Effects of shokyo (*Zingiberis Rhizoma*) and kankyo (*Zingiberis Processum Rhizoma*) on prostaglandin E_2 production in lipopolysaccharide-treated mouse macrophage RAW264.7 cells

Toshiaki Ara [Corresp., 1], **Hiroyuki Kitamura** [2], **Norio Sogawa** [1]

[1] Department of Pharmacology, Matsumoto Dental University, Shiojiri, Nagano, Japan

[2] Matsumoto Dental University Hospital, Shiojiri, Nagano, Japan

Corresponding Author: Toshiaki Ara
Email address: toshiaki.ara@mdu.ac.jp

We previously reported that shokyo and kankyo, which are water-extracted fractions of ginger, reduced LPS-induced PGE_2 production in human gingival fibroblasts. In this study, we examined the effects of these herbs on LPS-treated mouse macrophage RAW264.7 cells. Both shokyo and kankyo reduced LPS-induced PGE_2 production in a concentration-dependent manner, but the effects of kankyo were stronger than those of shokyo. Shokyo and kankyo did not inhibit cyclooxygenase (COX) activity, nor did they alter the expression of molecules in the arachidonic acid cascade. In addition, these herbs did not alter NF-κB p65 or ERK phosphorylation. These results suggest that shokyo and kankyo inhibit $cPLA_2$ activity. Although 6-shogaol produced similar results to those of shokyo and kankyo, the concentration of 6-shogaol required for the reduction ofPGE_2 production were higher than those of 6-shogaol in shokyo and kankyo. Therefore, several gingerols and shogaols other than 6-shogaol may play a role in the reduction of LPS-induced PGE_2 production. Thus, 6-shogaol, and other gingerols and shogaols inhibit $cPLA_2$ activity and reduce LPS-induced PGE_2 production via a different mechanism from traditional anti-inflammatory drugs. Moreover, kampo medicines that contain shokyo or kankyo are considered to be effective for inflammatory diseases.

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Toshiaki Ara[1], Hiroyuki Kitamura[2], and Norio Sogawa[1]

[1]**Department of Pharmacology, Matsumoto Dental University, 1780 Gobara Hirooka, Shiojiri, Nagano 399-0781, Japan**
[2]**Matsumoto Dental University Hospital, 1780 Gobara Hirooka, Shiojiri, Nagano 399-0781, Japan**

Corresponding author:
Toshiaki Ara[1]

Email address: toshiaki.ara@mdu.ac.jp

ABSTRACT

We previously reported that shokyo and kankyo, which are water-extracted fractions of ginger, reduced LPS-induced PGE_2 production in human gingival fibroblasts. In this study, we examined the effects of these herbs on LPS-treated mouse macrophage RAW264.7 cells. Both shokyo and kankyo reduced LPS-induced PGE_2 production in a concentration-dependent manner, but the effects of kankyo were stronger than those of shokyo. Shokyo and kankyo did not inhibit cyclooxygenase (COX) activity, nor did they alter the expression of molecules in the arachidonic acid cascade. In addition, these herbs did not alter NF-κB p65 or ERK phosphorylation. These results suggest that shokyo and kankyo inhibit $cPLA_2$ activity. Although 6-shogaol produced similar results to those of shokyo and kankyo, the concentration of 6-shogaol required for the reduction of PGE_2 production were higher than those of 6-shogaol in shokyo and kankyo. Therefore, several gingerols and shogaols other than 6-shogaol may play a role in the reduction of LPS-induced PGE_2 production. Thus, 6-shogaol, and other gingerols and shogaols inhibit $cPLA_2$ activity and reduce LPS-induced PGE_2 production via a different mechanism from traditional anti-inflammatory drugs. Moreover, kampo medicines that contain shokyo or kankyo are considered to be effective for inflammatory diseases.

INTRODUCTION

Japanese traditional medicines (kampo medicines) are used for the treatment of several inflammatory diseases. We focused on the inflammatory effects of these kampo medicines, and found that lipopolysaccharide (LPS)-induced PGE_2 production by human gingival fibroblasts (HGFs) were reduced by several kampo medicines, including shosaikoto (TJ-9) (Ara et al., 2008), orento (TJ-120) (Ara et al., 2010), hangeshashinto (TJ-14) (Nakazono et al., 2010), kakkonto (TJ-1) (Kitamura et al., 2014), shinbuto (TJ-30), and ninjinto (TJ-32) (Ara and Sogawa, 2017). Moreover, among the herbs contained in kakkonto, shokyo (*Zingiberis Rhizoma*), kanzo (*Glycyrrhizae Radix*), keihi (*Cinnamomi Cortex*) (Ara and Sogawa, 2016), and kankyo (*Zingiberis Processum Rhizoma*) reduced PGE_2 production (Ara and Sogawa, 2017). In particular, shokyo and kankyo strongly reduced LPS-induced PGE_2 production. These results suggested that kampo medicines

that include shokyo or kankyo have anti-inflammatory effects for periodontal disease.

Both shokyo and kankyo are contained in almost all kampo medicines, and are the aqueous extracts of ginger (*Zingiber offinale* Roscoe). As described in the recent our review (Ara et al., 2018), shokyo is the powdered rhizome of ginger, and kankyo is the steamed and powdered rhizome of ginger. Many reports have demonstrated that ginger possesses anti-inflammatory effects as below. Ginger is clinically used as a treatment for rheumatoid arthritis, fever, emesis, nausea, and migraine headache (Afzal et al., 2001), and a systematic review revealed that the extracts of ginger are clinically effective as hypoanalgesic agents (Lakhan et al., 2015). In an animal model, oral- or intraperitoneal-administrated aqueous extract of ginger reduced the serum PGE_2 level in rats (Thomson et al., 2002). Moreover, the crude hydroalcoholic extract of ginger reduced LPS-induced PGE_2 serum level, and improved tracheal hyperreactivity and lung inflammation in rats (Aimbire et al., 2007). Furthermore, ethanol extract of ginger reduced the tissue level of PGE_2 and improved acetic acid-induced ulcerative colitis in rats (El-Abhar et al., 2008).

