

Effects of shokyo (*Zingiberis Rhizoma*) and kankyo (*Zingiberis Processum Rhizoma*) on prostaglandin E₂ production in lipopolysaccharide-treated mouse macrophage RAW264.7 cells

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We previously reported that shokyo and kankyo, which are water-extracted fractions of ginger, reduced LPS-induced PGE₂ 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 PGE₂ production in a concentration-dependent manner. 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 translocation into nucleus, or phosphorylation of p65 or ERK. These results suggest that shokyo and kankyo inhibit cPLA₂ activity. Although 6-shogaol produced similar results to those of shokyo and kankyo, the concentration of 6-shogaol required for the reduction of PGE₂ 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 PGE₂ production. Thus, 6-shogaol, and other gingerols and shogaols inhibit cPLA₂ activity and reduce LPS-induced PGE₂ 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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ABSTRACT

We previously reported that shokyo and kankyo, which are water-extracted fractions of ginger, reduced LPS-induced PGE₂ 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 PGE₂ production in a concentration-dependent manner. 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- κ B p65 translocation into nucleus, or phosphorylation of p65 or ERK. These results suggest that shokyo and kankyo inhibit cPLA₂ activity. Although 6-shogaol produced similar results to those of shokyo and kankyo, the concentration of 6-shogaol required for the reduction of PGE₂ 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 PGE₂ production. Thus, 6-shogaol, and other gingerols and shogaols inhibit cPLA₂ activity and reduce LPS-induced PGE₂ 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 PGE₂ 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 PGE₂ production (Ara and Sogawa, 2017). In particular, shokyo and kankyo strongly reduced LPS-induced PGE₂ 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 officinale* 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, orally- or intraperitoneal-administrated aqueous extract of ginger reduced the serum PGE₂ level in rats (Thomson et al., 2002). Moreover, the crude hydroalcoholic extract of ginger reduced LPS-induced PGE₂ 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 PGE₂ and improved acetic acid-induced ulcerative colitis in rats (El-Abhar et al., 2008).

In recent review (Alsherbiny et al., 2019), the effects of the aqueous extract of ginger are summarized. For example, the aqueous extract of ginger reduced ultraviolet B-induced inflammatory cytokines production in human keratinocyte HaCaT cells and mice (Guahk et al., 2010) and LPS-induced inflammatory cytokines in mice (Choi et al., 2013). Moreover, the aqueous extract of ginger has the protective effects against various organs such as liver, kidney, neuron, and heart in mice and rats. However, there are few reports on the effects on PGE₂ production and the arachidonic acid cascade (Thomson et al., 2002; Ara and Sogawa, 2016, 2017). Therefore, we examined the effects of shokyo and kankyo themselves on PGE₂ 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. 3-D HPLC profiles of shokyo and kankyo were shown in Supplemental Figure 1. 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). NF-κB inhibitor, BAY 11-7082, was purchased from Wako. Mitogen-activated protein kinase kinase (MAPKK/MEK) inhibitor, PD98059, were purchased from Sigma (St. Louis, MO). 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 % CO₂.

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) (50000 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 CO₂ incubator.

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

91 **Measurement of prostaglandin E₂ (PGE₂)**

92 RAW264.7 cells were seeded in 96-well plates (50000 cells/well) and incubated in culture
93 medium at 37 °C overnight. For simultaneous treatment, cells were treated with varying concen-
94 trations of each herb in the absence or presence of LPS (100 ng/ml) for 24 h (200 μ l per well)
95 in triplicate or quadruplicate for each sample. For sequential treatment, cells were treated with
96 medium or LPS for 30 min, and thereafter treated with medium or each herb for 24 h. After
97 the culture supernatants were collected, viable cell numbers were measured using WST-8 as
98 described above.

99 The concentrations of PGE₂ in the culture supernatants were measured by enzyme-linked
100 immunosorbent assay (ELISA) according to the manufacturer's instructions (Cayman Chemical),
101 and were adjusted by the number of viable cells. Data are presented as pg per 10000 cells (mean
102 \pm SD).

