Oral administration of natural occurring phosphatidic acid and lysophosphatidic acid protect against nonsteroidal anti-inflammatory drug-induced gastric ulcer

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Abbreviations

PL	Phospholipids
LPA	lysophosphatidic acid
LPAR	lysophosphatidic acid receptor
PA	phosphatidic acid
PC	phosphatidylcholine
LPC	lysophosphatidylcholine
GI	gastrointestinal
PLA_2	phospholipase A_2
PLD	phospholipase D
COX-2	cyclooxygenase-2
PGE_2	prostaglandin E_2
NSAID	nonsteroidal anti-inflammatory drug
mTOR	mammalian target of rapamycin
TLC	thin layer chromatography
MALDI−TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
LC/MS/MS	liquid chromatography-tandem mass spectrometry
CMC	carboxymethyl cellulose
PTX	Pertussis toxin
BrdU	bromo-2-deoxy-uridine
LDH	lactate dehydrogenase

General introduction

General introduction

Phospholipids (PLs) are structurally diverse and ubiquitous group of lipid. There are two classes of PLs. The first group is glycerophospholipids (GPLs), which composed with glycerol and two fatty acyl residues. The second group is sphingophospholipids. Sphingophospholipids have long-chain sphingoid base, to which a fatty acid is linked by an amide bond. The hydrophilic part of these phospholipids is phosphate diester, or phosphate monoester. Besides a role of biomembrane component, PLs serves as signaling molecule or precursor of signaling molecule that evokes cellular function or cellular responses by interaction with target proteins.

Lysophosphatidic acid (LPA) is a simple GPL that mediates diverse cellular responses (Fig. 1) such as cell proliferation, migration, anti-apoptosis and cytoskeletal rearrangement [1]. LPA signaling involves in diverse physiological and pathophysiological condition. It has been reported that LPA signaling involves in the regulation of cardiac function such as regulation of cardiac myocytes contractility and protection of cardiac myocyte under ischemic conditions [2, 3]. LPA induces contraction of vascular smooth muscle cell (SMC) [4] and elevates arial blood pressure in rats [5]. The physiological role of LPA in animal reproduction has been reported. These include regulation of the ovarian function, spermatogenesis, fertilization, and pregnancy maintenance [6]. It is well reported that LPA has diverse role in gastrointestinal (GI) tract. Intra-rectal administration of LPA has been reported to ameliorate colitis in rats [7]. It is well known that LPA protect intestinal epithelial cells from radiation and chemotherapy-induced apoptosis [8]. LPA has wound healing activity in intestinal cells [9]. Our previous study revealed the ameliorative effect of LPA against stress- and aspirin-induced gastric ulcer [10, 11]. Besides with beneficial effect, LPA has been reported to associate with human disease such as cancer progression, pulmonary fibrosis and neuropathic pain [12].

LPA exerts its action via at least six types of G-protein-coupled receptor (GPCR), $LPA_{1–6}$. Expression patterns of these LPA receptors (LPAR) are diverse in various organ and cells. LPA_1 is highly expressed in the nervous system, LPA_2 in the thymus, spleen, stomach and LPA_3 in reproductive organs such as the ovary and uterus [13, 14]. On the other hand, LPA_4, LPA_5, and LPA_6 are expressed widely but most often at relatively low level [13].

In mammals, LPA has been detected in various biological fluids such as serum and plasma, tears, ascites, seminal plasma, and follicular fluid [15-20]. Moreover, it can also be produced in various cell types like endometrial cells, ovarian cells, mast cells, erythrocytes, neurons, and many others [21-24]. Two general pathways of LPA production have been demonstrated [13]. In the first pathway, autotaxin (ATX) converts lysophosphatidylcholine (LPC) and other lysophospholipids to LPA by its lysophospholipase D activity. This is a primary mechanism of LPA production in plasma/serum. The second pathway of LPA production is the hydrolysis of phosphatidic acid (PA) by phospholipase A_2 (PLA_2). Genetic deletion of ATX and resulting decrease of LPA production were shown to be impaired embryonic development due to inability of angiogenesis [25]. It has been reported that LPA receptor KO mice showed obvious physical defects, including retardation of physical growth and prominent craniofacial abnormalities [26, 27]. These results are direct evidence on physiological significance of LPA and LPA receptors.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used for relief of pain and inflammation associated with arthritis and musculoskeletal injury. Approximately 20% of people older than 65 years have been prescribed an NSAID [28]. Their use is associated with a significant risk of upper GI complications, including GI bleeding, ulcer perforation, gastric outlet obstruction, and symptomatic peptic ulcer disease. About 5 to 20% of long term NSAID users develop peptic ulcer [29]. Various mechanism of NSAID induced gastric injury include: a) damage of the gastric epithelium by intracellular accumulation of these drugs in an ionized state, b) reducing the hydrophobicity of the mucous gel layer by changing the action of surface-active phospholipids, c) suppression of the prostaglandin synthesis, d) uncoupling of mitochondrial oxidative phosphorylation and inhibition of the electron transport chain, leading to depletion of intracellular ATP, e) injury due to neutrophils adherence to the endothelium of gastric micro-circulation [30].

Medicinal herbs and various foods are considered to be a potential source to control various diseases including gastric ulcer [31]. Treatment of gastric ulcer by natural sources has advantage of being safer, inexpensive and having limited side effects. Our previous study revealed that LPA and PA ameliorate aspirin-induced stomach mucosal injury of mice [11]. Our study also found that PA and LPA are abundant in some vegetables [32, 33]. These results indicated a possibility that the LPA or PA-rich food or herbs protect against NSAIDs-induced gastric ulcer. The present

study aimed to identified PA and LPA-rich natural source and evaluates their ameliorative effect against NSAID-induced gastric ulcer. This study also aimed to evaluate their possible mechanism of action for gastric mucosal protection.

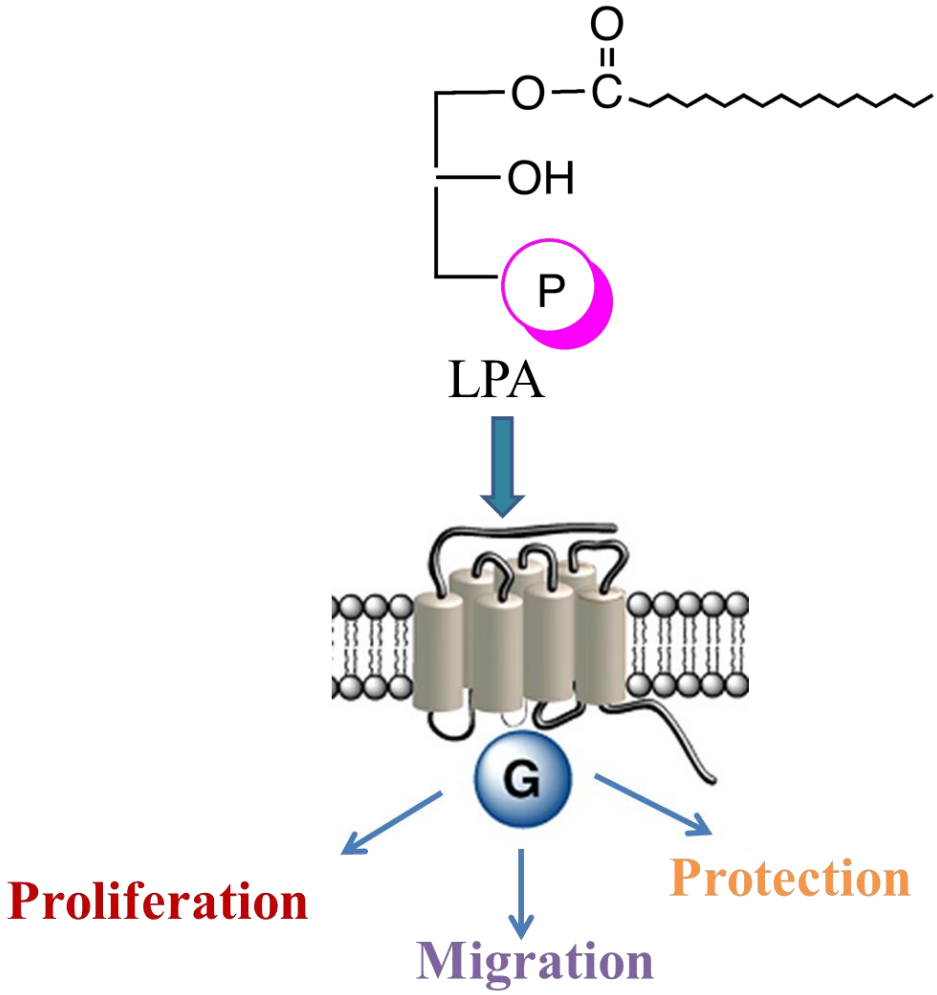

Figure 1. LPA/LPAR signaling axis. LPA signals through multiple G protein-coupled receptors to stimulate the proliferation, migration, and protection of many cell types.

CHAPTER-1

Concentrated phosphatidic acid in cereal brans as potential protective agents against indomethacin-induced stomach ulcer

1.1 Introduction

NSAIDs are widely used for reduction of inflammation and risk of deep vein thrombosis. Chronic NSAID users develop peptic ulcer disease [34]. Proton pump inhibitors (PPIs) are the first-line drugs for gastric ulcer treatment. However, long term use of PPIs has been associated with development of iron deficiency, anemia, [35] owing to impaired mineral absorption. Gastro protective agents that are easy to take, are inexpensive, and have fewer side effects can resolve this NSAID-associated problem.

PA is a natural phospholipid that is predominantly present in plant foodstuffs [32]. Recently, we found that oral administration of synthetic PA and LPA ameliorated aspirin-induced stomach mucosal injury in mice and PA was converted to LPA in the stomach by PLA_2 [11]. LPA is a bioactive lipids with diverse biological properties. LPA elicits various cellular responses; including cell proliferation, migration, and protection against apoptotic cell death in numerous types of cells through its specific G-protein-coupled receptors ($LPA_{1–6}$) [1]. Recent studies have revealed the roles of LPA signaling in maintaining the epithelial integrity of the GI tract of mammals [36, 37]. Previously, we demonstrated that orally administered LPA prevented water-immersion stress-induced gastric ulcer in rats [10]. Our recent study found that LPA_2 was localized on the apical side of gastric surface mucous cells and enhanced production of prostaglandin (PG) E_2, a cytoprotective PG that augments the mucous gel barrier in the GI tract [14]. These results suggest the possibility that gastric epithelia respond to luminal LPA formed from ingested PA and that PA-rich foods contribute to epithelial integrity of the stomach by augmentation of the cytoprotective function. However, it remains unknown whether a PA-rich food actually protects against peptic ulcer induced by NSAIDs.

Previously, we examined the PA content in various foods and found that cruciferous vegetables, such as cabbage and radish, are PA-rich foodstuffs [32]. In this study, we focused on cereals because they are staple foods that are often eaten daily in large amounts. Several investigators reported lipid compositions of cereals [38-41]. However, there are few reports on the PA content of cereals. We also examined the effect of materials prepared from PA-rich foods on the acute NSAID-induced stomach lesions in mice.

1.2 Materials and methods

1.2.1 Food Sources.

Flour of common buckwheat endosperm (*Fagopyrum esculentum*) grown in Hokkaido, Japan, was obtained from a mail-order grocery. Seeds of buckwheat grown in Tokushima, Japan, were a gift from a local farmer. The buckwheat seeds were from the same species but of different strains. In fact, seeds from Hokkaido were bigger than those from Tokushima. The milling company Tani Syokuryou Co. (Tokushima, Japan) kindly produced the powder from different parts of the buckwheat seeds grown in Tokushima. Flour of tartary buckwheat endosperm (*Fagopyrum tartaricum*) grown in Hokkaido, Japan, was purchased from a mail-order grocery. Flours from wheat (*Triticum durum*) grown in Ontario (Canada), Kumamoto (Japan), and Hokkaido (Japan) were purchased from a mail-order grocery, and the names of the strains are Supernova, Minaminomegumi, and Haruyutaka, respectively. All flours were from durum wheat species. Flours of whole wheat and wheat bran were prepared from the Haruyutaka strain grown in Hokkaido, Japan. Flour of rye (*Secale cereale*) grown in Hokkaido, Japan, was obtained from a mail-order grocery. The name of the strain is unknown. Brown rice (*Oryza sativa*) and its polished form (white rice) were purchased from a local market. Flours of the brown and white rice were prepared by milling in a mill mixer. Flour of rice bran was obtained from the brown rice using a rice mill. The first milling flour was collected and used as rice bran. Cornmeal (*Zea mays*), soybean flour (*Glycine max*), and peanuts (*Arachis hypogaea*) were purchased from a local market. Flours of these nuts were prepared by milling in a mill mixer. Cabbage (*Brassica oleracea*), radish (*Raphanus sativus*), and carrot (*Daucus carota*) were obtained from a local market.

1.2.2 Reagents.

1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphate (16:0/18:2 PA), 1-palmitoyl-2-lyso-sn-glycero-3-phosphate (16:0 LPA), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (16:0/18:2 PC), and egg yolk PA were purchased from Avanti Polar Lipids (Alabaster, AL). Egg yolk lecithin was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Phos-tag was obtained from Wako Pure Chemical Industries (Osaka, Japan). Carboxymethyl cellulose (CMC)

and indomethacin were obtained from Kanto Chemical Co. (Tokyo, Japan) and NACALAI TESQUE, Inc. (Kyoto, Japan), respectively.

1.2.3 Extraction of lipids and isolation of PA from cereal lipids

Lipids were extracted from vegetables and cereal flours using the Bligh and Dyer method [42] as described previously [33]. In brief, 2 g of cereal flours or minced vegetables were added to an equal amount of water and homogenized. The homogenates were heated in hot water for 3 min to increase the efficacy of lipid extraction and inactivate lipolytic enzymes. After heating, the homogenates were added to an appropriate volume of chloroform, methanol, and water to yield a mixture consisting of chloroform/methanol/water (7.5:15:6, v/v/v). Then, the suspension was homogenized again and centrifuged. After the supernatant was collected, the pellet was added to 11.4 ml of solvent consisting of chloroform/methanol/water (3:6:2.4, v/v/v), mixed vigorously, and centrifuged. The supernatant was combined and added to an appropriate volume of chloroform and water to make chloroform/methanol/water (21:21:18.9, v/v/v). After acidification with 0.2 ml of 5N HCl, the suspension was centrifuged. Lipids were obtained from the chloroform phase (lower phase). PLs in the extracted lipids were quantified on the basis of phosphomolybdenum-malachite green formation [43]. PA in lipid extracts was isolated by thin-layer chromatography (TLC). The mobile phase used in chromatography was chloroform/methanol/28% aqueous ammonia (60:35:8, v/v/v). After development, the plate was sprayed with primuline for visualization of lipids under ultraviolet (UV) light. PA was extracted from the silica gel using the Bligh and Dyer method [42] and quantified [43]. Mole percentages of PA in total PLs were calculated from the amounts of PA and total PLs. Because the amounts of PLs were determined on the basis of lipid phosphorus, calculations were required for conversion of the weight of lipid phosphorus (mg of Pi/g) to the weight of phospholipids (mg/g). In this calculation, 25 were used as the conversion factor for total PLs. In the case of PA, 21.8 were used as the conversion factor. The factors used were in accordance with the official method recommended by the American Oil Chemists' Society (AOCS) [39].

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

PA and LPA molecular species were determined by MALDI-TOF MS with Phos-tag, as described previously [44]. In brief, aliquots of PA and LPA were dissolved in 100 μl of methanol containing 0.1% aqueous ammonia. This solution (10 μl) was mixed with 5 μl of 0.1 mM ^{68}Zn Phos-tag solution, and 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 was layered on the mixture as a matrix solution and 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, pressure in the ion chamber, and accelerating voltage were 337 nm, $3.7 \times 10^{-}7$ Torr, and 20 kV, respectively. To enhance the reproducibility, 300 laser shots were averaged for each mass spectrum.

1.2.5 Gas chromatography-mass spectrometer (GS-MS)

GC-MS was performed for analysis of fatty acid composition of PA in cereals. The fatty acyl moiety of PA was converted to fatty acid methylesters using 5% methanolic HCl and the resultant fatty acid methylesters were subjected to GC-MS using a JEOL (Tokyo, Japan) automass mass spectrometer equipped with a Hewlett Packard (Wilmington, DE, USA) GC5890 gas chromatograph. The capillary column used for GC was a non-polar DB-1 column (30 m length× 0.25 mm internal diameter, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA).

1.2.6 Indomethacin-induced stomach lesions in mice

Indomethacin was suspended in 3% (w/v) CMC and then sonicated for 30 s. Synthetic PA, LPA, PC, or natural lipids extracted from cereal bran were mixed with 3% CMC, shaken vigorously, and sonicated for 1 min. The 5-week-old male ICR mice (35 g body weight) fasted overnight were administered 0.2 ml of the lipid suspension intragastrically. After 30 min, 0.2 ml of the indomethacin suspension (22.9 mg/kg of body weight) was administered intragastrically. The mice were anesthetized with diethyl ether and sacrificed 5 h after the indomethacin administration. Ulcer severity was evaluated on the basis of the total lesion lengths, as described previously [11]. Briefly, the isolated stomach was immersed in 2% formalin for 15 min. Then,

the stomach was cut along the greater curvature, and the lengths of lesion on the stomach wall were measured using a millimeter scale and a magnifying glass. The total lengths of lesion were expressed as a lesion index. The care and handling of mice were in accordance with the National Institution of Health guidelines. All experimental procedures described above were approved by the Tokushima University Animal Care and Use Committee.