Although there are many reports on hydrophobic solvent-extracts of ginger, as described above, there are few reports on the effects of shokyo or kankyo (Thomson et al., 2002; Ara and Sogawa, 2016, 2017). Therefore, we examined the effects of shokyo and kankyo themselves on PGE_2 production and the arachidonic acid cascade in mouse macrophage RAW264.7 cells. We also investigated the effects of 6-shogaol at a concentration corresponding to that of these herbs.

MATERIALS AND METHODS

Reagents and cells

Powders of shokyo and kankyo were provided by Tsumura & Co. These powders were suspended in Dulbecco's modified Eagle's medium (D-MEM, Wako, Osaka, Japan) containing 10 % heat-inactivated fetal calf serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin (culture medium), and were rotated at 4 °C overnight. Then, the suspensions were centrifuged and the supernatants were filtered through a 0.45 µm pore membrane. Lipopolysaccharide (LPS) from *Porphyromonas gingivalis* 381 was provided by Professor Nobuhiro Hanada (School of Dental Medicine, Tsurumi University, Japan). Arachidonic acid and 6-shogaol were purchased from Cayman Chemical (Ann Arbor, MI). Other reagents were purchased from Nacalai Tesque.

The mouse macrophage cell line RAW264.7 (RIKEN BioResource Research Center, Tsukuba, Japan) was cultured in culture medium at 37 °C in a humidified atmosphere of 5 % CO_2.

Measurement of cell viability

The numbers of viable cells were measured using WST-8 (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. In brief, cells were seeded onto 96-well plates (AGC Techno Glass Co., Chiba, Japan) (50 000 cells/well), and treated with shokyo or kankyo for 24 h. Then, the media were removed by aspiration and the cells were treated with a 100 µl mixture of WST-8 with culture medium for 2 h at 37 °C in a CO_2 incubator.

The optical density was measured (measured wavelength at 450 nm and reference wavelength at 655 nm) using an iMark microplate reader (Bio-Rad, Hercules, CA), and the mean background value was subtracted from each value. Data are presented as mean ± SD (n = 4).

Measurement of prostaglandin E_2 (PGE_2)

RAW264.7 cells were seeded in 96-well plates (50 000 cells/well) and incubated in culture medium at 37 °C overnight. For simultaneous treatment, cells were treated with varying concentrations of each herb in the absence or presence of LPS (100 ng/ml) for 24 h (200 µl per well) in triplicate or quadruplicate for each sample. For sequential treatment, cells were treated with medium or LPS for 30 min, and thereafter treated with medium or each herb for 24 h. After

the culture supernatants were collected, viable cell numbers were measured using WST-8 as described above.

The concentrations of PGE_2 in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Cayman Chemical), and were adjusted by the number of viable cells. Data are presented as pg per 10000 cells (mean $\pm$ SD).

Measurement of cyclooxygenase (COX)-2 activity

COX-2 activity was evaluated as reported previously (Wilborn et al., 1995), with slight modification. In brief, to estimate COX-2 activity, RAW264.7 cells (50000 cells/well in 96-well plate) were treated with LPS and each herb for 6 h (simultaneous treatment) or LPS for 6 h and thereafter with each herb for 1 h (sequential treatment). Then, the cells were washed and incubated in culture medium containing exogenous arachidonic acid (10 μM) for 30 min. The concentrations of PGE_2 in the supernatants were measured by ELISA. Data are presented as pg per 10000 cells (mean $\pm$ SD).

Preparation of cell lysates

RAW264.7 cells were cultured in 60-mm dishes, and treated with combinations of LPS and herbs for the indicated times. Then, the cells were washed twice with Tris-buffered saline, transferred into microcentrifuge tubes, and centrifuged at 6000 $\times$ g for 5 min at 4 °C. Supernatants were aspirated and cells were lysed on ice in lysis buffer [50 mM Tris-HCl, pH 7.4, 1 % Nonidet P-40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM ethyleneglycol bis(2-aminoethylether)tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1/100 volume of protease inhibitor cocktail (Nacalai tesque), and 1/100 volume of phosphatase inhibitor cocktail (Nacalai tesque)] for 30 min at 4 °C. Samples were next centrifuged at 12000 $\times$ g for 15 min at 4 °C, and supernatants were collected. The protein concentration was measured using a BCA Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL).

Western blotting

The samples (50 μg of protein) were fractionated in a polyacrylamide gel under reducing conditions and transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P; GE Healthcare, Uppsala, Sweden). The membranes were blocked with 5 % ovalbumin for 1 h at room temperature and incubated with the primary antibody for an additional 1 h. The membranes were further incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized with an ECL kit (GE Healthcare). Densitometric values of each band were calculated using ImageJ software.

Antibodies against COX-2 (sc-1745, 1:500 dilution), cytosolic PLA_2 ($cPLA_2$) (sc-438, 1:200 dilution), LOX-5 (LO-5, sc-515821, 1:250 dilution), annexin 1 (sc-11387, 1:1000 dilution), actin (sc-1616, 1:1000 dilution), NF-κB p65 (sc-372, 1:1000 dilution), and phosphorylated p65 (Ser536) (p-NF-κB p65; sc-101752, 1:200 dilution) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against extracellular signal-regulated kinase (ERK; p44/42 MAP kinase antibody, 1:1000 dilution) and phosphorylated ERK [Phospho-p44/42 MAPK (Thr202/Tyr204) (E10) monoclonal antibody, 1:2000 dilution] were from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated anti-goat IgG (sc-2020, 1:20000 dilution) was from Santa Cruz, and anti-rabbit IgG (1:20000 dilution) and anti-mouse IgG (1:20000 dilution) were from DakoCytomation (Glostrup, Denmark).