103 **Measurement of cytosolic phospholipase A₂ (cPLA₂) activity**

104 cPLA₂ activity was evaluated using cPLA₂ Assay kit (Cayman Chemical) according to the
105 manufacturer's instructions. RAW264.7 cells were cultured in 100-mm dishes, and treated
106 with 100 ng/ml of PgLPS for 2 h. Then, the cells were washed twice with Tris-buffered saline
107 (TBS), transferred into microcentrifuge tubes, and centrifuged at 6000 \times g for 5 min at 4 °C.
108 Supernatants were aspirated, and cells were resuspended in TBS with 1/100 volume of protease
109 inhibitor cocktail (Nacalai tesque) and 1/100 volume of phosphatase inhibitor cocktail (Nacalai
110 tesque), and were homogenized with Dounce tissue grinder (Sansyo, Tokyo, Japan). Then, cells
111 were centrifuged at 12000 \times g for 15 min at 4 °C. The supernatant was collected and used
112 as sample. To detect only cPLA₂ activity, samples were pretreated with 20 μ M of bromoenol
113 lactone [calcium-independent PLA₂ (iPLA₂)-specific inhibitor, Cayman Chemical] and 20 μ M
114 of thioetheramide-PC [secretory PLA₂ (sPLA₂)-specific inhibitor, Cayman Chemical] for room
115 temperature for 15 min. Bee venom PLA₂ was used as positive control.

116 **Measurement of cyclooxygenase (COX)-2 activity**

117 COX-2 activity was indirectly evaluated as reported previously (Wilborn et al., 1995), with
118 slight modification. In brief, to estimate COX-2 activity, RAW264.7 cells (50000 cells/well in
119 96-well plate) were treated with LPS and each herb for 6 h (simultaneous treatment) or LPS for
120 6 h and thereafter with each herb for 1 h (sequential treatment). Then, the cells were washed and
121 incubated in culture medium containing exogenous arachidonic acid (10 μ M) for 30 min. The
122 concentrations of PGE₂ in the supernatants were measured by ELISA. Data are presented as
123 100 % at LPS alone (mean \pm SD).

124 **Preparation of cell lysates**

125 RAW264.7 cells were cultured in 60-mm dishes, and treated with combinations of LPS and
126 herbs for the indicated times. Then, the cells were washed twice with TBS, transferred into mi-
127 crocentrifuge tubes, and centrifuged at 6000 \times g for 5 min at 4 °C. Supernatants were aspirated
128 and cells were lysed on ice in lysis buffer [50 mM Tris-HCl, pH 7.4, 1 % Nonidet P-40, 0.25 %
129 sodium deoxycholate, 150 mM NaCl, 1 mM ethyleneglycol bis(2-aminoethylether)tetraacetic
130 acid (EGTA), 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1/100 volume of protease
131 inhibitor cocktail, and 1/100 volume of phosphatase inhibitor cocktail] for 30 min at 4 °C. Sam-
132 ples 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). Protein levels were quantified using image analysis software ImageJ (National Institutes of Health [NIH], Bethesda, MD).

Antibodies against COX-2 (sc-1745, 1:1 000 dilution), cPLA₂ (sc-438, 1:500 dilution), LOX-5 (LO-5, sc-515821, 1:250 dilution), annexin 1 (sc-11387, 1:1 000 dilution), actin (sc-1616, 1:1 000 dilution), NF-κB p65 (sc-372, 1:1 000 dilution), and phosphorylated p65 (Ser536) (p-NF-κB p65; sc-101752, 1:500 dilution) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against extracellular signal-regulated kinase (ERK; p44/42 MAP kinase antibody, 1:1 000 dilution) and phosphorylated ERK [Phospho-p44/42 MAPK (Thr202/Tyr204) (E10) monoclonal antibody, 1:2 000 dilution] were from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated anti-goat IgG (sc-2020, 1:50 000 dilution) was from Santa Cruz, and anti-rabbit IgG (1:50 000 dilution) and anti-mouse IgG (1:50 000 dilution) were from DakoCytomation (Glostrup, Denmark).

Immunofluorescence staining

To detect p65 localization RAW264.7 cells were directly cultured on Lab-Tek chamber slides (16 well, Thermo Fisher Scientific, Waltham, MA, USA) overnight. The cells were treated with shokyo, kankyo (100 µg/ml), 6-shogaol (0, 0.1, 1, or 10 µM), or BAY 11-7082 (10 µM) for 2 h, and further treated with LPS (100 ng/ml) and shokyo, kankyo, 6-shogaol, or BAY 11-7082 for 30 min. Then, the cells were fixed with ice-cold methanol for 30 min at −20 °C. Subsequently, the cells were washed with PBS for 3 times, blocked with 1 % BSA in PBS for 1 h, incubated with anti-p65 antibody (1:50 dilution) for 1 h at room temperature. After washing with PBS for 3 times, cells were incubated for 1 h at room temperature with Alexa Fluor488-conjugated secondary antibody (Thermo Fisher Scientific, 1:500 dilution). After washing with PBS for 3 times, cells were mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Fluorescence was visualized using Axioplan2 imaging (Carl Zeiss, Inc., Oberkochen, Deutschland).

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 × 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.