1.2.7 Formation of LPA from buckwheat bran lipids in *ex-vivo* (an isolated mouse stomach)

The buckwheat bran lipid (600 nmol of PLs) suspension was injected into an isolated mouse stomach in which both ends of the stomach were ligated and then incubated at 37 °C with continuous aeration. After incubation, the lipids were washed and then extracted using the method of Bligh and Dyer, with acidification of the aqueous phase [33]. The lipids were separated by two-dimensional TLC. The mobile phases for the first and second chromatography were chloroform/methanol/28% ammonia (60:35:8, v/v/v) and chloroform/methanol/acetone/acetic acid/water (50:10:20:13:5, v/v/v/v/v), respectively. The resulting LPA was isolated and quantified, as described above. The molecular species of PA and LPA were determined by MALDI−TOF MS, as Phos-tag complexes.

1.2.8 Formation of LPA from buckwheat bran lipids *in vitro* (stomach lavage)

Stomach lavage fluid was collected from five-week-old male ICR mice stomach as described previously [11]. The PLA_2 activity of the stomach lavage fluid was determined toward buckwheat bran lipid as described previously [11]. The resulting LPA were isolated by two-dimensional TLC and quantified as described above.

1.3 Results

1.3.1 PA in cereals

We quantified amounts of total PLs and PA, focusing on cereals. Percentages of PA in total PLs were calculated from these values (Fig. 2). Wheat, rye, and rice contained PLs at levels of approximately 140 to 340 mg/100 g. PA accounted only for 2 to 4% of the total PLs in these flours. Amounts of PA in these cereal flours (2−7 mg/100 g) were low relative to those in other foodstuffs examined. There were no significant differences in the amounts of PLs or PA among the different strains of wheat. Common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*Fagopyrum tartaricum*) are different species belonging to the same genus. Although the amounts of PLs were similar between these two species of buckwheat flours, the PA content in tartary buckwheat flour (41 mg/100 g) was 2.4 to 3.7 times that of common buckwheat flours (11−17 mg/100 g). The PA level of the tartary buckwheat flour was comparable to that of cabbage (53 mg/100 g wet weight), which was identified as PA-rich foodstuff in our previous study [32]. The percentage of PA in the total PLs of cornmeal (15%) was considerably higher than those of wheat and rice. As a result, PA was much higher in cornmeal (20 mg/100 g) than in wheat and rice. Peanut flour and soybean flour characteristically contained abundant PLs; therefore, the amounts of PA in these nuts flours were relatively high (21−45 mg/100 g). However, the percentages of PA in the total phospholipids in peanut and soybean flours were not so high (3%).

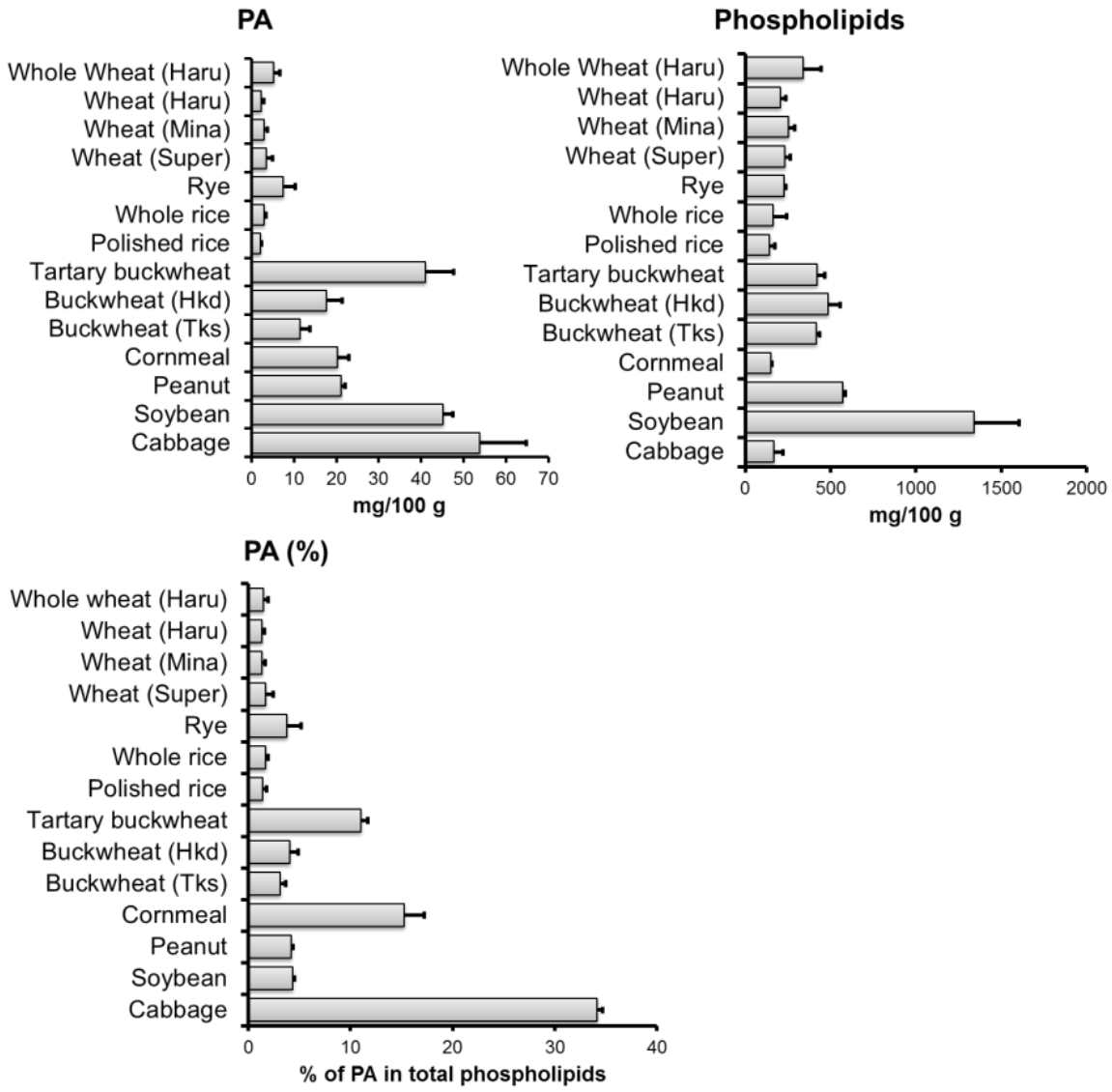

Figure 2. Amounts of PL, PA, and percentage of PA in cereal flours. Lipids were extracted from cereal flours and plant foodstuffs. PA was isolated by TLC from the extract. The amounts of PA and total PL were determined by measurement of lipids phosphorus. Percentage of PA was calculated from the amounts of total PL and PA. Names of strains or producing areas were indicated in parenthesis. Haru, Haruyutaka strain; Mina, Minaminomegumi strain; Super, Supernova strain; Hkd, Hokkaido area; Tks, Tokushima area.

1.3.2 PA content in different parts of the cereal seeds

We found that rice bran contained 50 times as much PA (104 mg/100 g) as that of rice endosperm (2.1 mg/100 g). Similarly, the PA content in buckwheat bran (188 mg/100 g) was 10

times that of buckwheat endosperm (19 mg/100 g). The percentages of PA in total PLs were also high in rice bran (12%) and buckwheat bran (25%). A similar tendency was observed in wheat seeds. However, the absolute amounts of PA and percentages of PA in total PLs in wheat bran were considerably lower than those of rice bran and buckwheat bran (Fig. 3).

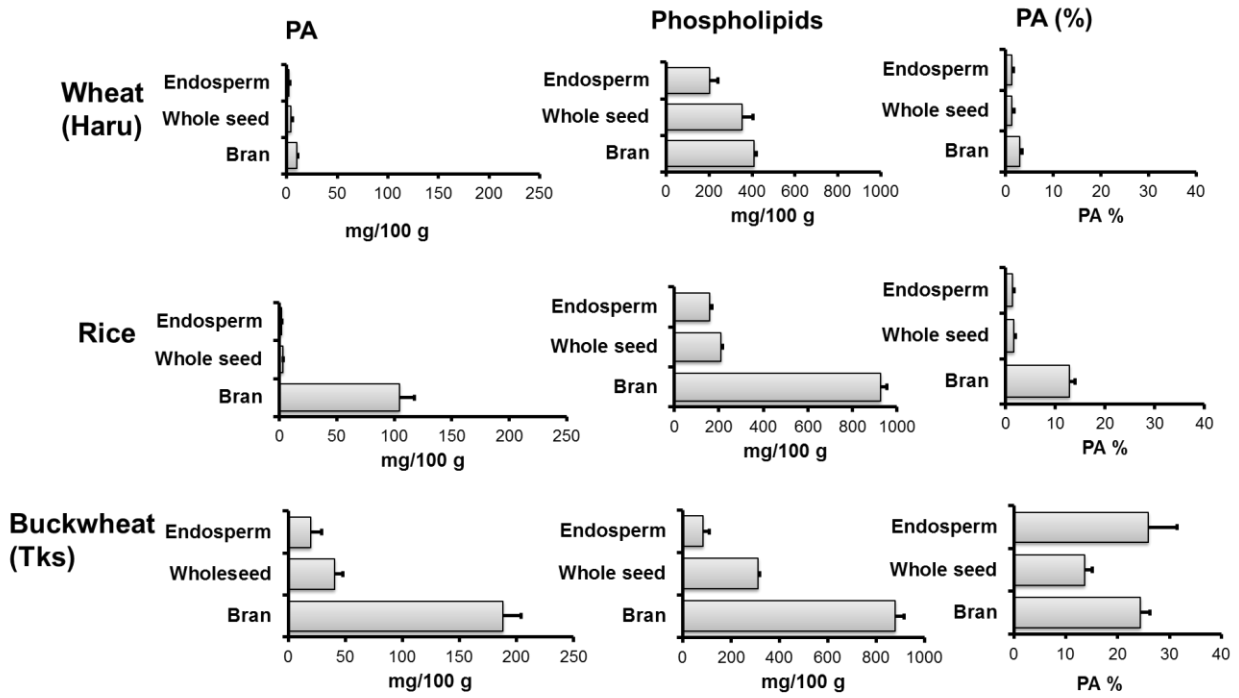

Figure 3. Amounts of PL and PA and percentages of PA in whole seed, endosperm, and bran of cereal seeds. Lipids were extracted from each part of cereal seeds. PA was isolated by TLC from the lipid extracts. Haru, Haruyutaka strain; Tks, Tokushima area

1.3.3 PA content in boiled and non-boiled cereals

Previously, we showed that cutting or mashing of raw cabbage leaves induced PA formation in the leaves [45] which was caused by hydrolysis of endogenous PLs, including PC by activated phospholipase D (PLD) during tissue destruction. Consistent with our previous findings, levels of PA prepared from boiled cabbage leaves, radish roots, and carrot roots were low relative to those prepared from raw ones. By contrast, levels of PA prepared from boiled brans of buckwheat and rice were similar to those of PA prepared from non-boiled brans (Fig. 4), which

indicated the absence of active PLD enzyme and presence of abundant PA in buckwheat and rice brans.

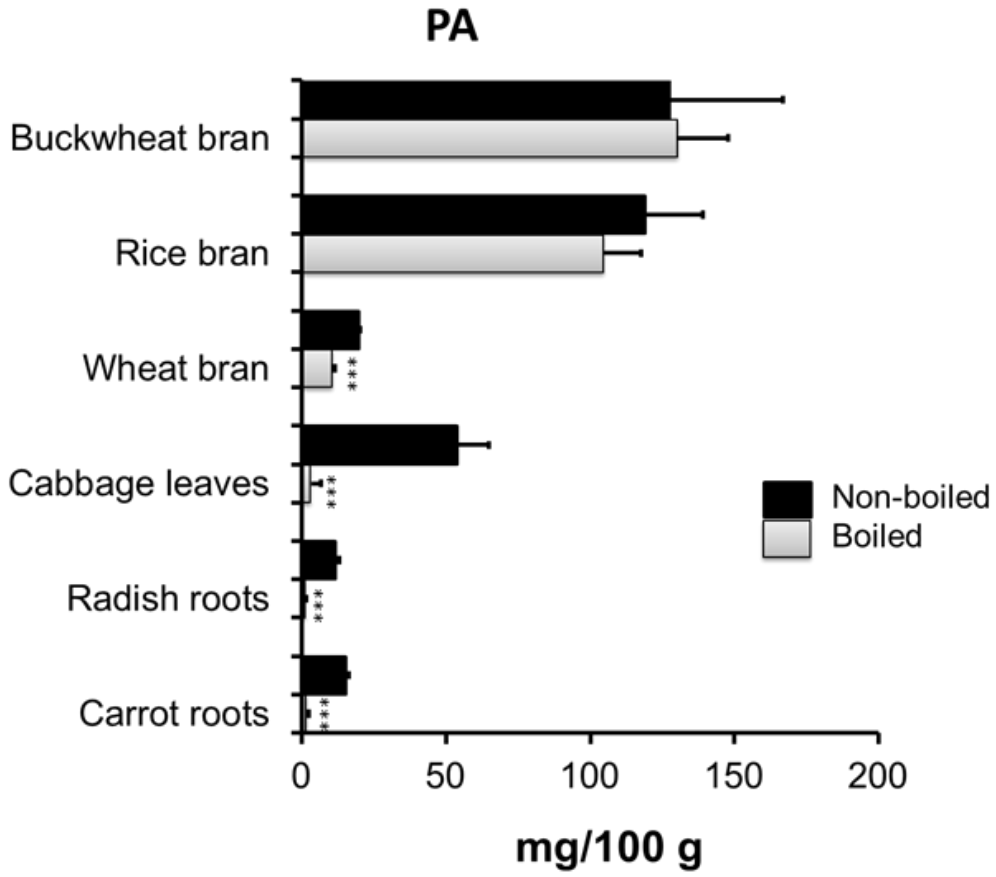

Figure 4. **Amounts of PA in non-boiled and boiled foodstuffs.** Lipids were extracted from boiled or non-boiled vegetables or cereal brans. PA was isolated by TLC from the lipid extracts. Significantly different from values of non-boiled foodstuffs, ***P <0.0005.

1.3.4 Molecular species and fatty acid composition of buckwheat bran PA

In buckwheat bran PA, five major ion at m/z 1259.6, 1261.6, 1283.6, 1285.6 and 1287.7 (Fig. 5) were detected, indicating the presence of 34:2 (16:0/18:2), 34:1 (16:0/18:1), 36:4 (18:2/18:2 or 18:1/18:3), 36:3 (18:1/18:2), 36:2 (18:1/18:1) PA, respectively.

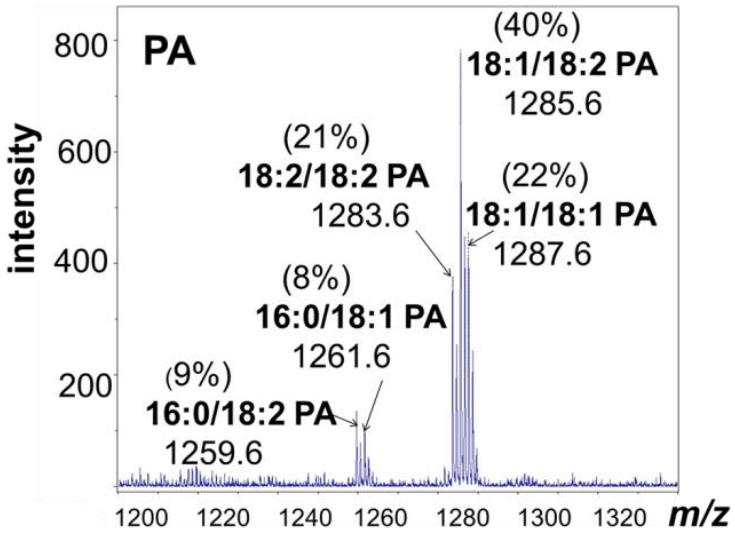

Figure 5. MALDI-TOF mass spectra of PA from buckwheat bran. PA from buckwheat bran was analyzed by MALDI-TOF MS as their Phos-tag complexes.

Fatty acid composition of buckwheat bran PA was determined by gas chromatography as shown in table 1. The most abundant fatty acids of buckwheat bran PA were oleic acid (18:1). Linoleic acid (18:2) was present in moderate amount. Palmitic acid (16:0) was present in very low percentage.

Table 1. Fatty acid composition of buckwheat bran PA

Fatty acid	% of fatty acid
Palmitic acid (16:0)	17.1 ± 0.9
Oleic acid (18:1)	47.9 ± 0.6
Linoleic acid (18:2)	35 ± 0.4

1.3.5 Establishment of indomethacin-induced acute gastric ulcer

Our previous study established an aspirin-induced gastric ulcer mice model [11]. In this study, we established an indomethacin induced gastric-ulcer mice model. Oral administration of

indomethacin produced gastric mucosal lesions in the mice. The lesion was linear and extended from the fundic area to the pyloric area as erosion (Fig. 6A). We used the total length of lesions as the lesion index. We found that indomethacin dose and time dependently cause the lesion formation in the mice stomach (Fig. 6B, C). We fixed the dose of indomethacin to be 22.9 mg/kg and time about 5 h because of stable lesion formation in this group of mice (Fig. 6A).