Quantification of 6-shogaol in shokyo and kankyo

The quantification of 6-shogaol in each herb was performed by the Nagano Prefecture Pharmaceutical Association Analytical Examination Center (Nagano, Japan). In brief, 1 ml of samples

(herbs in culture medium) was absorbed to a reverse-phase system cartridge (SepPak tC18, Waters, Milford, MA). Columns were washed with 2 ml of 40 % MeOH and 1 ml of 70 % MeOH. Then, samples were eluted with 100 % MeOH and concentrated to 1 ml. These samples were subjected to high-performance liquid chromatography (HPLC) with LC-20A (SHIMADZU, Kyoto, Japan). The conditions were as follows: column, X-Bridge 2.1 $\times$ 150 mm, 3 µm (Waters); solvent, 70 % aqueous acetonitrile; flow rate, 0.15 min/ml; column oven, 40 °C; detection, 228 nm; and injection, 10 µl.

Statistical analysis

Differences between the control group and experimental groups were evaluated by a two-tailed Dunnett's test. All computations were performed with the statistical program R (R Development Core Team, 2018). Dunnett's test was performed using the 'glht' function in the 'multcomp' package (Hothorn et al., 2008). The IC_{50} value and its 95 % confidence interval (CI) were calculated using the 'drm' function in the 'drc' package (Ritz et al., 2015). Values with $P < 0.05$ were considered significantly different.

RESULTS

Effects of shokyo and kankyo on cell viability

We first examined the effects of shokyo and kankyo on RAW264.7 cell viability. Both shokyo and kankyo reduced the cell viability in a concentration-dependent manner (Figure 1A and 1B). These effects were significant but weak at 1000 µg/ml, demonstrating that shokyo and kankyo have low cytotoxicity. Therefore, concentrations up to 100 µg/ml of shokyo and kankyo were used in further experiments because we used the same concentration of herbs in previous studies (Ara and Sogawa, 2016, 2017).

Effects of shokyo and kankyo on prostaglandin E_2 (PGE_2) production

We next examined whether shokyo and kankyo affect the production of PGE_2 by RAW264.7 cells. The time schedule of treatment is shown in Figure 2A. In the simultaneous treatment experiment, RAW264.7 cells treated with 100 ng/ml of LPS produced PGE_2. Shokyo and kankyo (both 100 µg/ml) strongly reduced LPS-induced PGE_2 production (Figure 2B), and the effects of kankyo were stronger than those of kankyo.

To exclude the possibility that components in these herbs non-specifically bind the LPS receptor and inhibit LPS signaling, we performed a sequential treatment experiment. In this experiment, the cells were treated with LPS first, and the LPS receptor was not inhibited. The same results (Figure 2C) as in the simultaneous treatment experiment were obtained, suggesting that the reduction of PGE_2 production is due to non-specific binding of the LPS receptor by components in shokyo and kankyo. Therefore, we performed simultaneous treatment in the following experiments.

We investigated the concentration-dependent effects of shokyo and kankyo on LPS-induced PGE_2 production. Both herbs reduced LPS-induced PGE_2 production in a concentration-dependent manner (Figure 2D and 2E).

We also examined leukotriene B_4 (LTB_4) production, but we were unable to detect LTB_4 regardless of LPS treatment (data not shown).

Effects of kankyo and shokyo on the arachidonic acid cascade

To clarify the mechanism by which shokyo and kankyo reduce LPS-induced PGE_2 production more directly, we assessed the effects of these two herbs on the arachidonic acid cascade. First, we examined the effects of shokyo and kankyo on COX activity. In order to bypass PLA_2, we added exogenous arachidonic acid to RAW264.7 cells treated with LPS alone or LPS plus herbs

(simultaneous treatment experiment). Then, we measured the PGE_2 level produced by COX. Both shokyo and kankyo increased LPS-induced PGE_2 production (Figure 3B), suggesting that these two herbs increase COX-2 activity. Next, to exclude the effects of the change in COX-2 expression, we performed the sequential treatment experiment. As the cells were treated with LPS first in this experiment, COX-2 protein levels were considered to be comparable. Kankyo slightly increased LPS-induced PGE_2 production (Figure 3C). Based on these results, shokyo and kankyo do not inhibit COX-2 activity.

Next, we examined whether shokyo and kankyo affect the expression of molecules in the arachidonic acid cascade. Shokyo and kankyo slightly reduced $cPLA_2$ expression (Figure 3D). COX-2 was not expressed in the absence of LPS, and the treatment with LPS alone increased COX-2 expression. Shokyo and kankyo did not alter LPS-induced COX-2 expression (Figure 3D). Moreover, shokyo and kankyo did not alter annexin 1 expression (Figure 3D).

The expression of COX-2 is well known to be regulated by NF-κB. Therefore, we analyzed NF-κB activation by the level of phosphorylation of p65, a subunit of NF-κB. Phosphorylated p65 (p-p65) was not detected without LPS treatment, and treatment with LPS alone increased the p-p65 level. Pretreatment of shokyo or kankyo for 1 h did not alter LPS-induced p65 phosphorylation (Figure 3E), demonstrating that shokyo and kankyo did not inhibit NF-κB activity.

Moreover, we evaluated the effects of shokyo and kankyo on ERK phosphorylation. $cPLA_2$ is directly phosphorylated and activated by phosphorylated ERK (Lin et al., 1993; Gijón et al., 1999). Therefore, we examined whether shokyo and kankyo suppress LPS-induced ERK phosphorylation. LPS treatment increased ERK phosphorylation at 0.5 h and its phosphorylation was later attenuated. However, 100 μg/ml of shokyo or kankyo only slightly reduced LPS-induced ERK phosphorylation (Figure 3F).

