–
Amomum seed	–	–	–	–
Zedoary rhizome	–	–	–	–
Sophora root	–	–	–	–
Coptis rhizome	–	–	–	–

The fatty acyl moieties of LPA are designated in terms of the number of carbon atoms and double bonds. The horizontal dashes lines indicate "not detectable". Our detection limit of LPA in MALDI-TOF MS method is around 3 nmol/g herb.

Highlights

- The concentration of LPA in mouse stomach fluid was determined to be 2.4 μM.
- Peony root powder, a medicinal herb used for the treatment of gastrointestinal disorders, contained highly concentrated LPA.
- The lipid of peony root showed an ameliorative effect against indomethacin-induced gastric ulcer in mice.
- LPA and LPA-rich herbal lipid enhanced PGE_2 production and reduce cytotoxicity of NSAIDs in gastric cells.