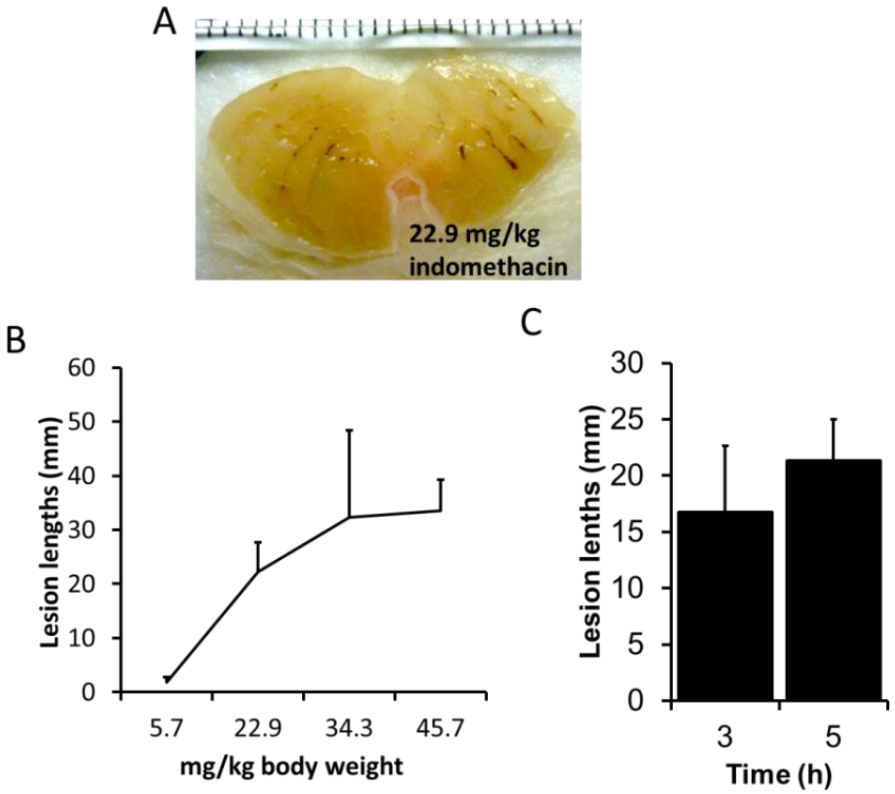

Figure 6: Dose and time dependent gastric lesion formation by indomethacin. (A, B) Fasted mice were intragastrically administrated with different dose of indomethacin and mice were sacrifice 5 h after indomethacin administration and the total length of lesions in the stomach was measured. (C) Mice were intragastrically administrated with 22.9 mg/kg indomethacin and scarified 3 or 5 h after indomethacin administration. The total length of lesions in the stomach was measured as lesion index.

1.3.6 Effect of synthetic PA and LPA in indomethacin-induced gastric ulcer

Previously, we found that pre-administration of synthetic PA and LPA significantly reduced aspirin-induced stomach lesions [11]. In this study, we also found that pre-administration of 1 mM synthetic PA

and LPA significantly reduced indomethacin-induced gastric lesions formation. PA reduced lesion formation more effectively than LPA at low concentrations (Fig. 7C). PC is the most abundant PL both in animal and plant foods. We found that synthetic PC showed marginal ameliorative effects at 1 mM. Egg yolk PA significantly reduced lesion formation at 0.3 mM and 1 mM. On the other hand, egg yolk lecithin did not reduce lesion formation in the same range of concentrations (Fig. 7D).

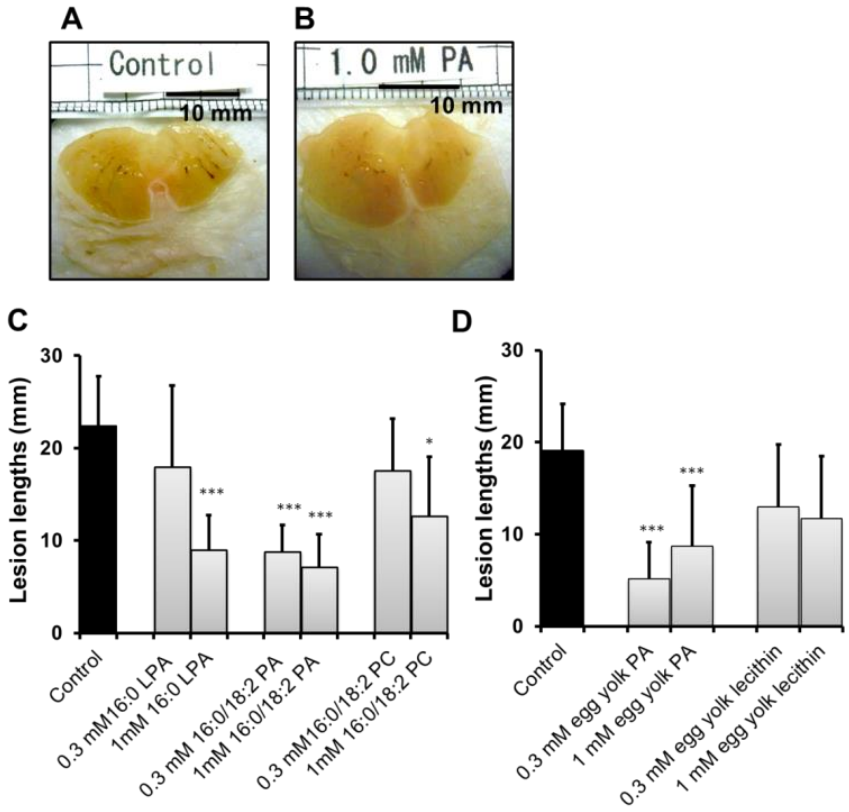

Figure 7. Effect of PA and LPA on indomethacin-induced gastric lesions. (A, B) Mice were intragastrically administered with 0.2 ml of 3% CMC (A) or 0.2 ml of 1 mM 16:0/18:2-PA suspension in 3% CMC (B). After 30 min, 0.2 ml of indomethacin in 3% CMC was intragastrically administered. The total lengths of lesions on the stomach wall were measured at 5 h after indomethacin injection. (C, D) Mice were intragastrically administered with 0.2 ml of 3% CMC, or 0.2 ml of PL suspensions in 3% CMC. Here, 0.3 mM and 1 mM egg yolk lecithin indicates administration of egg yolk lecithin containing 0.06 and 0.2 μmol of PLs in 0.2 ml of the suspension, respectively. After 30 min, 0.2 ml of indomethacin in 3% CMC was intragastrically administered. Results are expressed as means ± SDs. Amounts of LPA, PA, PC, egg yolk PA and egg yolk lecithin in 0.2 ml of a 1 mM suspension were 2.5, 4, 4.3, 4, 4.3 mg/kg body weight, respectively. The amount of indomethacin injected was 22.9 mg/kg body weight throughout the experiments. The numbers of experiments were 25 (control), and 5-10 (others). *P <0.05, ***P <0.0005 versus control.

1.3.7 Effect of PA-rich cereals in indomethacin-induced gastric ulcer

We prepared lipid extracts from cereal brans and soybean, and examined their anti-gastric ulcer activity. The percentages of PA in the lipid extracts from buckwheat bran, rice bran, wheat bran, and soybean were 25%, 12%, 4%, and 3%, respectively. The amounts of lipids administered were determined on the basis of their phospholipids content. For example, administration of 0.2 ml of CMC suspension of buckwheat bran lipids at 1 mM means administration of the lipid extracts containing 0.2 μmol of PLs in 0.2 ml. As shown in figure 8A, administration of buckwheat bran lipids and rice bran lipids at 1 mM significantly reduced indomethacin-induced gastric lesions. Neither wheat bran lipids nor soybean lipids at the same concentration significantly reduced gastric lesions. The maximum protection rate (49%) was observed at 1 mM of buckwheat bran lipids. A higher concentration (3 mM) of buckwheat bran lipids did not reduce gastric lesions (Fig. 8B). Similarly, a higher concentration of synthetic PA did not show an ameliorative effect (Fig. 8C).

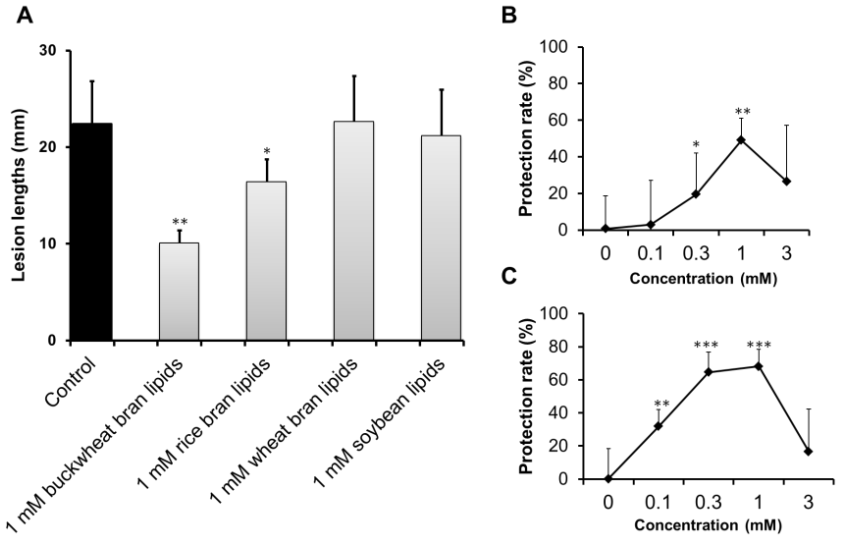

Figure 8. Effect of cereal bran lipids on indomethacin-induced gastric lesions. (A, B, C) Mice were intragastrically administered with 0.2 ml of 3% CMC (control and 0 mM) or 0.2 ml of lipid suspension in 3% CMC. After 30 min, 0.2 ml of indomethacin in 3% CMC was intragastrically administered. The total lengths of lesions on the stomach wall were measured at 5 h after injection of indomethacin. Indicated concentrations are based on the amounts of PL. (A) One mM indicates administration of bran lipids containing 0.2 □mol of PLs in 0.2 ml of the suspension. (B, C) Increasing concentrations of buckwheat bran lipids (B) or 16:0/18:2 PA (C) were administered as lipid suspensions in 3% CMC. Results are expressed as means ± SD. The amount of indomethacin injected was 22.9 mg/kg body weight throughout the experiments. The numbers of experiments were 15 (control), and 5-10 (others). *P <0.05, **P <0.005, ***P <0.0005 versus control.

1.3.8 Effect of LPA receptor antagonist on PA anti-ulcer effect

The anti-ulcer mechanism of PA is not well understood in our previous study [11]. We considered that formation of LPA from PA is one of the indirect mechanisms of PA to show ameliorative effect. To examine this possibility, we examined PA effect by using the LPA receptor antagonist, Ki-16425. As shown in Fig. 9, Ki-16425 partially suppresses the PA anti-ulcer effect. This result suggests that anti-ulcer effect of PA is partially mediated via formation of LPA.

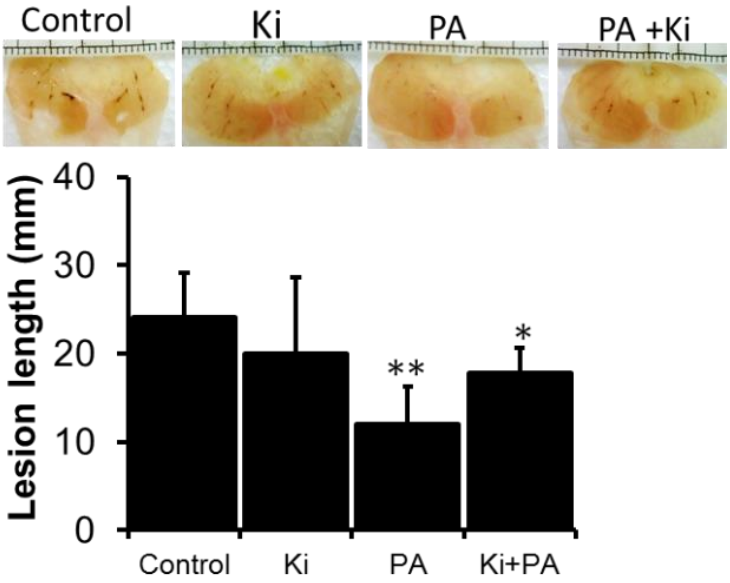

Figure 9. LPA receptor antagonist abolished PA anti-ulcer effect. Fasted mice were intragastrically administered with 0.2 ml of 3% CMC or 0.2 ml of 1 mM Ki-16425 or 0.2 ml of 0.3 mM 16:0/18:2 PA in presence or absence of 1mM Ki-16425 suspension in 3% CMC. After 30 min, 0.2 ml of indomethacin in 3% CMC was intragastrically administered. The total lengths of lesions on the stomach wall were measured at 5 h after indomethacin injection. Results are expressed as means ± SDs. The numbers of experiments were 5. *P <0.05, **P <0.005 versus control.

1.3.9 Formation of LPA from buckwheat bran lipids

To determine whether LPA was actually formed from PA during digestion of buckwheat bran lipids in mouse stomach, we conducted an *ex vivo* experiment by using isolated mouse stomach. Before digestion, LPA spot was scarcely detected in buckwheat bran lipids (Fig. 10A). After

incubation of buckwheat bran lipids for 30 min in an isolated stomach, LPA was detected as an apparent spot on TLC (Fig. 10B). Quantification of LPA and PA before and after incubation revealed that about 16 nmol LPA was formed with concomitant decrease of the amount of PA in an isolated stomach. The elevated level of LPA remained unchanged up to 1 h of incubation (Fig. 10C). The sum of the amounts of LPA and PA at the end of incubation was only 30% of the PA injected as buckwheat bran lipids. The lower recovery of PA plus LPA may have been due to incorporation or decomposition of PA and LPA during incubation in the stomach. The formation of LPA was confirmed by MALDI-TOF MS. The major molecular species of LPA that formed in stomach from buckwheat bran lipids were found to be 16:0, 18:2, and 18:1 LPA (Fig. 10E). This is consistent with the fact that major molecular species of buckwheat bran PA were 16:0/18:2, 16:0/18:1, 18:2/18:2, 18:1/18:2, and 18:1/18:1 PA (Fig. 10D).

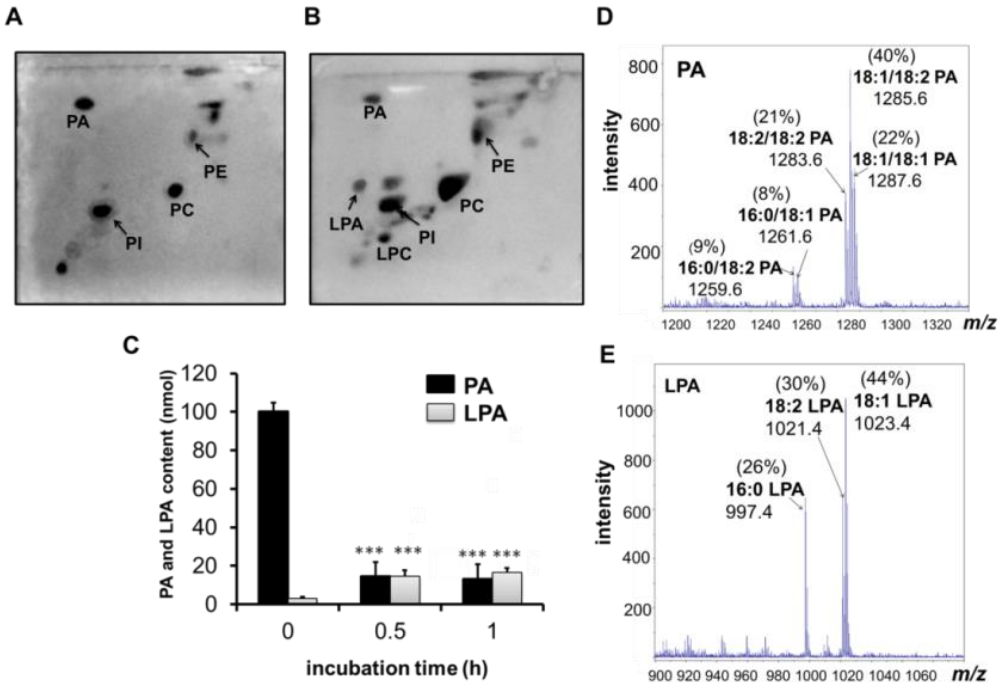

Figure 10. Formation of LPA from buckwheat bran lipids in mouse stomach. (A, B) Buckwheat bran lipid was incubated in an isolated mouse stomach at 37 ^{o}C for 30 min. Two-dimensional TLC of non-incubated (A) and incubated (B) buckwheat bran lipids are shown. (C) Buckwheat bran lipids were incubated in isolated mouse stomach at 37 ^{o}C with continuous aeration, and stomach contents were washed out at indicated times. Lipids were extracted for determination of PA and LPA. The numbers of experiments were 5. Significantly different from values obtained at 0 h, ***P <0.0005. (D, E) PA and LPA isolated from stomach-incubated buckwheat bran lipids were subjected to MALDI-TOF MS as their phos-tag complexes, for determination of molecular species composition.

We also treated buckwheat bran lipids with stomach lavage for confirmation of phospholipase A_2 activity. Buckwheat bran lipids containing 17 nmol of PA were treated with mice stomach lavage. We found that 8 nmol LPA was formed within 30 min of incubation (Fig. 11). These results are consistent with our previous results showing that stomach PLA_2 hydrolyzes PA to form LPA in stomach without sodium deoxycholate [11].