We also evaluated the effects of shokyo and kankyo on the lipoxygenase pathway. Lipoxygenase (LOX)-5 expression was not altered by LPS treatment. Moreover, LOX-5 expression was not affected by shokyo or kankyo (Supplemental Figure 1A). Shokyo and kankyo did not change LOX activity because LTB_4 production was not altered when arachidonic acid was added (Supplemental Figure 1B and 1C). Furthermore, the LTB_4 level was lower than that of PGE_2 (Figure 3B and 3C).

Quantification of 6-shogaol in shokyo and kankyo

6-Shogaol is one of the major and bioactive components in shokyo and kankyo. In order to assess the effects of 6-shogaol on PGE_2 production by RAW264.7 cells at a similar concentration to shokyo and kankyo, we quantified the amount of 6-shogaol in shokyo and kankyo. HPLC analysis revealed that the 5 mg/ml shokyo and kankyo solutions used in this study contained 2.97 μM and 4.87 μM 6-shogaol, respectively (Table 1 and Supplemental Figure 2). Thus, 100 μg/ml of shokyo and kankyo contained 59.4 nM and 97.4 nM 6-shogaol, respectively (Table 1).

Effects of 6-shogaol on PGE_2 production and molecular expression in the arachidonic acid cascade

We investigated the effects of 6-shogaol on LPS-induced PGE_2 production by RAW264.7 cells. 6-Shogaol reduced LPS-induced PGE_2 production in a concentration-dependent manner (Figure 4A). One hundred nM 6-shogaol reduced PGE_2 production to approximately 50 %, and 1000 nM (= 1 μM) 6-shogaol inhibited PGE_2 production. The IC_{50} value of 6-shogaol was 105 nM (95 % CI: 27.7–182 nM). However, the effects of 6-shogaol were weaker than those of shokyo and kankyo considering the concentration of 6-shogaol in shokyo and kankyo (red and blue lines in Figure 4A).

The effects of 6-shogaol on the expression of molecules in the arachidonic acid cascade

and intracellular signal transduction pathways were evaluated, but 10 μM 6-shogaol did not affect the expression of $cPLA_2$, annexin 1, or COX-2 (Figure 4B). Similarly, 6-shogaol did not affect NF-κB p65 phosphorylation (Figure 4C). In contrast, 6-shogaol slightly reduced ERK phosphorylation (Figure 4D).

DISCUSSION

There are few reports on the effects of shokyo or kankyo, which are aqueous extracts of ginger. We previously examined the effects of shokyo and kankyo on the arachidonic acid cascade in HGFs, and suggested that these herbs inhibit $cPLA_2$ activity because they did not inhibit COX-2 activity or suppress $cPLA_2$ and COX-2 expression (Ara and Sogawa, 2016, 2017). In this study, we examined the effects of these herbs in macrophage-like RAW264.7 cells and obtained similar results (Figure 3B–3D). In addition, shokyo and kankyo did not alter annexin 1 (also named lipocortin1) expression (Figure 3D), which is produced by glucocorticoids and inhibits $cPLA_2$ activity (Gupta et al., 1984; Wallner et al., 1986). Moreover, shokyo and kankyo did not alter NF-κB phosphorylation (Figure 3E), which is required to induce COX-2 expression and ERK phosphorylation (Figure 3F), which leads to the activation of $cPLA_2$. These results suggest that shokyo and kankyo inhibit $cPLA_2$ activity in RAW264.7 cells and in HGFs, and their effects may be cell type-non-specific.

As a possible mechanism by which shokyo and kankyo reduced LPS-induced PGE_2 production, components of shokyo and kankyo may bind to LPS receptors on the cell surface and inhibit LPS signaling. However, even after the removal of LPS, shokyo and kankyo reduced LPS-induced PGE_2 production in the sequential treatment experiment (Figure 2C). If some components in these herbs either competitively or noncompetitively block LPS receptors or reduce PGE_2 production, LPS-induced NF-κB p65 and ERK phosphorylation should have been inhibited. However, shokyo and kankyo did not suppress LPS-induced NF-κB and ERK phosphorylation (Figure 3E and 3F). These results therefore excluded this hypothesis, and the target sites of shokyo and kankyo are present intracellularly.

Gingerols and shogaols are the major components of ginger (reviewed in Ara et al., 2018); therefore, shokyo and kankyo contain these components. With prolonged storage or heating of ginger, gingerols are dehydrated and converted to shogaols (Afzal et al., 2001). Because kankyo is subjected to heat processing, kankyo contains a larger amount of shogaols than shokyo. Among them, 6-shogaol is one of the bioactive components, and was reported to reduce PGE_2 production (Ara et al., 2018). Indeed, kankyo contained 1.7-times the amount of 6-shogaol as shokyo (approximately 60 nM and 100 nM 6-shogaol in 100 μg/ml of shokyo and kankyo, respectively). Moreover, the effects of kankyo on the reduction of PGE_2 production were greater than those of shokyo (Figure 2D and 2E). Therefore, this difference was considered to be due to the amount of 6-shogaol. However, because shokyo and kankyo are water-extracts of ginger, the amount and effects of gingerols and shogaols are thought to be lower than those of organic solvent-extracts such as methanol.