177 **Statistical analysis**

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

184 **RESULTS**

185 **Effects of shokyo and kankyo on cell viability**

186 We first examined the effects of shokyo and kankyo on RAW264.7 cell viability. Both shokyo
187 and kankyo reduced the cell viability in a concentration-dependent manner (Figure 1A and 1B).
188 However, the cell viability is hardly affected at 100 $\mu\text{g/ml}$ of shokyo and kankyo, and is slightly
189 reduced at 1000 $\mu\text{g/ml}$. Therefore, concentrations up to 100 $\mu\text{g/ml}$ of shokyo and kankyo were
190 used in further experiments because we used the same concentration of herbs in previous studies
191 (Ara and Sogawa, 2016, 2017).

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

193 We next examined whether shokyo and kankyo affect the production of PGE_2 by RAW264.7 cells.
194 The time schedule of treatment is shown in Figure 2A. In the simultaneous treatment experiment,
195 RAW264.7 cells treated with 100 ng/ml of LPS produced PGE_2 . Shokyo and kankyo (both
196 100 $\mu\text{g/ml}$) strongly reduced LPS-induced PGE_2 production (Figure 2B).

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

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

207 **Effects of kankyo and shokyo on the arachidonic acid cascade**

208 To clarify the mechanism by which shokyo and kankyo reduce LPS-induced PGE_2 production
209 more directly, we assessed the effects of these two herbs on the arachidonic acid cascade. First,
210 we examined cPLA₂ activity using the homogenate of RAW264.7 cells. However, we did not
211 detect cPLA₂ activity because the activity was background level (data not shown). Next, we
212 examined the effects of shokyo and kankyo on COX activity. In order to bypass PLA₂, we
213 added exogenous arachidonic acid to RAW264.7 cells treated with LPS alone or LPS plus herbs
214 (simultaneous treatment experiment). Then, we measured the PGE_2 level produced by COX.
215 Both shokyo and kankyo increased LPS-induced PGE_2 production (Figure 3B), suggesting that
216 these two herbs increase COX-2 activity. Next, to exclude the effects of the change in COX-2
217 expression, we performed the sequential treatment experiment. As the cells were treated with
218 LPS first in this experiment, COX-2 protein levels were considered to be comparable. Kankyo
219 slightly increased COX-2 activity (Figure 3C). Based on these results, shokyo and kankyo do not
220 inhibit COX-2 activity.

221 Next, we examined whether shokyo and kankyo affect the expression of molecules in the
222 arachidonic acid cascade. Shokyo and kankyo slightly reduced cPLA₂ 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- κ B. Therefore, we analyzed NF- κ B activation by the translocation of p65, a subunit of NF- κ B, into nucleus. p65 was localized at cytoplasm in untreated RAW264.7 cells. When RAW264.7 cells were treated with LPS, p65 was mainly localized at nucleus although p65 was present in cytoplasm (Figure 4A). Shokyo and kankyo did not affect the p65 translocation into nucleus (Figure 4A). In addition, we analyzed NF- κ B activation by the level of phosphorylation of p65. 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 4B), demonstrating that shokyo and kankyo did not inhibit NF- κ B activity.

Moreover, we evaluated the effects of shokyo and kankyo on ERK phosphorylation. cPLA₂ 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 μ g/ml of shokyo or kankyo only slightly reduced LPS-induced ERK phosphorylation (Figure 4C).

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 2A). Shokyo and kankyo did not change LOX activity because LTB₄ production was not altered when arachidonic acid was added (Supplemental Figure 2B and 2C). Furthermore, the LTB₄ level was lower than that of PGE₂ (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 PGE₂ 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 μ M and 4.87 μ M 6-shogaol, respectively (Table 1 and Supplemental Figure 3). Thus, 100 μ g/ml of shokyo and kankyo contained 59.4 nM and 97.4 nM 6-shogaol, respectively (Table 1).

Effects of 6-shogaol on PGE₂ production and molecular expression in the arachidonic acid cascade

We investigated the effects of 6-shogaol on LPS-induced PGE₂ production by RAW264.7 cells. 6-Shogaol reduced LPS-induced PGE₂ production in a concentration-dependent manner (Figure 4A). One hundred nM 6-shogaol reduced PGE₂ production to approximately 50 %, and 1000 nM (= 1 μ M) 6-shogaol inhibited PGE₂ production. The IC₅₀ value of 6-shogaol was 105 nM (95 % CI: 27.7–182 nM). However, comparing with the calculated 6-shogaol concentration in shokyo and kankyo based on the results in Table 1, the effects of 6-shogaol on PGE₂ production were weaker than those of shokyo and kankyo (red and blue lines in Figure 5A, respectively).