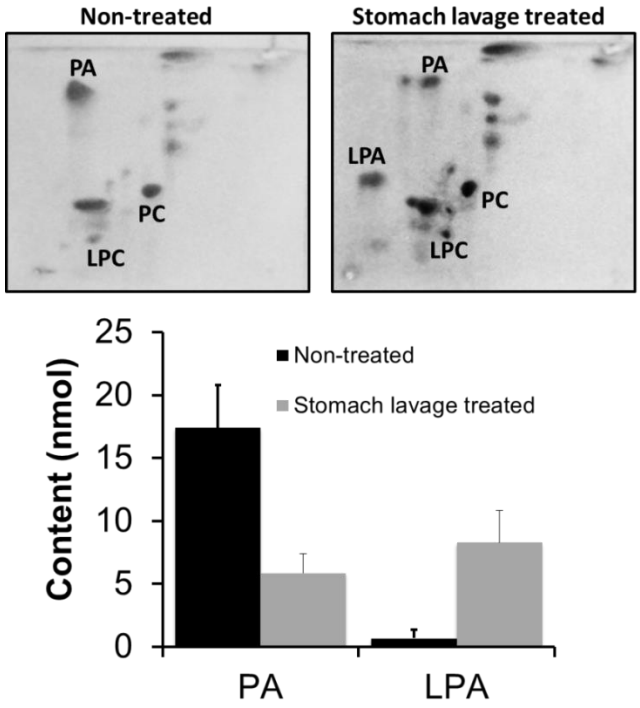

Figure 11. Formation of LPA from buckwheat bran lipids by stomach lavage fluid
Stomach lavage fluids that collected from ICR mice were centrifuged and the supernatant was used as an enzyme source. Buckwheat bran lipids (97 nmole) were treated with in absence (control) and presence of the stomach lavage fluid at pH 7.4 using tris/HCl buffer. The resulting lysophospholipids were isolated by TLC and their phosphorus contents were determined

1.4 Discussion

Previously, we reported that orally administrated PA ameliorates aspirin-induced stomach lesions in mice [11]. Here, we found that PA is concentrated in the bran parts of rice and buckwheat seeds, and that lipid extracts from these PA-rich cereal brans have ameliorative activity against indomethacin-induced gastric lesions over a limited range of concentrations.

Weihrauch and Son have compiled data on the total lipid and PL contents of 140 foods, including cereals [39]. The PL contents of cereals obtained in this study (wheat, rye, rice, and corn flours) were in a similar range as those of the data reported earlier. To our knowledge, PA contents of wheat, rye, and rice flours are unknown. Here, we found that the PA contents of these cereals were 2−3, 7, and 2.1 mg/100 g, respectively.

Buckwheat seeds are good sources of valuable nutrients, and their consumption has been associated with numerous beneficial effects on health [46]. The amounts of PLs in buckwheat flour have been reported by Mazza to be 0.3% on a dry weight basis (300 mg/100 g) [47]. Consistent with this data, we found that the PL contents in common buckwheat and tartary buckwheat flours were 417 to 486 mg/100 g and 421 mg/100 g, respectively. In addition, we found that tartary buckwheat flour contained a higher level of PA (41 mg/100 g) than common buckwheat flours (11−17 mg/100 g).

Enrichment of PLs in rice bran has been reported by Choudhury [48]. Enrichment of fats in buckwheat bran has been reported by Bonafaccia [49]. In this study, PA contents in rice bran and buckwheat bran were determined to be 104 mg/100 g and 188 mg/100 g, respectively. We also found that PA accounted for relatively higher percentages in the total PLs (12% and 25% in rice bran and buckwheat bran, respectively) in these brans. To our knowledge, this is the first study to show that PA is concentrated in the bran parts of rice and buckwheat seeds. The physiological significance of bran parts of seeds is protection of embryos from disease, pests, mechanical injury, and unlimited water penetration. It has been reported that PA plays a vital role in plant defense response against various environmental stresses [50-52]. Abundant PA in the bran parts of cereal seeds may have a role in protection of internal seed parts from such stresses.

Indomethacin is a widely used NSAID to relieve pain, swelling, and symptoms of arthritis. Like aspirin, indomethacin causes gastrointestinal adverse events, including bleeding and ulceration in the stomach. Previously, we demonstrated that pre-administration of synthetic PA and LPA ameliorated aspirin-induced acute gastric lesions in mice and those free fatty acids, LPC, tri- and diacylglycerol did not reduce ulcer lesions over the same range of concentrations [11]. Consistent with our previous study with aspirin, both synthetic PA and LPA exerted ameliorative activity against indomethacin-induced stomach lesions. We showed that egg yolk PA but not egg lecithin had an ameliorative effect on indomethacin-induced gastric lesions. These results indicated that pre-administration of purified PA effectively suppressed tissue damage caused by NSAIDs in stomach.

In the present study, we demonstrated that lipids containing high percentages of PA, such as buckwheat bran lipids significantly ameliorated indomethacin-induced gastric lesions. By contrast, lipids containing low percentages of PA, such as wheat bran and soybean lipids did not significantly reduce gastric lesions over the same range of concentrations. We also observed that the ameliorative effect was more potent for synthetic PA than for lipid extracts of buckwheat bran, especially at low concentrations. These results indicated that anti-ulcer potency of PA was affected by co-existing lipids. Lipid components other than PA present in the extract may interfere with the effects of PA and LPA. PC, lysoPC, free fatty acids, and other surface-active lipids in the extract may trap and hide PA as inclusion micelles or liposomes. The fact that buckwheat bran powder showed a lower ameliorative effect than those of buckwheat bran lipids and synthetic PA may be explained by this interference of the co-existing components.

Defensive strategies of gastric mucosa include suppression of gastric acid secretion, formation of a mucus gel layer of mucin, secretion of bicarbonate and enhancement of mucosal blood flow. PGE_2 is considered to play pivotal roles in these mucosal protections. Previously, we found that LPA_2 was localized on the apical side of gastric surface mucous cells and that LPA increase PGE_2 level by upregulation of cyclooxygenase (COX)-2 mRNA in human stomach cells [14]. Pre-administration of LPA is considered to strengthen a barrier function and keep the mucous cells vigorous. In addition, anti-apoptosis via LPA_2 [53] and enhancement of cell migration [33]

have been shown as LPA action in gastrointestinal cells. The protective effect of pre-administered LPA against NSAIDs-induced stomach ulcer can be partially explained by these LPA-receptor-mediated actions in the stomach. In fact, we demonstrated LPA formation from buckwheat bran lipids in isolated mouse stomach. In this experiment, amount of LPA formed was determined to be 16 nmol from buckwheat bran lipids containing 100 nmol PA (Fig. 10C). Based on this result, 6 nmol LPA is considered to be formed from effective dose of buckwheat bran lipids. Previously, we showed that gastric PLA_2 effectively hydrolyzes PA to LPA only at neutral pH [11]. Considering that neutral space in stomach cavity is mucous gel layer, formation of LPA is likely to proceed in the gel layer. LPA formed in such a limited space (estimated to approximately 0.4 ml/mouse stomach) may effectively interact with LPA_2 (Fig. 12).

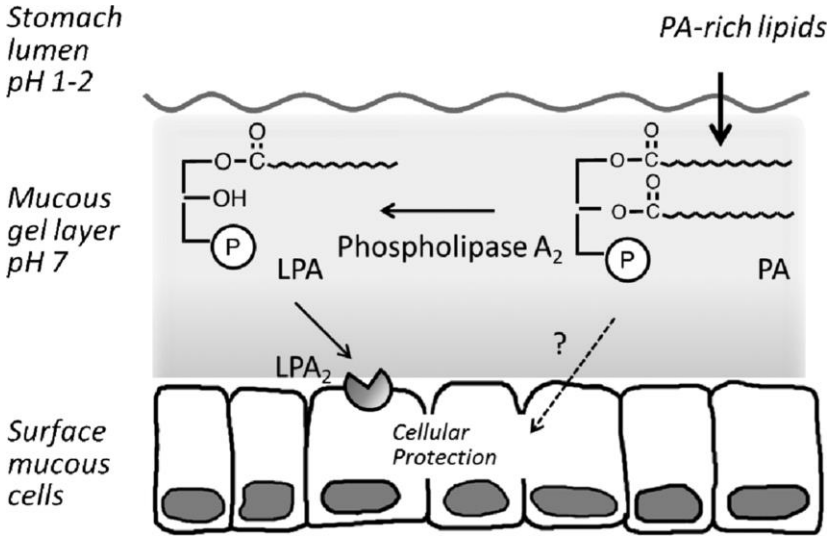

Figure 12. Hypothetical mechanism of the ameliorative effect of PA-rich lipids. LPA is formed from PA by gastric PLA_2 in the mucous gel layer. The resulting LPA activates LPA_2 that is expressed in the apical side of surface mucous cells in the stomach. LPA_2 has been known to associate with anti-apoptosis and upregulation of mRNA of COX-2. PA also has a possibility to enhance PGE_2 production and modulate the cell survival signal.

The elevated concentration of LPA in stomach gel layer would be retained up to 1 h, when PA-rich lipid was administered. Formation of LPA at the gel layer may explain why administration of small amount of PA is effective than that of much amount of LPA.

PA has been shown to modulate cellular function. It has been reported that exogenous PA enhanced calcium influx and calcium release from intracellular stores [54-56]. Schepp *et al*. have shown that increase of intracellular calcium concentration enhances PGE_2 production in human gastric mucous cells [57]. It has been reported that mammalian target of rapamycin (mTOR) is a critical target for cell survival and that both intracellular and extracellular PA activated mTOR signaling [58, 59]. In this regard, Pagano *et al*. have shown that exogenous PA can diffuse across the cell membrane through dephosphorylation and phosphorylation reactions [60]. These biological actions of PA may involve in the ameliorative effect of PA-rich foods.

Synthetic PA significantly reduced the total length of lesions at concentration from 0.1 to 1 mM, which corresponds to 0.4 to 4 mg/kg body weight. However, PA at 3 mM (12 mg/kg body weight) was found to have no effect. Ineffectiveness at high concentrations was also observed in an experiment with synthetic LPA. At present, the exact reasons for this phenomenon are unknown. A possible explanation is desensitization of the LPA receptor by supraphysiological concentration of LPA. Considering that LPA concentration in saliva has been reported to be approximately 0.9 μM [61], the LPA receptor expressed on the apical side of the stomach epithelia may respond to LPA at similar concentration. LPA administration at high concentrations may induce internalization of the LPA receptor and hamper LPA-receptor-mediated signaling.

In conclusion, we characterized buckwheat bran and rice bran as PA-rich foodstuffs. We also demonstrated that natural PA, such as egg yolk PA, and PA-rich lipids, such as buckwheat bran lipids, significantly reduced indomethacin-induced stomach lesions over a limited range of concentrations. Our results suggest that the purified PA or lipid extracts from PA-rich foodstuffs described here may be effective as anti-ulcer supplements.

CHAPTER-2

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

2.1 Introduction

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, $LPA_{1–6}$ [62]. Recent studies have revealed important actions of LPA in the mammalian GI tract. These include inhibition of diarrhea, regulation of intestinal electrolyte transport, protection of intestinal cells from apoptosis, and wound healing [36, 37].

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 [63, 64]. Infection with *Helicobacter pylori*, smoking, drinking alcohol, and chronic ingestion of drugs are major causes of peptic ulcer. Recently, the number of patients with NSAID-induced gastric ulcer is increasing due to increased use of NSAIDs for pain treatment and prevention of thrombosis [65]. NSAIDs cause gastric ulcer by inhibition of COX-1 and COX-2, which produce a predominant mucosal defense factor, PGE_2. Our recent study showed that orally administered synthetic LPA ameliorates aspirin-induced gastric ulcer in mice [11]. 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 [14]. 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 [66]. Considering that some medicinal herbs and vegetables contain LPA abundantly [10, 33], 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.

2.2 Materials and Methods

2.2.1 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.

2.2.2 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). 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).

2.2.3 Extraction of lipid and isolation of PA and LPA from herbs

Lipids were extracted from the medicinal herbs by the Bligh and Dyer method [42] with acidification of the water/methanol phase, as described previously [33]. 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 PA and LPA in the lipid extract were 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. PA and LPA was identified, extracted from the silica gel by the Bligh and Dyer method [39], and quantified by the colorimetric method based on phospho-molybdenum-malachite green formation [43]. 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 [39].

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

Molecular species of PA and LPA in herbs were determined by MALDI-TOF MS as described previously [44]. 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.

2.2.5 NSAIDs-induced gastric ulcer

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. Five week old ICR mice were fasted overnight. 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.

2.2.6 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 table 2. 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.

Table 2. 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'			

2.2.7 PGE_2 production assay

PGE_2 production in MKN74 cells was performed as described previously [14]. 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 min. The supernatant were collected and PGE_2 was evaluated by EIA kit according to the manufacturer's instructions.

2.2.8 Cell death assay of MKN74 cells

2.2.8a UV-induced cell death

MKN74 cells were seeded in 35 mm polystyrene dish in a density of $1x10^5$. The cells were serum starved for 16 h and exposed to UV light for different time periods. After UV exposure, the cells were incubated at indicated time periods. At the end of incubation, the cells were stained with propidium iodide (PI) for 30 min. The extent of cell death was determined by observation with a fluorescent microscope, Axiovert 200 M (Zeiss, Oberkochen, Germany). The dead cells and living cells were counted in different subfield of dish and measured the % of dead cells.

2.2.8b H_2O_2-induced cell death

MKN74 cells were seeded in 35 mm polystyrene dish in a density of $1x10^5$. Different dose of H_2O_2 were added to serum starved cells for different time periods. To examine the LPA effect, serum starved cells were pre-incubated with LPA. After 15 min, cells were treated with 250 μM of H_2O_2 for 8 h. The cells were stained with PI and cell death assessed as describe above.

2.2.8c Serum starvation induced cell death

The cells were seeded in 35-mm polystyrene dishes at a density of 1×10^5 cells, and incubated in 2 ml of 10% medium for 6 h. Then, the cells were incubated in the serum-free medium for 24, 48, 72 and 96 h. At the end of incubation, the cells were harvested and stained with trypan blue. The dead and living cells counted by trypan blue exclusion test.

2.2.8d Indomethacin-induced cell death

MKN74 cells were seeded at a density of $1x10^5$ in 35-mm polystyrene dishes and serum starved for 24 h. The serum starved cells were treated with different concentration of indomethacin for 24 h. At the end of incubation, the cells were stained with PI and observed in fluorescence microscope. To examine the LPA effect, we fixed the indomethacin concentration at 0.8 mM. LPA was added 2 h before addition of indomethacin solution. After 30 h, the cells

stained with PI and the extent of cell death was determined by observation with a fluorescent microscope as describe above. For the LDH assay, MKN74 cells were treated in the same manner as described above. At the end of incubation, 100 μl of the culture medium supernatant was added per well of a 96-well microplate. The LDH activity was measured according to the instructions of the LDH assay kit. The activity was expressed as % of maximum release of LDH that can be obtained in a sample of the lysis buffer-treated cells. The extent of cell death was also determined by flow cytometric analysis. The PI-stained cells were subjected to a flow cytometer (Becton Dickinson) that was operated using Cell Quest software, and at least 10,000 cells were analyzed for each sample.

2.2.9 Cell proliferation assay

MKN74 cells seeded in 35-mm polystyrene dishes were serum-starved for 24 h. Then they were cultured with or without synthetic or peony root-derived LPA in the presence or absence of PTX. After 24 h, cells were harvested by trypsinization, mixed with trypan blue dye, and the number of living cells was counted using a hemocytometer. The proliferation of MKN74 cells 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.

2.2.10 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 [67] 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, [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.

2.3 Results

2.3.1 Abundance of LPA in medicinal herbs

Our previous study [33] 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. 13A). 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. Surprisingly, the percentage of LPA in total PLs in peony root was 11%, which is 30 and 400 times of those in cabbage leaves and soybean seed powder, respectively (Table 3).

Table 3. LPA is highly concentrated in the lipid fraction of peony root

	Cabbage		Soybean		Peony root	
	μg/g	(%)	μg/g	(%)	μg/g	(%)
Total PLs	2300 ± 160	-	13000 ± 2700	-	2200 ± 140	-
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 percentages in total PL.

We previously revealed that PA, a diacyl derivative of LPA, serves as a source of LPA in the digestive tract [11]. The amounts of PA in these medicinal herbs were comparable (Fig. 13B) to those in cabbage and soybean, which were characterized as PA-rich foods previously [32].

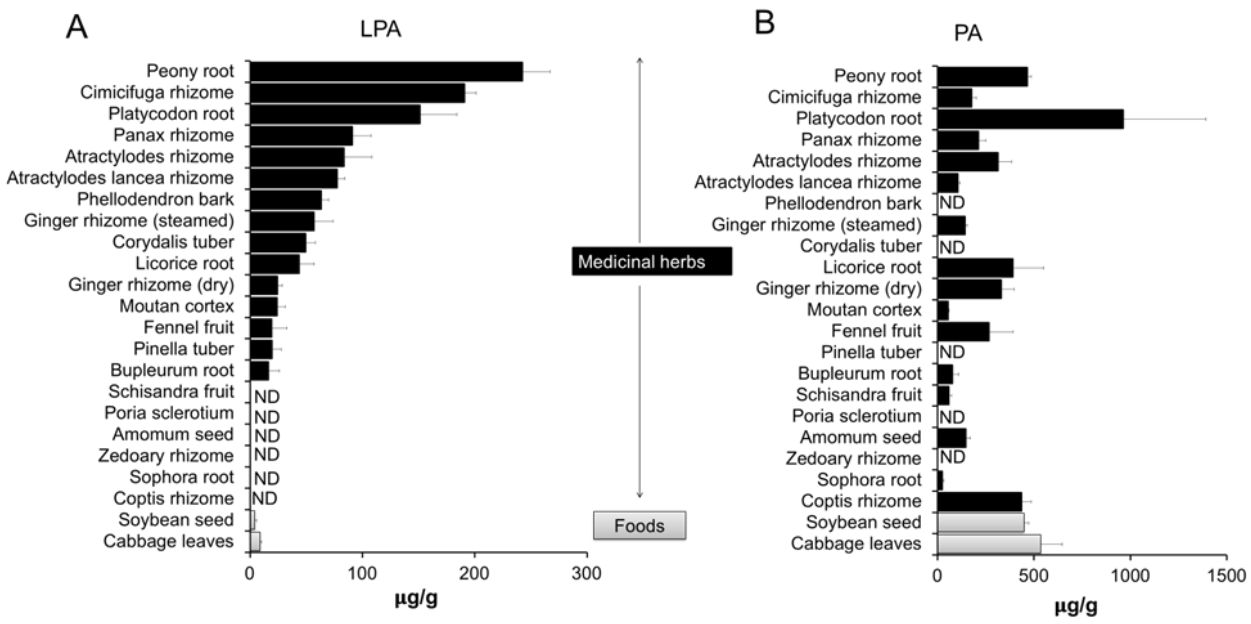

Figure 13. Abundance of LPA in medicinal herbs. Amounts of (A) LPA and (B) PA isolated from herbs were determined by measurement of their lipid phosphorus. Data represent means ± SD of three independent experiments. Herbs and foods except for cabbage leaves were dry weight. ND: Not detectable (less than 2 µg/g).