Next, we will discuss the effects of 6-shogaol on PGE_2 production. In this study, the IC_{50} value of 6-shogaol for PGE_2 production was approximately 100 nM (Figure 4A). This IC_{50} value is consistent with that in previous reports: approximately 100 nM in IL-1β-treated human oral keratinocytes (Kono et al., 2014) and approximately 60 μg/ml (= 217 nM) in LPS-treated U937 cells (Lantz et al., 2007). However, the IC_{50} value in this study was considered to be insufficient to inhibit the arachidonic acid cascade, as described below. The IC_{50} value of 6-shogaol for COX-2 activity is 2.1 μM in A549 cells (Tjendraputra et al., 2001). In another report, 6-shogaol did not inhibit COX-activity in a cell-free experimental model (van Breemen et al., 2011). Although we did not examine the effects of 6-shogaol on COX-2 activity, 100 nM 6-shogaol was considered to

not affect COX-2 activity because 100 µg/ml of kankyo, which contains approximately 100 nM 6-shogaol, did not inhibit COX-2 activity. Moreover, 0.17 µM 6-shogaol reduced COX-2 activity to approximately 70 % in IL-1β-treated human oral keratinocytes (Kono et al., 2014). The reported concentrations of 6-shogaol required for the reduction of COX-2 expression were higher than that in our study. The expression of COX-2 mRNA was reduced to approximately 70 % by 0.17 µM 6-shogaol (Kono et al., 2014). However, the expression of COX-2 protein was not affected by 1 µM 6-shogaol, was slightly reduced by 5 µM, and was significantly reduced by 10 µM in LPS-treated mouse microglial BV-2 cells (Ha et al., 2012). Therefore, our results that shokyo and kankyo did not inhibit COX activity are consistent with these previous reports. Similar results were observed in mouse skin (Kim et al., 2005). Similarly, a high concentration of 6-shogaol was reported to be required for the inhibition of NF-κB activation. PMA-induced NF-κB promoter activity was reduced to approximately 50 %, but p65 phosphorylation was not affected by 5 µM shogaol in human breast carcinoma cells (Ling et al., 2010). In this study, even 10 µM 6-shogaol did not affect $cPLA_2$ or COX-2 expression, or NF-κB phosphorylation (Figure 4B). Therefore, our results are consistent with these previous results. Our results suggested that 6-shogaol reduces LPS-induced PGE_2 production via the inhibition of $cPLA_2$ activity because the remaining and probable target site in the arachidonic acid cascade is $cPLA_2$

However, the effects of shokyo and kankyo cannot be accounted for by only 6-shogaol. Although 100 µg/ml of kankyo reduced LPS-induced PGE_2 production to approximately 10 % (Figure 2E), 100 nM 6-shogaol reduced it to approximately 50 % (Figure 4A). Therefore, some components other than 6-shogaol may be involved in the reduction of PGE_2 production. It was previously reported that 6-gingerol is the most abundant in hangeshashinto, which contains kankyo (Kono et al., 2014). Moreover, the amount of 6-shogaol is approximately half of that of 6-gingerol, and the amounts of 8- gingerol, 10-gingerol, 8-shogaol, and 10-shogaol are smaller (Kono et al., 2014). 6-Shogaol reduced PGE_2 production the most, followed by 6-gingerol, 8- and 10-gingerol, and slight reduction by 8- and 10-shogaol (Kono et al., 2014). Thus, the effects of 6-shogaol on the reduction of PGE_2 production are the strongest and those of other components are weak. Therefore, gingerols and shogaols may have additive effects on PGE_2 production. Next, we will discuss the effects of gingerols and shogaols on the arachidonic acid cascade. The IC_{50} values of these components for the inhibition of COX-2 activity are on the order of µM, similar to 6-shogaol (Tjendraputra et al., 2001; van Breemen et al., 2011). Moreover, 6-, 8-, and 10-gingerol reduced COX-2 expression at the µM order (Lantz et al., 2007). Among ginger extracts, 10 µM 10-gingerol, 6-shogaol, 8-shogaol, and 10-shogaol reduced $cPLA_2$ activity to approximately 50 % (Nievergelt et al., 2011). Although the concentrations of these components in shokyo and kankyo are lower than 10 µM, shokyo and kankyo may inhibit $cPLA_2$ activity by their additive effects.

We next evaluated the effects of shokyo and kankyo on the LOX pathway. However, RAW264.7 cells produced only a small amount of LTB_4 regardless of the presence of LPS. Moreover, shokyo and kankyo did not affect LOX-5 expression or LOX activity, suggesting that the LOX pathway is not active in macrophages, and that shokyo and kankyo do not inhibit the lipoxygenase pathway. Aspirin-induced asthma (AIA) is induced by the ingestion of acid nonsteroidal anti-inflammatory drugs (NSAIDs), and is considered to be caused by leukotrienes, which are increased by acid NSAIDs an contract the bronchus (Vaszar and Stevenson, 2001; Bochenek et al., 2002). Similarly, acid NSAIDs exacerbate general asthma. Based on our findings that (1) shokyo and kankyo inhibit upstream of the arachidonic acid cascade, (2) they did not inhibit the cyclooxygenase pathway, and (3) they did not enhance lipoxygenase pathways, shokyo and kankyo may reduce leukotriene production. Therefore, shokyo and kankyo may not exacerbate asthma, including AIA. As such, shokyo and kankyo may be safely used for patients with asthma, including AIA, instead of conventional anti-inflammatory drugs. Moreover, as

oral-administrated ginger protected aspirin-induced gastric ulcers in rats (Wang et al., 2011), shokyo and kankyo may be available as anti-inflammatory drugs instead of NSAIDs.

CONCLUSION

We demonstrated that shokyo and kankyo, which are water-extracted fractions of ginger, reduced LPS-induced PGE_2 production in RAW264.7 cells. Their mechanisms of action were suggested to via the inhibition of $cPLA_2$ activity because both herbs did not inhibit COX activity or suppress the expression of molecules in the arachidonic acid cascade.

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Table 1. Concentrations of 6-shogaol in shokyo and kankyo by HPLC analysis

	concentration of herb	
	5 mg/ml	100 μg/ml
shokyo	2.97 μM	59.4 μM
kankyo	4.87 μM	97.4 μM

Chromatography profiles are shown in Supplemental Figure 2

FIGURE LEGENDS

Figure 1

Cytotoxicity of shokyo (A) and kankyo (B). RAW264.7 cells were treated with each herb (0, 100, 300, or 1000 μg/ml) for 24 h. Then, the numbers of viable cells were measured by WST-8. *P*-values vs. without each herb were calculated by Dunnett's method. $^{*}P < 0.05$, $^{***}P < 0.001$.