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 and 16:0, 18:2 LPA (Table 4). Peony root PA and LPA also contained the predominant PA species as 16:0/18:2 PA and 18:2 LPA (Fig. 14).

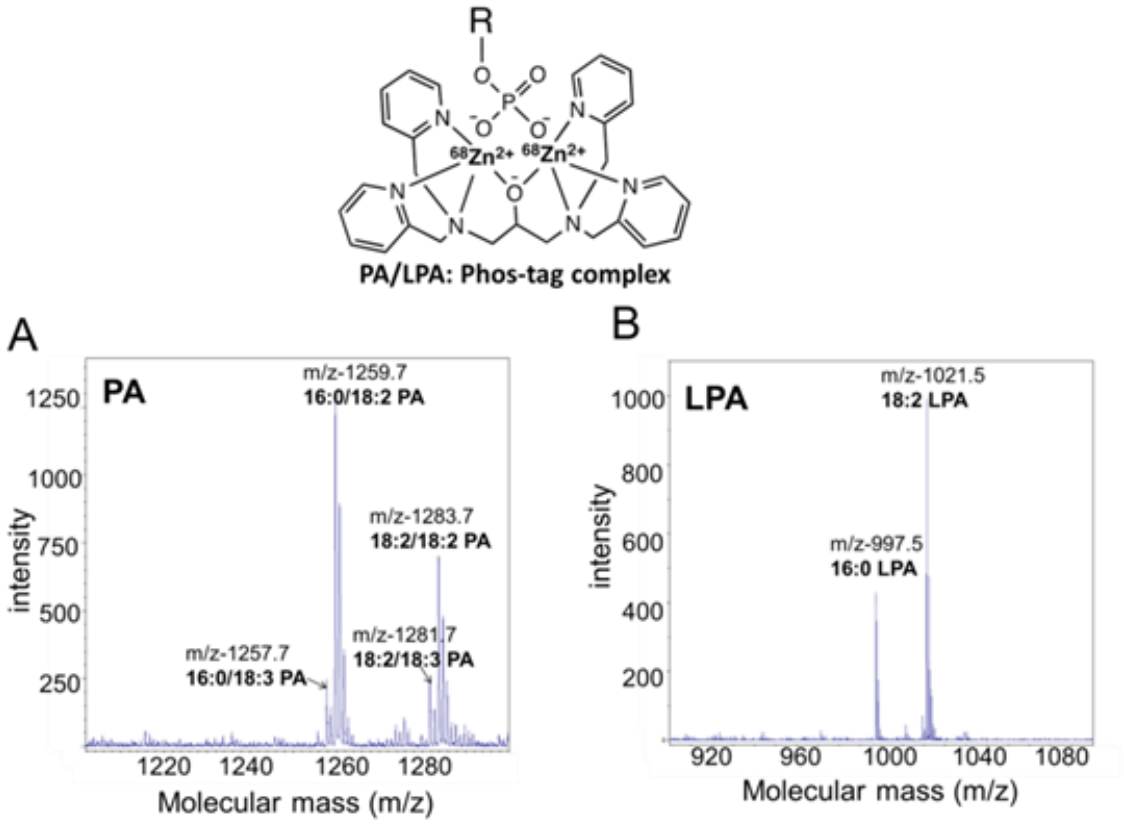

Figure 14. **MALDI TOF mass spectra of PA and LPA isolated from peony root.** (A) PA and (B) LPA from peony root were analyzed by MALDI-TOF MS as their Phos-tag complexes. Chemical structure shown is a complex of a phosphate monoester compound with Phos-tag.

Table 4. Molecular species composition of PA and LPA in medicinal herbs

	PA							LPA			
	*34:3	34:2	34:1	36:5	36:4	36:3	36:2	16:0	18:1	18:2	18:3
Peony root	9	53	–	10	28	–	–	29	–	71	–
Cimicifuga rhizome	–	75	–	–	25	–	–	42	8	41	9
Platycodon root	–	62	–	–	38	–	–	12	–	83	4
Panax rhizome	–	60	16	–	24	–	–	42	–	58	–
Atractylodes rhizome	–	67	–	–	33	–	–	33	–	67	–
Atractylodes lancea rhizome	–	66	–	–	34	–	–	41	–	58	–
Phellodendron bark	–	–	–	–	–	–	–	36	–	56	8
Ginger rhizome (steamed)	–	–	–	–	100	–	–	23	41	36	–
Corydalis tuber	–	–	–	–	–	–	–	35	–	59	6
Licorice root	–	62	–	–	38	–	–	17	14	58	11
Ginger rhizome (dry)	16	75	–	–	9	–	–	44	–	46	–
Moutan cortex	–	55	–	–	45	22	14	21	14	57	8
Fennel fruit	–	32	16	–	16	–	–	41	27	32	–
Pinellia tuber	–	–	–	–	–	–	–	41	–	58	–
Bupleurum root	–	65	–	–	35	28	–	100	–	–	–
Schisandra fruit	–	25	–	–	45	–	–	–	–	–	–
Poria sclerotium	–	–	–	–	–	–	–	–	–	–	–
Amomum seed	15	40	45	–	–	–	–	–	–	–	–
Zedoary rhizome	–	–	–	–	–	–	–	–	–	–	–
Sophora root	–	53	–	–	47	14	–	–	–	–	–
Coptis rhizome	–	47	–	9	30			–	–	–	–

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".

2.3.2 Amelioration of indomethacin-induced gastric ulcer by PA and LPA-rich herbal lipids

The anti-ulcer effects of herbal lipid on indomethacin-induced gastric ulcer were examined. Mice were intragastrically administered the lipid extract that corresponded to 2 g herb powder/kg body weight. We found that administration of PA and LPA-rich platycodon root and peony root lipids, but not moutan cortex, pinellia tuber, zedoary rhizome, and poria sclerotium lipids, significantly reduced the indomethacin-induced gastric lesion (Fig. 15). These results suggest that PA or LPA is one the active component in peony and platycodon root for the amelioration of indomethacin-induced gastric ulcer.

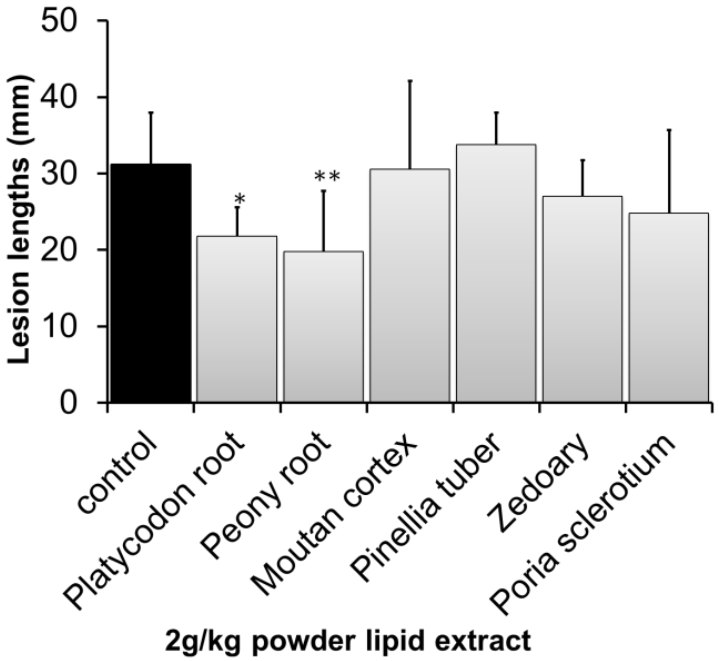

Figure 15. Effect of LPA and PA-rich herbs lipids on indomethacin-induced gastric ulcer. Lipids extracted from herbs were suspended in 3% CMC solution, and 0.2 ml of lipid suspension was intragastrically administered to mice. After 0.5 h, 0.2 ml of indomethacin-suspension (22.9 mg/kg) was administrated intragastrically. Five hour after indomethacin administration, mice were sacrificed. Total lesion lengths were measured. The amount of lipid administered to mice corresponded to 2 g powder per kg of body weight. The number of experiments was 10 (control), and 5–10 (others). *P<0.05, **P<0.005 versus control.

2.3.3 Anti-ulcer effect of peony root lipid and powder

Orally administered peony root lipid reduced indomethacin-induced lesion formation in a dose-dependent fashion (Fig. 16A, B). The maximum reduction was observed when mice were

administered 1 mM of peony root lipid (Fig. 16A, 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. 16B). 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. 16C). This dose of the powder corresponds to the administration of 1 mM peony root lipid.

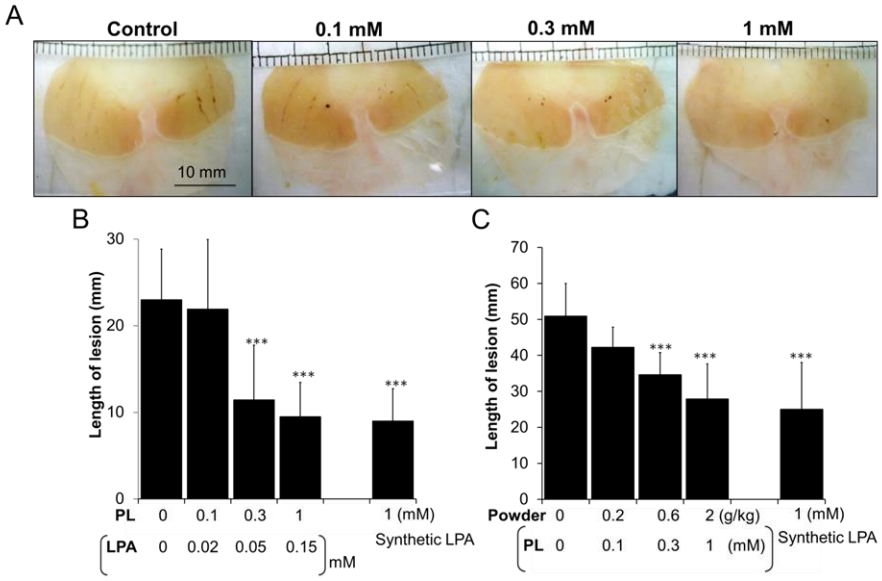

Figure 16. Anti-ulcer effect of peony root lipid and powder. (A, B) Peony root lipid was suspended in 3% CMC. Aliquots of the suspension (0.2 ml) were orally administered to mice. After 30 min, 0.2 ml of indomethacin (22.9 mg/kg) in 3% CMC was intragastrically administered. The total lengths of lesions 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. 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.

2.3.4 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. 17A). 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. 17B). This is consistent with our previous observation that LPA enhances PGE_2 production via up-regulation of COX-2 [14]. 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. 17C), suggesting the involvement of Gi-coupled receptor in their actions.

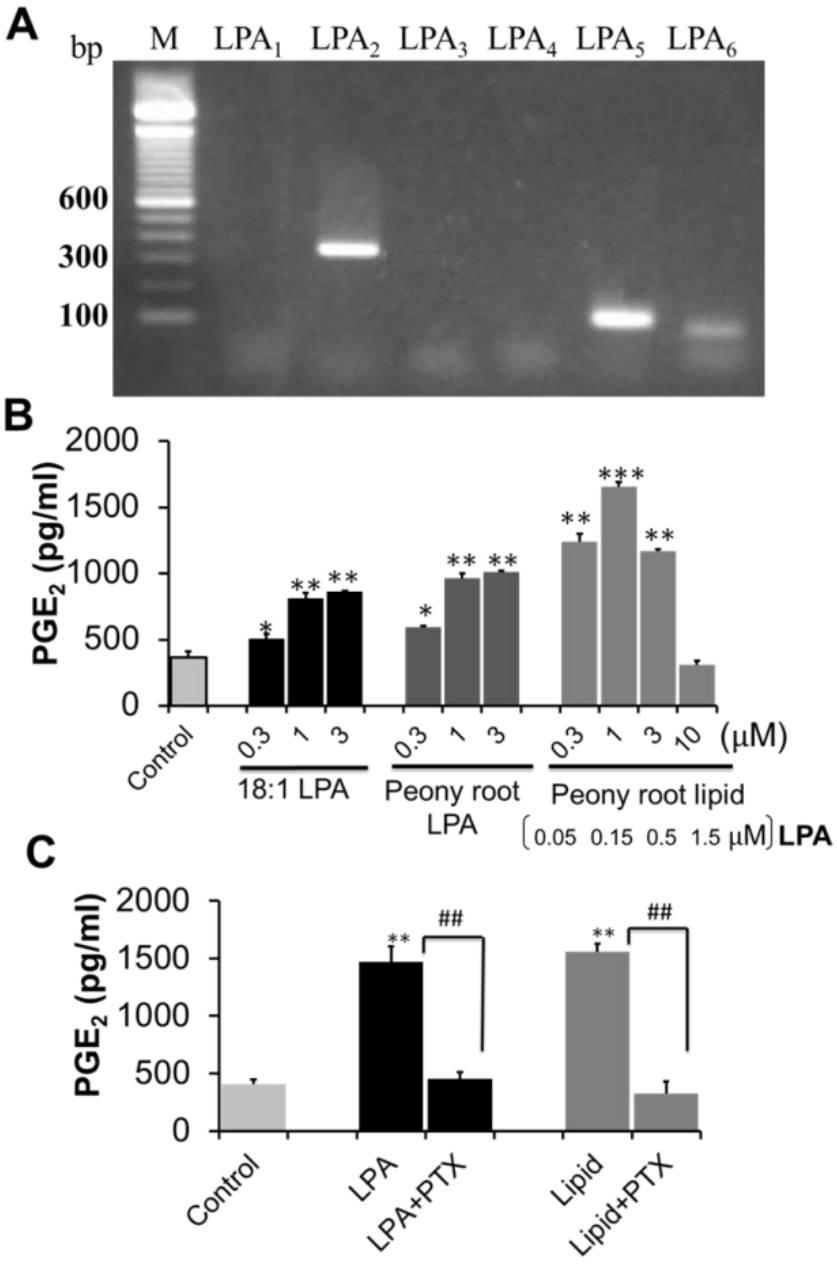

Figure 17. Enhancement of PGE_2 production by LPA and peony root lipid. (A) mRNA profile of LPARs (LPA_{1-6}) of MKN74 cells. (B) MKN74 cells repleted with arachidonic acid were preincubated with the indicated concentration of LPA or peony root lipid for 5 h before stimulation with 1 μM A23187 for 10 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.

2.3.5 Effect of LPA on various apoptotic stimuli in MKN74 cells

2.3.5a UV-induced cell death

UV irradiation is a powerful mutagenic, carcinogenic and induces apoptosis in various cells [68]. To know the LPA effect on UV-induced apoptosis, we first tried to establish UV-induced cell death of MKN74 cells. We could not establish a good apoptotic cell death induced by UV in MKN74 cells. High UV exposure is required for induction of cell death. Once it starts to death, cellular protection was not observed even in the presence of fetal bovine serum (FBS) (Fig. 18). Thus, LPA effect was not examined in this condition.

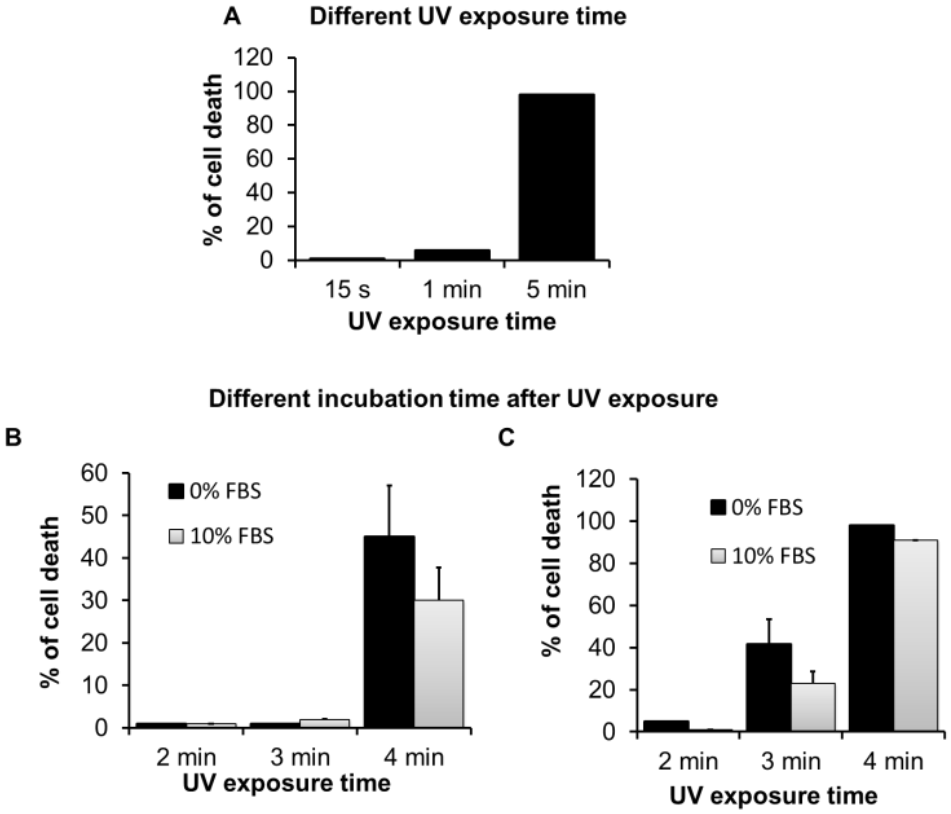

Figure 18. UV-induced cell death of MKN74 cells. (A) MKN74 cells were serum starved for 16 h and exposed to UV light for different time periods. After 4 h incubation, the cells were stained with PI and observed in florescence microscope. (B, C) MKN74 cells serum starved for 16 h and exposed to UV for different time periods. Cells were further incubated for 2 h (B) or 6 h (C). At the end of incubation, cells were stained with PI. In all experiment, cell dish to UV light distance were set to 9 cm.

2.3.5b H_2O_2-induced cell death

H_2O_2 induced cell death was observed by florescence microscope after staining the cells with PI. We found that H_2O_2 induce MKN74 cells death with dose and time dependent fashion (Fig. 19A, B). We fixed the H_2O_2 dose at 250 μM and incubation time about 8 h. This condition causes about 30-40% death of MKN74 cells. We found that up to 3 μM of LPA could not significantly prevent H_2O_2 induced cell death (Fig. 19C). However, there is tendency of LPA to prevent H_2O_2 induced cell death.

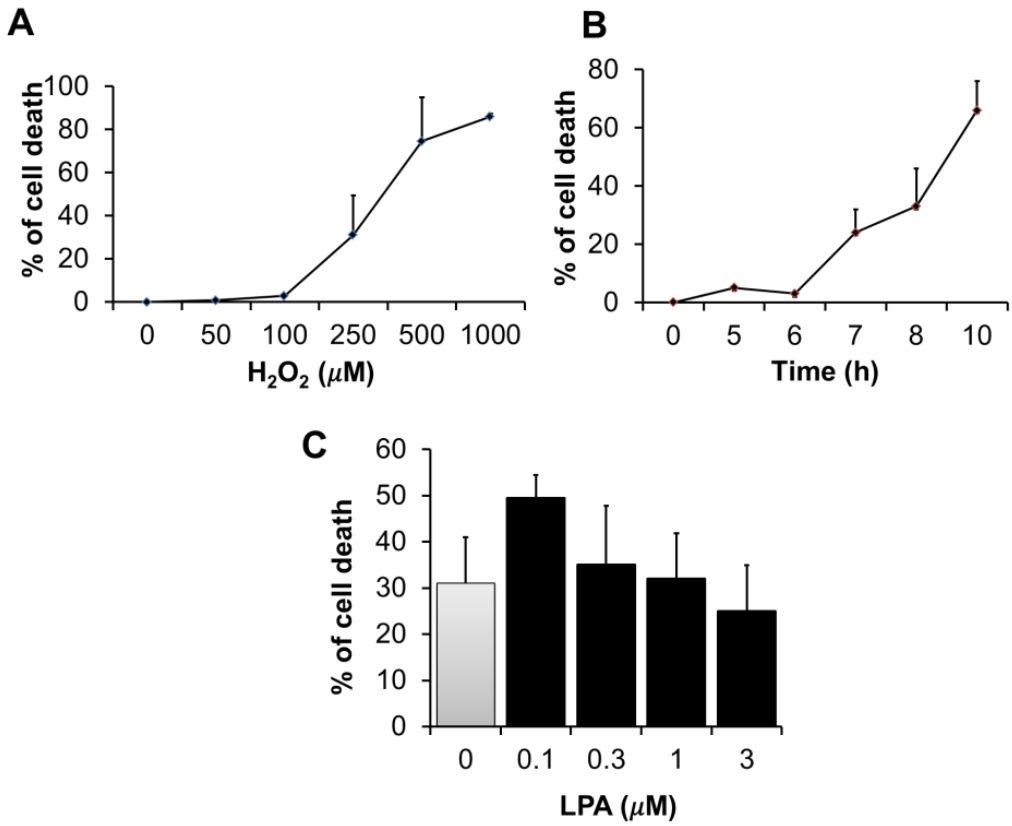

Figure 19. LPA effect on H_2O_2 induced cell death. (A) MKN74 cells were serum starved for 16 h and treated with indicated concentration of H_2O_2 for 8 h. At the end of incubation, cells were stained with PI and observed with florescence microscope. (B) MKN74 cells were serum starved for 16 h and treated with 250 μM of H_2O_2 for indicated time periods and observed the cell death by florescence microscope after staining with PI. (C) LPA was added in 16 h serum starved MKN74 cells. H_2O_2 (250 μM) were added 15 min after LPA addition and incubated for 8 h. At the end of incubation, cells were stained with PI and observed at fluorescence microscopy.

2.3.5c Serum starvation-induced cell death

MKN74 cell death was not observed in 24 h serum starved condition. At 48 and 72 h time periods, about 15-20% cell deaths occurred (Fig. 20A). We examined the LPA effect on 72 h serum starved condition. We found that LPA dose-dependently prevents serum starvation-induced cell death of MKN74 cells (Fig. 20B).

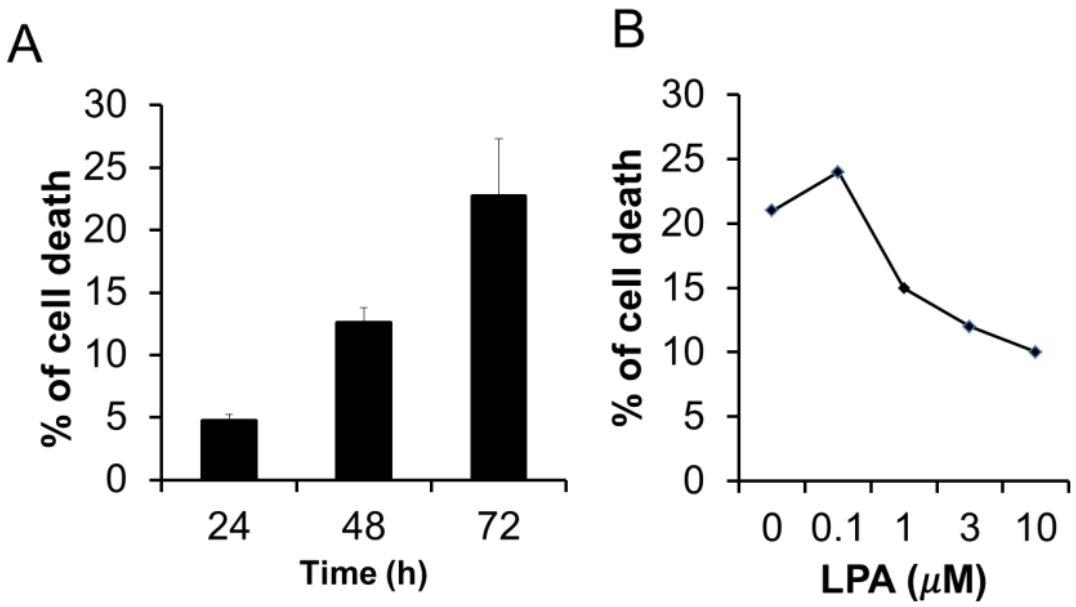

Figure 20. LPA effect on serum-starvation induced cell death. (A) MKN74 cells were seeded in 35 mm polystyrene dish in a density of $1x10^5$ and serum starved at indicated time periods. At end of the incubation, cells were harvested and mixed with trypan blue and living and dead cells were counted by hemocytometer as trypan blue exclusion test. (B) MKN74 cells were serum starved for 24 h and then treated with different concentration of 18:1 LPA for additional 48 h. The living and dead cells were counted by trypan blue exclusion test.

2.3.5d Indomethacin-induced cell death

Treatment of MKN74 cells with indomethacin for 24 h, dose dependently induced cell death (Fig 21A). To examine the LPA effect on indomethacin-induced cell death, we fixed the indomethacin dose at 0.8 mM and incubation time about 30 h based on our preliminary experiment. We found that synthetic 18:1 LPA dose-dependently prevent indomethacin-induced

cell death (Fig. 21B). We also showed that peony root LPA also have protective effect against indomethacin-induced cell death at 10 μM.

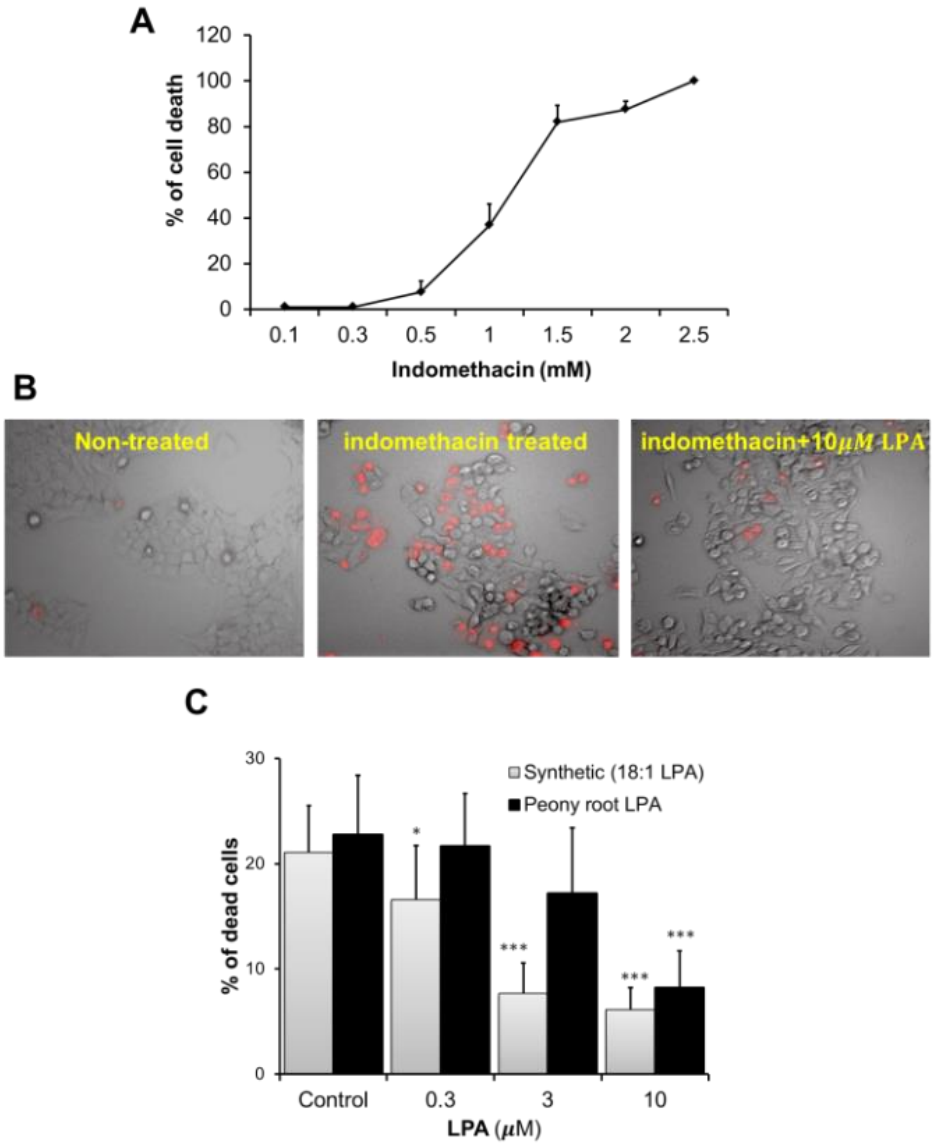

Figure 21. LPA protects indomethacin-induced cell injury in MKN74 cells. (A) Serum starved MKN74 cells were incubated with indicated concentration of indomethacin for 24 h. The living and dead cells were counted after staining of cells with PI. (B) Serum-starved MKN74 cells were incubated with 0.8 mM indomethacin with or without 10 μM 18:1 LPA. After 30 h, dead cells are stained with propidium iodide (PI). The image shown is phase-contrast microscopic photo merged with a fluorescent microscopic photo for indication of PI-positive cells. (C) Serum-starved MKN74 cells were incubated with 0.8 mM indomethacin in the absence or presence of increasing concentration of LPAs. The percentage of dead cells was calculated by counting PI-positive cells in several randomized subfields in each dish from three different experiments. **P <0.01, ***P <0.001 versus control.

2.3.5d∗ LPA prevent indomethacin-induced cytotoxicity

LDH is a cytosolic enzyme. When cell membrane was damaged, LDH is released into the surrounding extracellular space. The presence of this enzyme in the culture medium is an indicator of cellular toxicity. The amount of LDH released into the medium was assessed to determine the cytotoxicity. We found that LPA prevent indomethacin-induced cytotoxicity in dose-dependent fashion (Fig. 22).

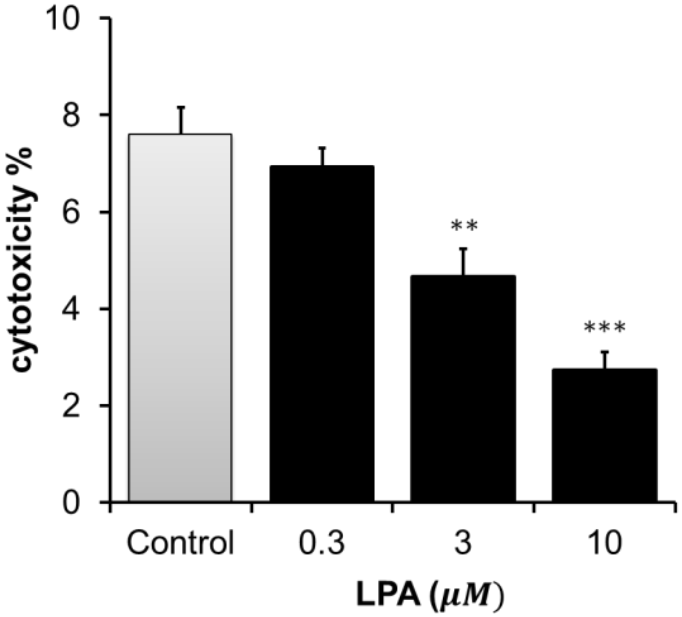

Figure 22. **LPA prevent indomethacin induced cytotoxicity.** Serum-starved MKN74 cells were incubated with 0.8 mM indomethacin in the absence or presence of increasing concentration of 18:1 LPA. LDH leakage in the culture media was determined to know the extent of the cellular damage. **P <0.01, ***P <0.001 versus control.

2.3.5d# Morphology of indomethacin treated cells

As shown in fig 23, non-treated MKN74 cells were tightly attached each other. We found that treatment of the cells with 0.8 mM indomethacin, loss of attachment and rounding of their cell shape, a typical morphological change in apoptotic cells [69, 70]. 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. 23).

Figure 23. Morphology of indomethacin-induced cell death. Serum starved MKN74 cells were treated with 0.8 mM indomethacin in the presence or absence of 10 μM of 18:1 LPA for 30 h. Cell morphology was observed in light microscopy.

2.3.5d■ Involvement of Gi-protein for LPA effect

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. 24). This is also confirmed by the morphological change of the cells. We found that LPC did not show a protective effect on indomethacin-induced cell death (Fig. 24). These results indicated that an LPA/LPA_2 axis operates in the protective effects against indomethacin-induced cellular damage.

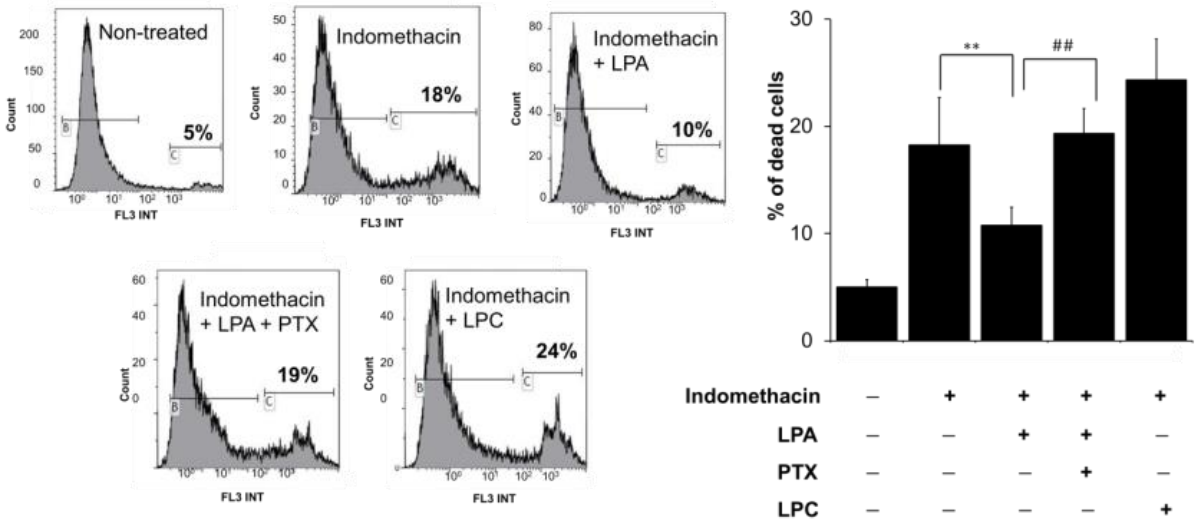

Figure 24. Involvement G-protein for LPA-induced prevention of indomethacin-induced cell death. Serum-starved MKN74 cells were treated with LPA (in the presence or absence of 100 ng/ml of PTX) or LPC and then treated with indomethacin (0.8 mM). Flow cytometry was performed 30 h after indomethacin treatment. Values in the flow cytometry chart indicate the % of dead cells. **P <0.01 versus control, ## P <0.01 versus LPA.