Figure 2

Effects of shokyo and kankyo on PGE_2 production. (A) Time schedule of treatment with LPS and/or each herb. Simultaneous treatment: Cells were treated with combinations of LPS (0 or 100 ng/ml) and medium or each herb (100 μg/ml) for 24 h. Sequential treatment: Cells were treated with medium or LPS (100 ng/ml) for 30 min, washed, and further treated with medium or each herb (100 μg/ml) for 24 h. (B, C) Concentrations of PGE_2 were measured by ELISA, adjusted by cell number, and expressed as per 10000 cells (mean ± SD, n = 3) in simultaneous (B) and sequential (C) treatment experiments. med: medium, s: shokyo, k: kankyo. (D, E) Concentration-dependent effects of shokyo (D) and kankyo (E) on LPS-induced PGE_2 production. Cells were treated with combinations of LPS (100 ng/ml) and each herb (0, 1, 10, or 100 μg/ml) for 24 h. *P*-values vs. with LPS alone were calculated by Dunnett's method. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 3

Effects of shokyo and kankyo on the arachidonic acid cascade and intracellular signal transduction. (A) Time schedule of treatment with LPS and/or each herb. Simultaneous treatment: RAW264.7 cells were treated with LPS (100 ng/ml) and medium or each herb (100 μg/ml) for 6 h, washed, and then treated with 10 μM arachidonic acid for 30 min. Sequential treatment: RAW264.7 cells were treated with LPS (100 ng/ml) for 6 h, and further treated with medium or each herb (100 μg/ml) for 1 h. Then, the cells were washed and treated with 10 μM arachidonic acid for 30 min. (B, C) Effects of shokyo and kankyo on COX activity. Concentrations of PGE_2 were measured by ELISA, adjusted by cell number, and expressed as per 10000 cells (mean ± SD, n = 4) in simultaneous (B) and sequential (C) treatment experiments. *P*-values vs. with LPS alone by Dunnett's test are indicated. (D) Effects of herbs on $cPLA_2$, annexin 1, and COX-2 expression. RAW264.7 cells were treated with a combination of LPS (0 or 100 ng/ml) and medium or each herb (100 μg/ml) for 8 h, and protein levels were examined by Western blotting. (E, F) Effects of herbs on NF-κB and ERK phosphorylation. RAW264.7 cells were treated with each herb (100 μg/ml) for 1 h and further treated with LPS (100 ng/ml) for 15 min. NF-κB p65 and phosphorylated p65 levels (E), and ERK and phosphorylated ERK (pERK) levels (F) were examined by Western blotting. The upper band indicates ERK1 (p44 MAPK) and the lower band indicates ERK2 (p42 MAPK) in (F). med: medium, s: shokyo, k: kankyo.

Figure 4

Effects of 6-shogaol on LPS-induced PGE_2, the arachidonic acid cascade, and intracellular signal transduction. (A) Effects of 6-shogaol on LPS-induced PGE_2, RAW264.7 cells were treated with LPS (100 ng/ml) and each herb (100 μg/ml) for 24 h. Concentrations of PGE_2 were measured by ELISA, adjusted by cell number, and expressed as per 10000 cells (mean ± SD, n = 3). *P*-values vs. with LPS alone were calculated by Dunnett's method. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Red and blue lines represent the effects of shokyo and kankyo in Figure 2D and 2E, respectively. The concentrations of 6-shogaol were calculated using the results shown in Table 1. (B) Effects of 6-shogaol on $cPLA_2$, annexin 1, and COX-2 expression. RAW264.7 cells were treated with a combination of LPS (0 or 100 ng/ml) and 6-shogaol (0, 0.1, 1, or 10 μM) for 8 h, and protein levels were examined by Western blotting. (C, D) Effects of 6-shogaol on NF-κB and ERK phosphorylation. RAW264.7 cells were treated with 6-shogaol (0, 0.1, 1, or 10 μM) for 1 h and further treated with LPS (100 ng/ml) for 15 min. NF-κB p65/phosphorylated p65 levels (C), and ERK/phosphorylated ERK (pERK) levels (D) were examined by Western blotting.

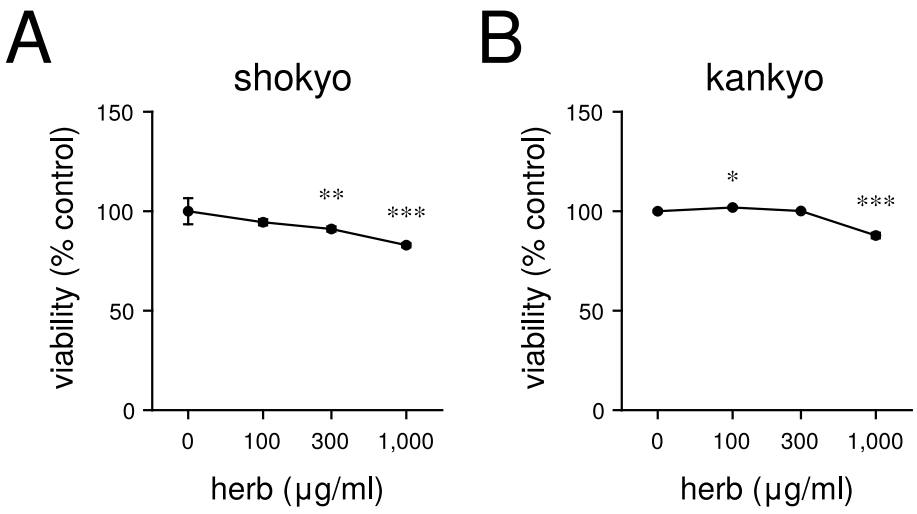

Figure 1. Cytotoxicity of shokyo and kankyo

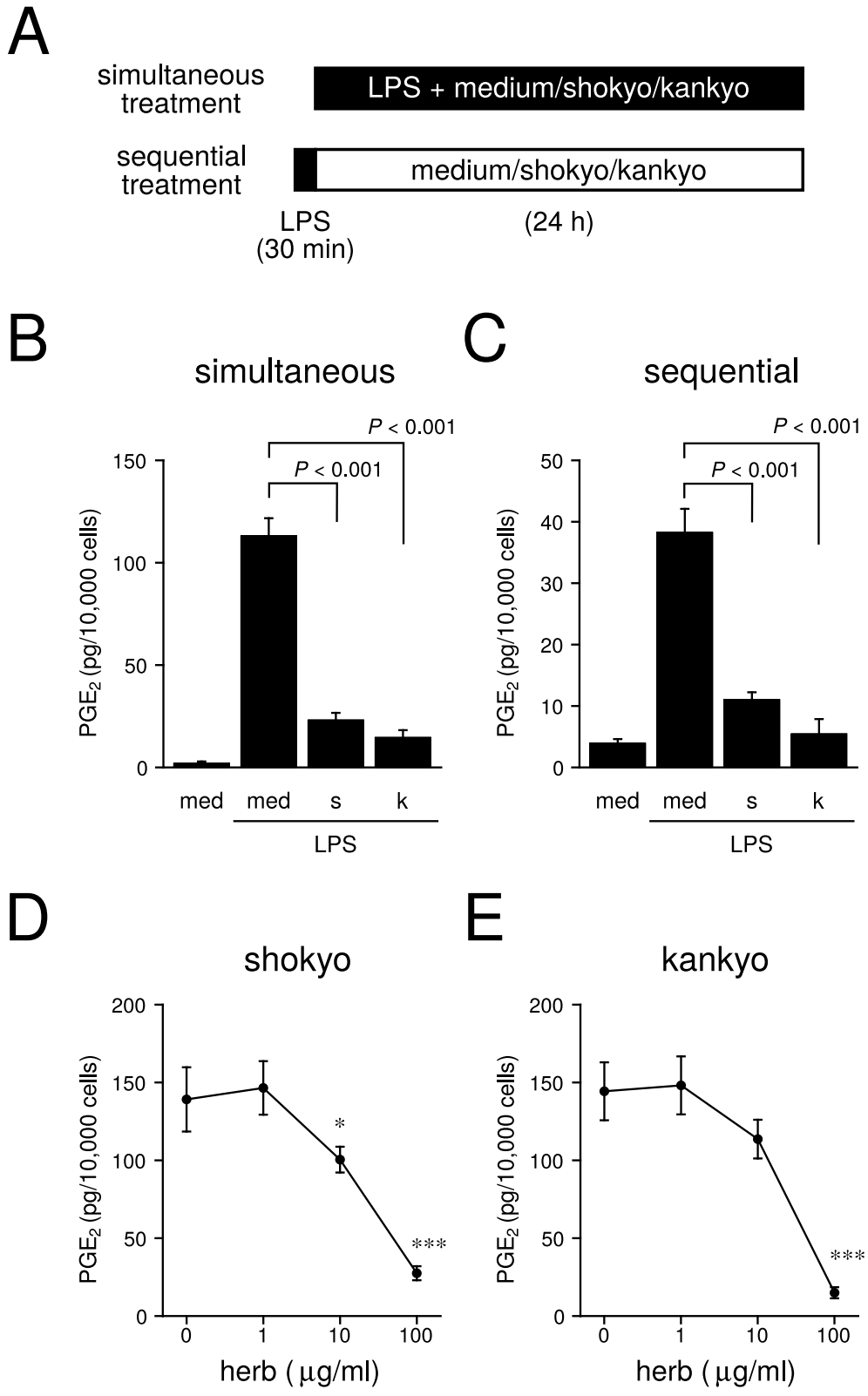

Figure 2. Effects of shokyo and kankyo on PGE_2 production

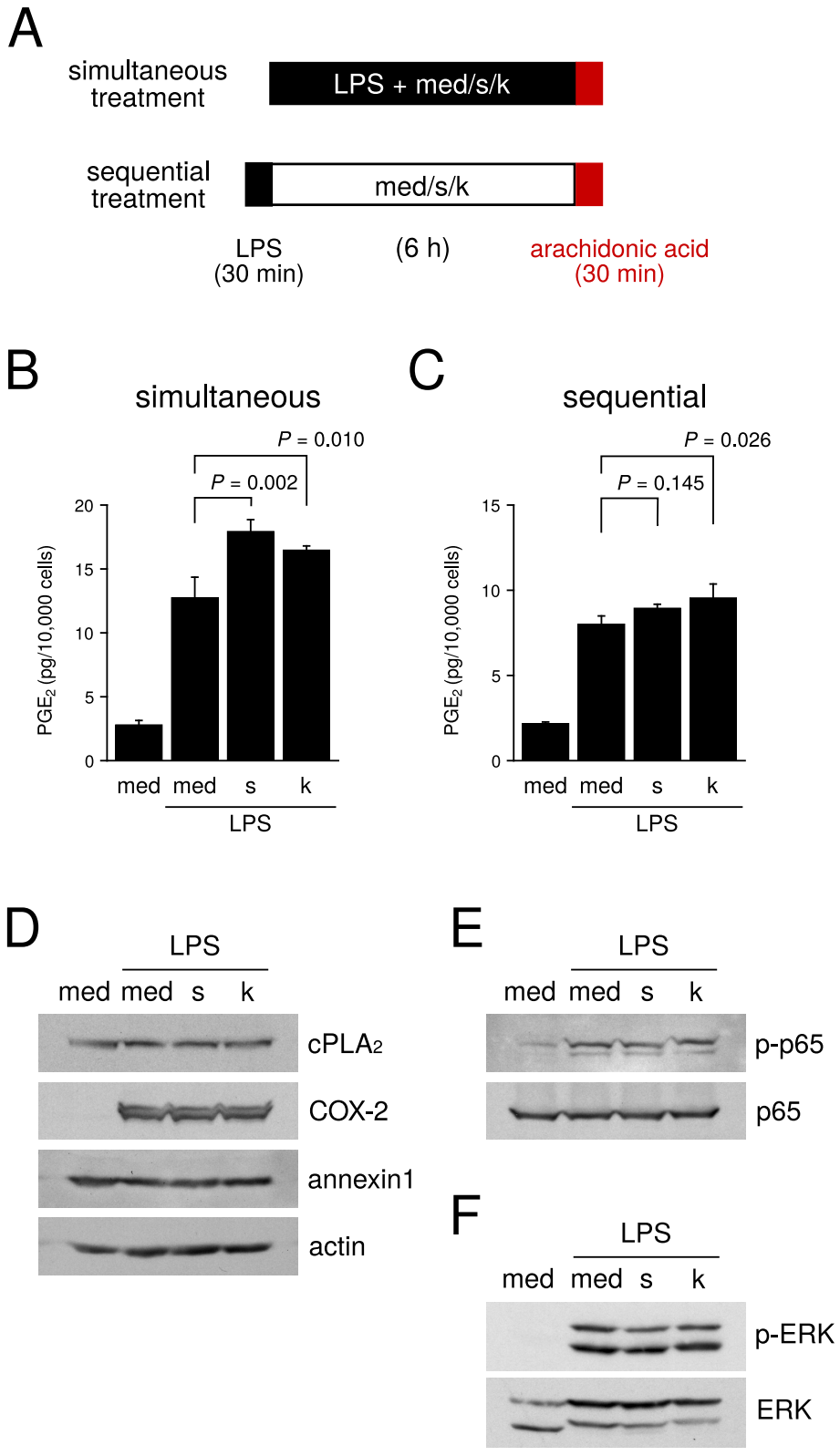