2.3.6 LPA stimulated proliferation of MKN74 cells

Treatment of MKN74 cells with LPA induced proliferation in a dose-dependent manner (Fig. 25A). LPA-induced cell proliferation was also confirmed by measuring incorporation of the thymidine analog BrdU into the cells (Fig. 25B). Peony root LPA also stimulated the proliferation of MKN74 cells (Fig. 25A, B). LPA-induced cell proliferation was partially and significantly inhibited by PTX (Fig. 25C). Again, LPC had no proliferative effect.

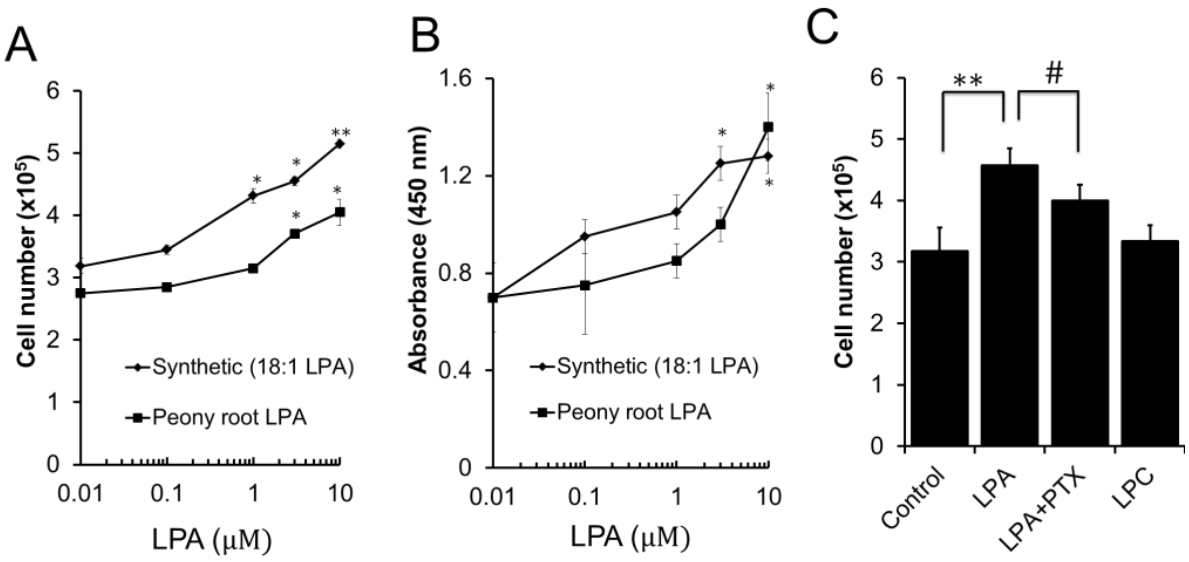

Figure 25. LPA stimulates proliferation of MKN74 cells. (A) MKN74 cells were serum starved for 24 h and then treated with different concentrations of LPA. After 24 h, cells were collected by trypsinization, and living cells were counted using a hemocytometer. *P <0.05, **P <0.01 versus control. (B) MKN74 cells were serum starved for 48 h and then treated with LPA for 24 h. BrdU was added 4 h before of termination of incubation. BrdU incorporation was assessed using a BrdU cell proliferation assay kit. *P <0.05 versus control. (C) The proliferation assay was conducted in the presence or absence of 100 ng/ml PTX or with 16:0 LPC (10 μM) instead of LPA. After 24 h, cells were collected by trypsinization and living cells were counted using a hemocytometer. **P <0.01 versus control, $^{\#}$P <0.05 versus LPA.

2.3.7 LPA concentration in a mouse stomach fluid

In order to better understanding 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 [71]. We found that the total LPA concentration in the stomach fluid was 2.4 μM (Fig. 26). The major species of LPA in the stomach fluid were found to be 16:0, 18:0, 18:1, and 18:2 LPAs (Fig. 26). 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, 20:0, 20:1, 20:2, 20:4, 22:0, 22:4, and 22:6, were present in low levels (0.01–0.1 μM).

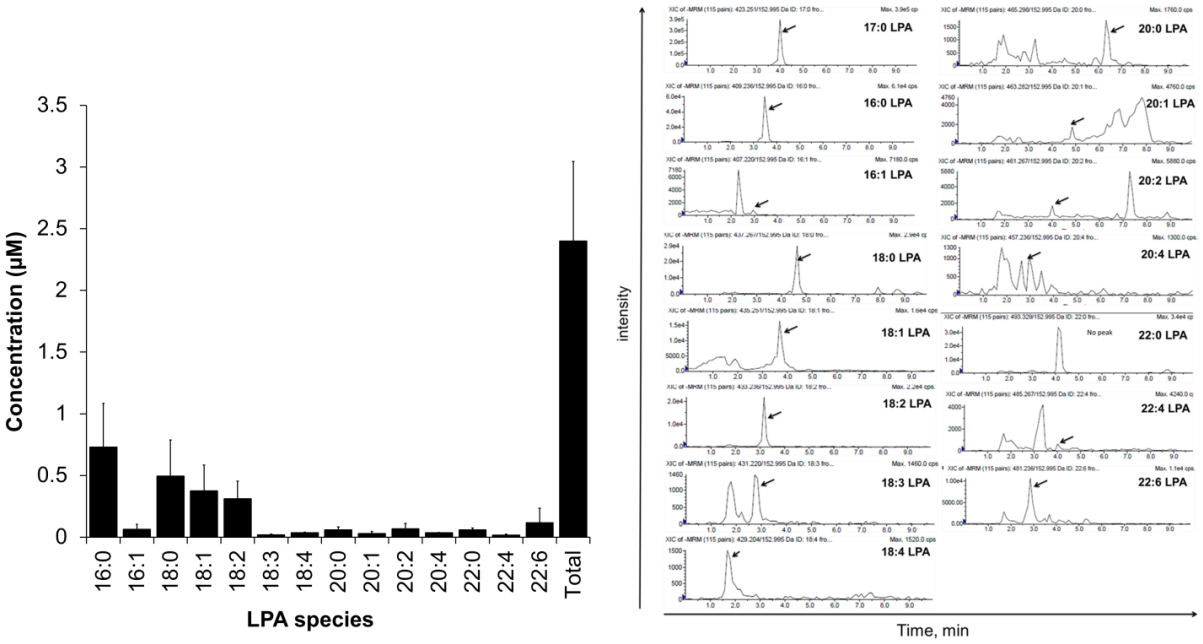

Figure 26. LPA concentration in mouse stomach fluid. Stomachs of fasted mice were isolated, and the stomach was cut along the greater curvature. The stomach surface was washed with a small amount of PBS. Lipids were extracted from the stomach washing solution and subjected to LC/MS/MS by using 17:0 LPA as an internal standard.

2.4 Discussion

Research in medicinal herbs has identified many active components that exert anti-ulcer effects with diverse mechanisms of action. These include compounds belonging to flavonoids, alkaloids, tannins, and saponins [72, 73]. Dietary phospholipids from soy and milk are also shown as ameliorative agents for NSAIDs-induced stomach ulcer [74]. In this study, we found that LPA in medicinal herbs is a potential component for prevention of gastric mucosal injury. This notion is based on several observations. Firstly, synthetic LPA and herbal LPA showed ameliorative activity against cytotoxic effect of indomethacin. Secondly, LPAs and LPA-rich lipid enhanced PGE_2 production, an important cytoprotective factor in GI mucosa. Thirdly, peony root lipid, an LPA-rich herbal lipid identified here, significantly ameliorated indomethacin-induced gastric lesions in mice. Lastly, there were many LPA-rich herbs in Chinese traditional medicines used for the treatment of GI disorders. We discuss on mechanisms of these effects in detail.

A well-known mechanism of NSAID-induced gastric mucosal lesions is the inhibition of COX-1 and COX-2 enzymes and a resulting decrease in gastroprotective PGE_2. The importance of PGE_2 in the integrity of stomach mucosa is evident from the fact that common anti-ulcer drugs, such as rebamipide and geranylgeranylacetone, up-regulate COX-2, leading to the enhancement of PGE_2 production [75, 76]. Consistent with our previous study [14], LPA from medicinal herb was found to enhance PGE_2 production in human gastric cancer cell line, MKN74 cells. We also showed that peony root lipid, which has concentrated LPA, enhanced PGE_2 production with Gi-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 [69, 70]. 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 [30]. It is also reported that NSAIDs chemically interact with cell membrane phospholipids, disrupt membrane permeability, and form membrane pores [30]. 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 [77]. 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 [78].

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 [53, 79, 80]. On the other hand, LPA_5 seems to be coupled with Gq rather than Gi in many cells [81, 82]. 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 [14] 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) [61] and similar or relatively higher level to that in human plasma or serum LPA (0.1–2.4 μM) [15, 83, 84]. The presence of LPA in stomach fluid is reasonable because LPA receptors are expressed in the apical side of gastric mucosal cells [14]. 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*. 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 [85] and showed potent gastro protective effects *in vivo* and *in vitro*. 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.

Conclusion

Conclusion

LPA is a naturally occurring signaling lipid. Recent investigation revealed that LPA plays important roles in epithelial integrity of GI tract [36, 37]. Those include protection against radiation-induced apoptosis [8], inhibition of secretory diarrhea by modulating chloride channel activity [37] and amelioration of intestinal epithelial injury in colitis [7]. Our previous study demonstrated ameliorative effects of LPA on stress- or NSAIDs-induced stomach ulcer in rats [10, 11]. All these effects are observed when LPA is administered orally or rectally, indicating that LPA coming from apical side can affect epithelial activity in GI tract. Among LPA receptors identified so far ($LPA_{1\sim6}$), LPA_2 has been known to abundantly expressed in GI tract as shown by a database [The Human protein atlas, http:/www.proteinatlas.org/]. Previously, we examined localization of LPA_2 in mouse stomach, and found that LPA_2-expressing stomach epithelial cells are not cells located basolateral side but cells located just below lumen, those are surface mucous cells [14]. Importantly, LPA_2 is localized on apical membrane of surface mucous cells, indicating that this LPA receptor exists to respond LPA coming from apical side. In fact, LPA has been shown to present not only in blood but also in saliva. In this study, for the first time, we demonstrated existence of LPA in the stomach fluid at biologically significant levels. These observations led a notion that surface mucous cells received vital signal from stomach fluid via LPA/LPA_2 axis. Furthermore, a hypothesis that LPA-rich diet contribute epithelial integrity by strengthen LPA/LPA_2 signal is considered. In this study, we examined these possibilities.

To examine a possibility that LPA/LPA_2 axis is involved for the vital signal in stomach, we examined the LPA effect in LPA_2 expressing gastric cancer cells, MKN74 cells. We found that LPA enhances PGE_2 production, prevents cell death and stimulates proliferation of MKN74 cells at relevant concentration found in stomach fluid. These results suggest that gastric epithelial cells respond to endogenous LPA via LPA/LPA_2 axis.

In this study, we found that PA is abundant in several cereals such as buckwheat, cornmeal, and peanut. We found significant ameliorative effect of PA-rich buckwheat bran lipids against indomethacin-induced gastric ulcer. However, when we tested the food itself, the PA-rich buckwheat powder did not significantly prevent the gastric lesions. In this regard, dose of indomethacin used in this study was 16 times higher compared to chronic indomethacin exposure

in human. In vivo assay system with low dose NSAID as chronic stomach ulcer model should be established. Effect of PA-rich food should be examined in such chronic model.

In this study, we found several LPA-rich herbs that traditionally used for the treatment of GI disorder. We found that purified LPA and lipid fraction prepared from the LPA-rich herbs enhanced PGE_2 production and stimulate proliferation of gastric cells. Herbs LPA also prevented gastric cell injury against indomethacin-induced toxicity both in *in vivo* and *in vitro* model. These results suggest that LPA is one of the active components in traditional herbs to contribute epithelial integrity by strengthen LPA/LPA_2 signal in human stomach (Fig. 27).

Anti-ulcer drugs are not always safe for reducing the risk of side-effect of NSAIDs. In this view points, ingestion of PA or LPA-rich diet identified here could be good remedies for the prevention of NSAID-induced gastric ulcer with low cost and no side effect.

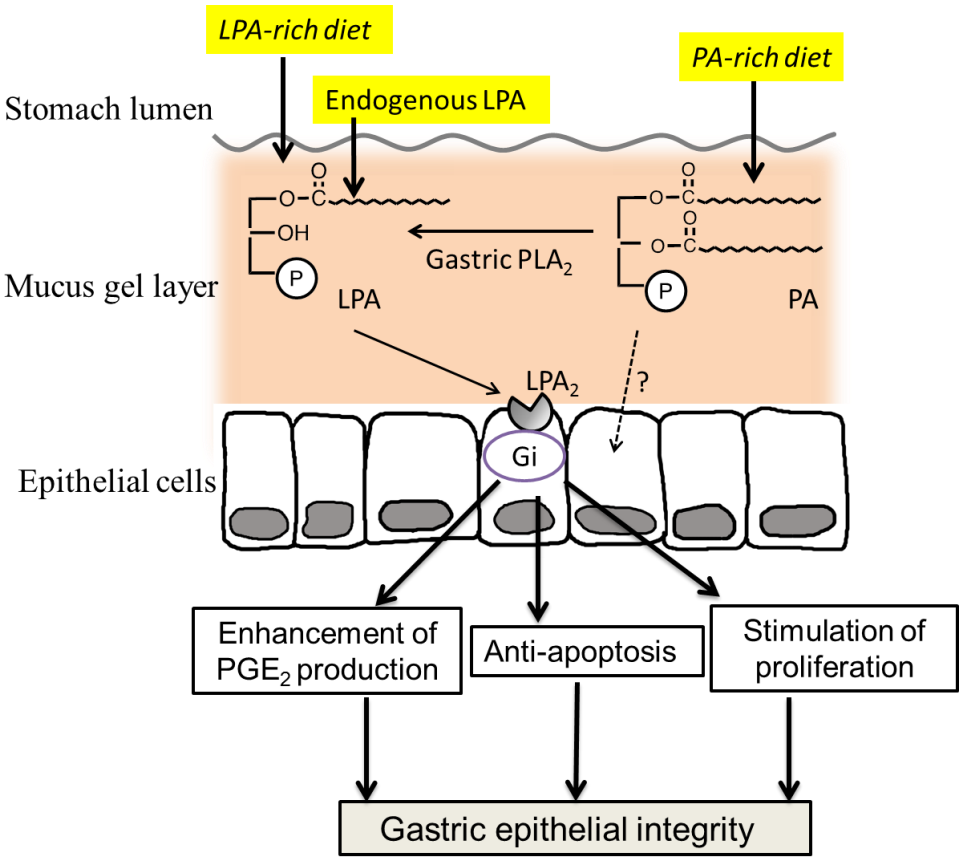

Figure 27. LPA contributes epithelial integrity by strengthening LPA/LPA_2 signal. LPA (dietary, endogenous) enhanced PGE_2 production, prevents cell death and stimulates proliferation of gastric cells via LPA/LPA_2 signal.

References

References

[1]. Moolenaar WH, Van Meeteren LA, Giepmans BN. (2004). The ins and outs of lysophosphatidic acid signaling. Bioessays, 26: 870–881.

[2] Karliner JS, Honbo N, Summers K, Gray MO, Goetzl EJ. (2001). The lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid enhance survival during hypoxia in neonatal rat cardiac myocytes. J. Mol. Cell Cardiol., 33: 1713–1717.

[3] Cremers B, Flesch M, Kostenis E, Maack C, Niedernberg A, Stoff A, Sudkamp M, Wendler O, Bohm M. (2003). Modulation of myocardial contractility by lysophosphatidic acid (LPA). J. Mol. Cell Cardiol., 35: 71–80.

[4] Ainslie K, Shi ZD, Garanich JS, Tarbell JM. (2004). Rat aortic smooth muscle cells contract in response to serum and its components in a calcium independent manner. Ann. Biomed. Eng., 32: 1667–1675.

[5] Tokumura A, Fukuzawa K, Tsukatani H. (1978). Effects of synthetic and natural lysophosphatidic acids on the arterial blood pressure of different animal species. Lipids, 13: 572–574.

[6] Ye X, Chun J. (2010). Lysophosphatidic acid (LPA) signaling in vertebrate reproduction. Trends Endocrinol. Metab., 21: 17.

[7] Sturm A, Zeeh J, Sudermann T, Rath H, Gerken G, Dignass AU. (2002). Lisofylline and lysophospholipids ameliorate experimental colitis in rats. Digestion, 66: 23–29.

[8] Deng W, Balazs L, Wang DA, Van Middlesworth L, Tigyi G, Johnson LR. (2002). Lysophosphatidic acid protects and rescues intestinal epithelial cells from radiation- and chemotherapy-induced apoptosis. Gastroenterol., 123: 206−216.

[9] Sturm A, Sudermann T, Schulte KM, Goebell H, Dignass AU. (1999). Modulation of intestinal epithelial wound healing in vitro and in vivo by lysophosphatidic acid. Gastroenterol., 117: 368–377.

[10] Adachi M, Horiuchi G, Ikematsu N, Tanaka T, Terao J, Satouchi K, Tokumura A. (2011). Intragastrically administrated lysophosphatidic acid protect against gastric ulcer in rats under water-immersion restraint stress. Dig. Dis. Sci., 56: 2252−2261.

[11] Tanaka T, Morito K, Kinoshita M, Ohmoto M, Urikura M, Satouchi K, Tokumura A. (2013). Orally administrated phosphatidic acid and lysophosphatidic acids ameliorate aspirin-induced stomach mucosal injury in mice. Dig. Dis. Sci., 58: 950−958.

[12] Lin ME, Herr DR, Chun J. (2010). Lysophosphatidic acid (LPA) receptors: signaling properties and disease relevance. Prostaglan. Lipid Mediat., 91: 130–138.

[13] Potocka IW, Rawińska P, Zieba I, Boruszewska D, Sinderewicz E, Waśniewski T, Skarzynski DJ. (2014). Lysophosphatidic acid (LPA) signaling in human and ruminant reproductive tract. Mediat. Inflamm., 2014: 1–14.