Figure 3. Effects of shokyo and kankyo on the arachidonic acid cascade and intracellular signal transduction

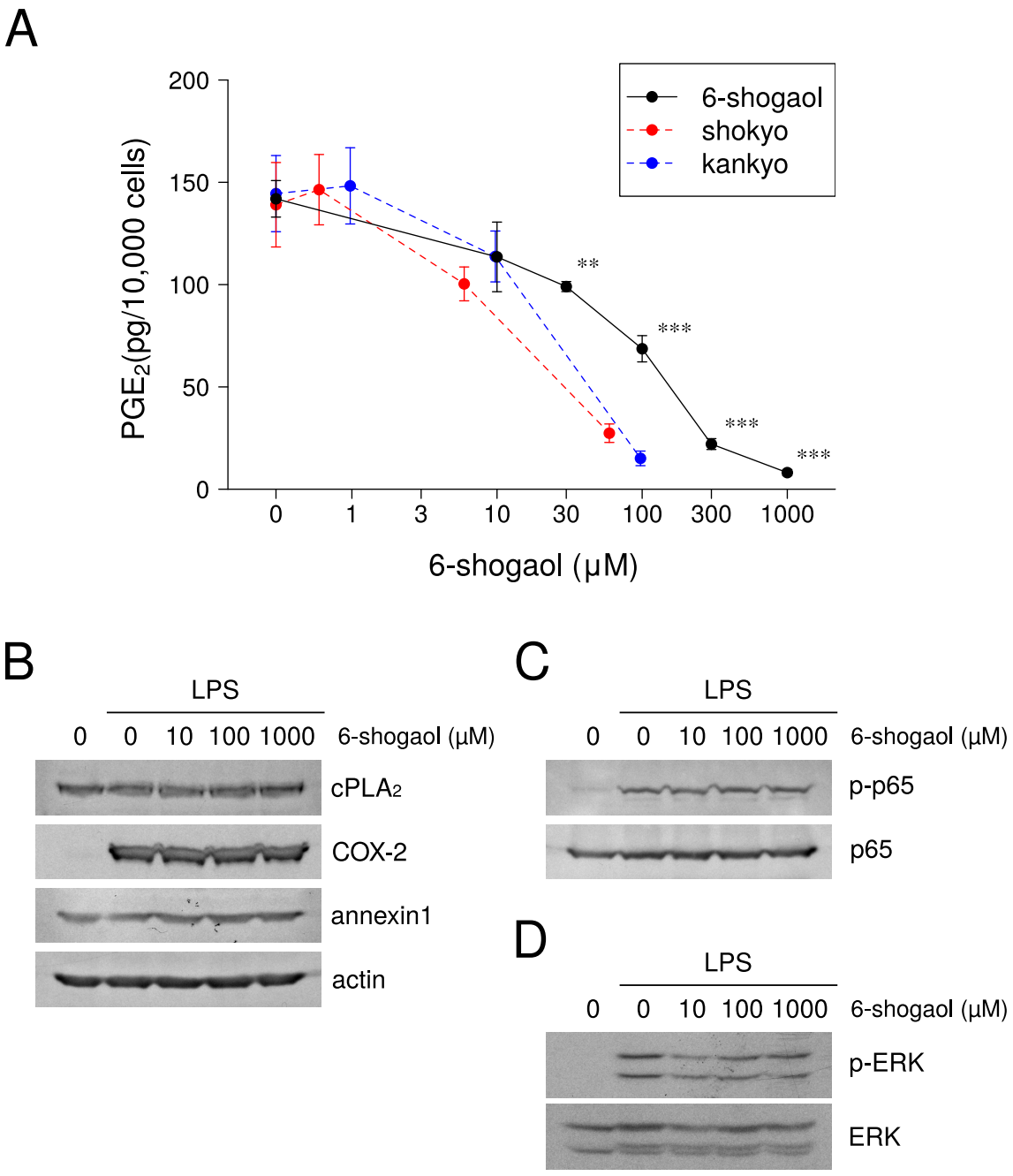

Figure 4. Effects of 6-shogaol on LPS-induced PGE_2, the arachidonic acid cascade, and intracellular signal transduction