[14] Tanaka T, Ohmoto M, Morito K, Kondo H, Urikura M, Satouchi K, Tokumura A. (2014). Type 2 lysophosphatidic acid receptor in gastric surface mucous cells: Possible implication of prostaglandin E2 production. Biofactors, 40: 355−361.

[15] Aoki J, Taira A, Takanezawa Y, Kishi Y, Hama K, Kishimoto T, Mizuno K, Saku K, Taguchi R, Arai H. (2002). Serum lysophosphatidic acid is produced through diverse phospholipase pathways. J. Biol. Chem., 277: 48737–48744.

[16] Sano T, Baker D, Virag T, Wada A, Yatomi Y, Kobayashi T, Igarashi Y, Tigyi G. (2002). Multiple mechanisms linked to platelet activation result in lysophosphatidic acid and sphingosine 1-phosphate generation in blood. J. Biol. Chem., 277: 21197–21206.

[17] Woclawek-Potocka I, Komiyama J, Saulnier-Blache JS, Brzezicka E, Bah MM, Okuda K, Skarzynski DJ. (2009). Lysophosphatic acid modulates prostaglandin secretion in the bovine uterus. Reproduction, 137: 95–105.

[18] Liliom K, Guan Z, Tseng JL, Desiderio DM, Tigyi G. Watsky MA. (1998). Growth factor-like phospholipids generated after corneal injury. American J. Physiol. Cell Physiol., 274: C1065–C1074.

[19] Tokumura A, Kume T, Fukuzawa K, Tahara M, Tasaka K, Aoki J, Arai H, Yasuda K, Kanzaki H. (2007). Peritoneal fluids from patients with certain gynecologic tumor contain elevated levels of bioactive lysophospholipase D activity. Life Sci., 80: 1641–1649.

[20] Tokumura A, Miyake M, Nishioka Y, Yamano S, Aono T, Fukuzawa K. (1999). Production of lysophosphatidic acids by lysophospholipase D in human follicular fluids of in vitro fertilization patients. Biol. Reprod., 61: 195–199.

[21] Woclawek-Potocka I, Brzezicka E, Skarzynski DJ. (2009). Lysophosphatic acid modulates prostaglandin secretion in the bovine endometrial cells differently on days 8–10 of the estrous cycle and early pregnancy. J. Reprod. Devel., 55: 393–399.

[22] Shen Z, Belinson J, Morton RE, Xu Y, Xu Y. (1998). Phorbol 12-myristate 13-acetate stimulates lysophosphatidic acid secretion from ovarian and cervical cancer cells but not from breast or leukemia cells. Gynecol. Oncol., 71: 364–368.

[23] Mori K, Kitayama J, Aoki J, Kishi Y, Shida D, Yamashita H, Arai H, Nagawa H. (2007). Submucosal connective tissue-type mast cells contribute to the production of lysophosphatidic acid (LPA) in the gastrointestinal tract through the secretion of autotaxin (ATX)/lysophospholipase D (lysoPLD). Virchows Archiv., 451: 47–56, 2007.

[24] Fukushima N, Weiner JA, Chun J. (2000). Lysophosphatidic acid (LPA) is a novel extracellular regular of cortical neuroblast morphology. Develop. Biol., 228: 6–18.

[25] Tanaka M, Okudaira S, Kishi Y, Ohkawa R, Iseki S, Ota M, Noji S, Yatomi Y, Aoki J, Arai H. (2006). Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. J. Biol. Chem., 281: 25822–30.

[26] Contos JJ, Fukushima N, Weiner JA, Kaushal D, Chun J. (2000). Requirement for the LPA_1 lysophosphatidic acid receptor gene in normal suckling behavior. Proc. Natl. Acad. Sci., 97: 13384–13389.

[27] Gennero I, Laurencin-Dalicieux S, Conte-Auriol F, Briand-Mésange F, Laurencin D, Rue J, Beton N, Malet N, Mus M, Tokumura A, Bourin P, Vico L, Brunel G, Oreffo RO, Chun J, Salles

JP. (2011). Absence of the lysophosphatidic acid receptor LPA_1 results in abnormal bone development and decreased bone mass. Bone, 49: 395–403.

[28] Kasman N. Badley E. (2006). Arthritis-related prescription medications. Public Health Agency of Canada, Ottawa, Ontario, Canada, Vol. 2017: Chapter 5.

[29] Linder JD, Wilcox CM. (2001). Acid peptic disease in the elderly. Gastroenterol Clin North Am., 30: 363–376.

[30] Musumba C, Pritchard DM, Pirmohamed M. (2009). Review article: cellular and molecular mechanisms of NSAID-induced peptic ulcers. Aliment. Pharmacol. Ther., 30: 517–531.

[31] Amani SA, Awaada RM, Gamal AS. (2013). Natural products in treatment of ulcerative colitis and peptic ulcer. J. S. Chem. Soci., 17: 101-124.

[32] Tanaka T, Kassai A, Ohmoto M, Morito K, Kashiwada Y, Takaishi Y, Urikura M, Morishige J, Satouchi K, Tokumura A. (2012). Quantification of phosphatidic acid in foodstuffs using a thin-layerchromatography-imaging technique. J. Agric. Food Chem., 60: 4156−4161.

[33] Tanaka T, Horiuchi G, Matsuoka M, Hirano K, Tokumura A, Koike T, Satouchi K. (2009). Formation of lysophosphatidic acid, a wound healing lipid, during digestion of cabbage leaves. Biosci. Biotechnol. Biochem., 73: 293−1300.

[34] Lanza FL, Chan FK, Quigley EM, Practice Parameters Committee of the American College of Gastroenterology. (2009). Guidelines for prevention of NSAID-related ulcer complications. Am. J. Gastroenterol., 104: 728−738.

[35] Sarzynski E, Puttarajappa C, Xie Y, Grover M, Laird-Fick H. (2011). Association between proton pump inhibitor use and anemia: A retrospective cohort study. Dig. Dis. Sci., 56: 2349−2353.

[36] Tokumura A. (2011). Physiological significance of lysophospholipids that act on the lumen side of mammalian lower digestive tracts. J. Health Sci., 57: 115−128.

[37] Yun CC, Kumar A. (2015). Diverse roles of LPA signaling in the intestinal epithelium. Exp. Cell Res., 333: 201−207.

[38] Morrison WR. (1977). Cereal lipids. Proc. Nutr. Soc., 36: 143−148.

[39] Weihrauch JL, Son YS. (1983). Phospholipid content of foods. J. Am. Oil Chem. Soc., 60: 1971−1978.

[40] Parsons JG, Price PB. (1979). Phospholipids of barley grain. J. Agric. Food Chem., 27: 913−915.

[41] González-Thuillier I, Salt L, Chope G, Penson S, Skeggs P, Tosi P, Powers SJ, Ward JL, Wilde P, Shewry PR, Haslam RP. (2015). Distribution of lipids in the grain of Wheat (cv. Hereward) determined by lipidomic analysis of milling and pearling fractions. J. Agric. Food Chem., 63: 10705−16.

[42] Bligh EG, Dyer WJ. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37: 911−917.

[43] Chalvardjian A, Rudnicki E. (1970). Determination of lipid phosphorus in the nanomolar range. Anal. Biochem., 36: 225−226.

[44] Morishige J, Urikura M, Takagi H, Hirano K, Koike T, Tanaka T, Satouchi K. (2010). A clean-up technology for the simultaneous determination of lysophosphatidic acid and sphingosine-1-phosphate by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a phosphate capture molecule, Phos-tag. Rapid Commun. Mass Spectrom., 24: 1075−1084.

[45] Urikura M, Morishige J, Tanaka T, Satouchi K. (2012). Phosphatidic acid production in the processing of cabbage leaves. J. Agric. Food Chem., 60: 11359−11365.

[46] Giménez-Bastida JA, Zieliński H. (2015). Buckwheat as a functional food and its effects on health. J. Agric. Food Chem., 63: 7896−913.

[47] Mazza G. (1988). Lipid content and fatty acid composition of buckwheat seed. Cereal Chem., 65: 122−126.

[48] Choudhury NH, Juliano BO. (1980). Lipids in developing and mature rice grain. Phytochemistry., 19: 1063−1069.

[49] Bonafaccia G, Marocchini M, Kreft I. (2003). Composition and technological properties of the flour and bran from common and tartary buckwheat. Food Chem., 80: 9−15.

[50] Testerink C, Munnik T. (2005). Phosphatidic acid: A multifunctional stress signaling lipid in plants. Trends Plant Sci., 10: 368−375.

[51] Wang X, Devaiah SP, Zhang W, Welti R. (2006). Signaling functions of phosphatidic acid. Prog. Lipid Res., 45: 250−278.

[52] Klimecka M, Szczegielniak J, Godecka L, Lewandowska E, Dobrowolska G, Muszynska G. (2011). Regulation of wound-responsive calcium-dependent protein kinase from maize (ZmCPK11) by phosphatidic acid. Acta Biochim. Polym., 58: 589−595.

[53] Deng W, Shuyu E, Tsukahara R, Valentine WJ, Durgam G, Gududuru V, Balazs L, Manickam V, Arsura M, Van Middlesworth L, Johnson LR, Parrill AL, Miller DD, Tigyi G. (2007). The lysophosphatidic acid type 2 receptor is required for protection against radiation-induced intestinal injury. Gastroenterol., 132: 1834−1851.

[54] Ohsako S, Dequchi T. (1981). Stimulation of phosphatidic acid of calcium influx and cyclic GMP synthesis in neuroblastoma cells. J. Biol. Chem., 256: 10945−10948.

[55] Nayar R, Mayer LD, Hope MJ, Cullis PR. (1984). Phosphatidic acid as a calcium ionophore in large unilamellar vesicle systems. Biochim. Biophys. Acta Biomembr., 777: 343−346.

[56] Liu P, Xu Y, Hopfner RL, Gopalakrishnan V. (1999). Phosphatidic acid increases inositol-1, 4, 5,-trisphosphate and [Ca^{2+}] levels in neonatal rat cardiomyocytes. Biochim. Biophys. Acta Mol. Cell Biol. Lipids, 1440: 89−99.

[57] Schepp W, Steffen B, Schusdziarra V, Classen M. (1986). Calcium, calmodulin, and cyclic adenosine monophosphate modulate prostaglandin E_2 release from isolated human gastric mucosal cells. J. Clin. Endocrinol. Metab., 63: 886−891.

[58] Fang Y, Vilella M, Bachmann R, Flanigan A, Chen J. (2001). Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science, 294: 1942−1945.

[59] Joy JM, Gundermann DM, Lowery RP, Jäger R, McCleary SA, Purpura M, Roberts MD, Wilson SMC, Hornberger TA, Wilson JM. (2014). Phosphatidic acid enhances mTOR signaling and resistance exercise induced hypertrophy. Nutr. Metab., 11: 29.

[60] Pagano RE, Longmuir KJ. (1985). Phosphorylation, transbilayer movement, and facilitated intracellular transport of diacylglycerol are involved in the uptake of a fluorescent analog of phosphatidic acid by cultured fibroblasts. J. Biol. Chem., 260: 1909−1916.

[61] Sugiura T, Nakane S, Kishimoto S, Waku K, Yoshioka Y, Tokumura A. (2002). Lysophosphatidic acid, a growth factor-like lipid, in the saliva. J. Lipid Res., 43: 2049−2055.

[62] Yung YC, Stoddard NC, Chun J. (2014). LPA receptor signaling: pharmacology, physiology, and pathophysiology. J. Lipid Res., 55: 1192–1214.

[63] Kaunitz JD, Akiba Y. (2004). Gastroduodenal mucosal defense: role of endogenous mediators. Curr. Opin. Gastroenterol., 20: 526–532.

[64] Laine L, Takeuchi K, Tarnawski A. (2008). Gastric mucosal defense and cytoprotection: bench to bedside. Gastroenterol., 135: 41–60.

[65] Halverson PB. (1999). Nonsteroidal anti-inflammatory drugs: benefits, risks, and COX-2 selectivity. Orthop. Nurs., 18: 21–26.

[66] Bi WP, Man HB, Man MQ. (2014). Efficacy and safety of herbal medicines in treating gastric ulcer: a review. World J. Gastroenterol., 20: 17020–17028.

[67] Tsuboi K, Okamoto Y, Ikematsu N, Inoue M, Shimizu Y, Uyama T, Wang J, Deutsch DG, Burns MP, Ulloa NM, Tokumura A, Ueda N. (2011). Enzymatic formation of N-acylethanolamines from N-acylethanolamine plasmalogen through N-acylphosphatidylethanolamine-hydrolyzing phospholipase D-dependent and -independent pathways. Biochim. Biophys. Acta, 1811: 565–577.

[68] Dunkern TR, Fritz G, Kaina B. (2001). Ultraviolet light-induced DNA damage triggers apoptosis in nucleotide excision repair-deficient cells via Bcl-2 decline and caspase-3/-8 activation. Oncogene, 20: 6026 – 6038.

[69] Zhu GH, Wong BC, Eggo MC, Ching CK, Yuen ST, Chan EY, Lai KC, Lam SK. (1999). Non-steroidal anti-inflammatory drug-induced apoptosis in gastric cancer cells is blocked by protein kinase C activation through inhibition of c-myc. Br. J. Cancer, 79: 393–400.

[70] Tomisato W, Tsutsumi S, Rokutan K, Tsuchiya T, Mizushima T. (2001). NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. Am. J. Physiol. Gastrointest. Liver Physiol., 281: G1092–1100.

[71] Bickel M, Kauffman JGL. (1981). Gastric gel mucus thickness: effect of distention, 16, 16-dimethyl prostaglandin E_2, and carbenoxolone. Gastroenterol., 80: 770–775.

[72] Junior LCK, Santin JR, Niero R, Andrade SF, Filho VC. (2012). The therapeutical lead potential of metabolites obtained from natural sources for the treatment of peptic ulcer. Phytochem. Reviews, 11: 567–616.

[73] Jesus NZ, Falcao H, Gomes IF, Laite TJ, Lima GR, Filho JM, Tavares JF, Silva MS, Filho P F, Batista LM. (2012). Tannins, peptic ulcers and related mechanisms. Int. J. Mol. Sci., 13: 3203–3228.

[74] Küllenberg D, Taylor LA, Schneider M, Massing U. (2012). Health effects of dietary phospholipids. Lipids Heal. Dis., 11: 3.

[75] Sun WH, Tsuji S, Tsujii M, Gunawan ES, Kawai N, Kimura A, Kakiuchi Y, Yasumaru M, Iijima H, Okuda Y, Sasaki Y, Hori M, Kawano S. (2000). Induction of cyclooxygenase-2 in rat gastric mucosa by rebamipide, a mucoprotective agent. J. Pharmacol. Exp. Ther., 295: 447–452.

[76] Nishida T, Yabe Y, Fu HY, Hayashi Y, Asahi K, Eguchi H, Tsuji S, Tsujii M, Hayashi N, Kawano S. (2007). Geranylgeranylacetone induces cyclooxygenase-2 expression in cultured rat gastric epithelial cells through NF-kappaB. Dig. Dis. Sci., 52: 1890–1896.

[77] Ramachandran S, Shida D, Nagahashi M, Fang X, Milstien S, Takabe K, Spiegel S. (2010). Lysophosphatidic acid stimulates gastric cancer cell proliferation via ERK1-dependent upregulation of sphingosine kinase 1 transcription. FEBS Lett., 584: 4077– 4082.

[78] Radhika V, Ha JH, Jayaraman M, Tsim ST, Dhanasekaran DN. (2005). Mitogenic signaling by lysophosphatidic acid (LPA) involves G alpha 12. Oncogene, 24: 4597– 4603.

[79] Jeong KJ, Park SY, Seo JH, Lee KB, Choi WS, Han JW, Kang JK, Park CG, Kim YK, Lee HY. (2008). Lysophosphatidic acid receptor 2 and Gi/Src pathway mediate cell motility through cyclooxygenase 2 expression in CAOV-3 ovarian cancer cells. Exp. Mol. Med., 40: 607–616.

[80] Yun CC, Sun H, Wang D, Rusovici R, Castleberry A, Hall RA, Shim H. (2005). LPA_2 receptor mediates mitogenic signals in human colon cancer cells. Am. J. Physiol. Cell Physiol., 289: C2–C11.

[81] Lee CW, Rivera R, Gardell S, Dubin AE, Chun J. (2006). GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP. J. Biol. Chem., 281: 23589–23597.

[82] Kotarsky K, Boketoft A, BristulfJ J, Nilsson E, Norberg A, Hansson S, Owman C, Sillard R, Leeb-Lundberg LM, Olde B. (2006). Lysophosphatidic acid binds to and activates GPR92, a G protein-coupled receptor highly expressed in gastrointestinal lymphocytes. J. Pharmacol. Exp. Ther., 318: 619–628.

[83] Murph M, Tanaka T, Pang J, Felix E, Liu S, Trost R, Godwin AK, Newman R, Mills G. (2007). Liquid chromatography mass spectrometry for quantifying plasma lysophospholipids: potential biomarkers for cancer diagnosis. Methods Enzymol., 433: 1–25.

[84] Hosogaya S, Yatomi Y, Nakamura K, Ohkawa R, Okubo S, Yokota H, Ohta M, Yamazaki H, Koike T, Ozaki Y. (2008). Measurement of plasma lysophosphatidic acid concentration in healthy subjects: strong correlation with lysophospholipase D activity. Ann. Clin. Biochem., 45: 364–368.

[85] Bandoh K, Aoki J, Taira A, Tsujimoto M, Arai H, Inoue K. (2000). Lysophosphatidic acid (LPA) receptors of the EDG family are differentially activated by LPA species. Structure-activity relationship of cloned LPA receptors. FEBS Lett., 478: 159–165.

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