The effects of 6-shogaol on the expression of molecules in the arachidonic acid cascade and intracellular signal transduction pathways were evaluated, but 10 μ M 6-shogaol did not affect the expression of cPLA₂, annexin 1, or COX-2 (Figure 5B). Moreover, 6-shogaol did not affect LPS-induced p65 translocation into nucleus (Figure 6A), or p65 phosphorylation (Figure 6B). Furthermore, 6-shogaol did not alter ERK phosphorylation (Figure 6C).

DISCUSSION

There are few reports on the effects of shokyo or kankyo, which are aqueous extracts of ginger, on the arachidonic acid cascade. We previously examined the effects of shokyo and kankyo on the arachidonic acid cascade in HGFs, and suggested that these herbs inhibit cPLA₂ activity because they did not inhibit COX-2 activity or suppress cPLA₂ 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 cPLA₂ activity (Gupta et al., 1984; Wallner et al., 1986). Moreover, shokyo and kankyo did not alter NF- κ B p65 translocation into nucleus (Figure 4A), p65 phosphorylation (Figure 4B), or ERK phosphorylation (Figure 4C). Because NF- κ B activation is required to induce COX-2 expression, our results that shokyo and kankyo did not alter COX-2 expression (Figure 3D) are consistent with those in NF- κ B activation. In addition, because ERK phosphorylation is required to activate cPLA₂, our results suggest that shokyo and kankyo did not alter cPLA₂ activation. Unfortunately, we could not directly evaluate the effects of shokyo, kankyo, and 6-shogaol on cPLA₂ activity in this study, because cPLA₂ activity of RAW264.7 cells were not detected. However, these results suggest that shokyo and kankyo inhibit cPLA₂ activity in RAW264.7 cells and in HGFs, and their effects may be cell type-nonspecific.

As a possible mechanism by which shokyo and kankyo reduced LPS-induced PGE₂ 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 PGE₂ production in the sequential treatment experiment (Figure 2C). If some components in these herbs either competitively or noncompetitively block LPS receptors or reduce PGE₂ production, LPS-induced NF- κ B p65 and ERK phosphorylation should have been inhibited. However, shokyo and kankyo did not suppress LPS-induced NF- κ B and ERK phosphorylation (Figures 4B and 4C). 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 as shown in Supplemental Figure 1. Among them, 6-shogaol is one of the bioactive components, and was reported to reduce PGE₂ 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 μ g/ml of shokyo and kankyo, respectively). 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 PGE₂ production. In this study, the IC₅₀ value of 6-shogaol for PGE₂ production was approximately 100 nM (Figure 5A). This IC₅₀ value is consistent with that in previous reports: approximately 100 nM in IL-1 β -treated human oral keratinocytes (Kono et al., 2014) and approximately 60 μ g/ml (= 217 nM) in LPS-treated U937 cells (Lantz et al., 2007). However, the IC₅₀ value in this study was considered to be insufficient to inhibit the arachidonic acid cascade, as described below. The IC₅₀ value of 6-shogaol for COX-2 activity is 2.1 μ M 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 μ g/ml of kankyo, which contains approximately 100 nM

6-shogaol, did not inhibit COX-2 activity. Moreover, 0.17 μ M 6-shogaol reduced COX-2 activity to approximately 70 % in IL-1 β -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 μ M 6-shogaol (Kono et al., 2014). However, the expression of COX-2 protein was not affected by 1 μ M 6-shogaol, was slightly reduced by 5 μ M, and was significantly reduced by 10 μ M 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- κ B activation. PMA-induced NF- κ B promoter activity was reduced to approximately 50 %, but p65 phosphorylation was not affected by 5 μ M shogaol in human breast carcinoma cells (Ling et al., 2010). In this study, even 10 μ M 6-shogaol did not affect cPLA₂ or COX-2 expression, p65 translocation to nucleus (Figure 6A), or p65 phosphorylation (Figure 6B) Therefore, our results are consistent with these previous results. Our results suggested that 6-shogaol reduces LPS-induced PGE₂ production via the inhibition of cPLA₂ activity because the remaining and probable target site in the arachidonic acid cascade is cPLA₂

However, the effects of shokyo and kankyo cannot be accounted for by only 6-shogaol. Although 100 μ g/ml of kankyo reduced LPS-induced PGE₂ production to approximately 10 % (Figure 2E), 100 nM 6-shogaol reduced it to approximately 50 % (Figure 5A). Therefore, some components other than 6-shogaol may be involved in the reduction of PGE₂ 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 PGE₂ 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 PGE₂ production are the strongest and those of other components are weak. Therefore, gingerols and shogaols may have additive effects on PGE₂ production. Next, we will discuss the effects of gingerols and shogaols on the arachidonic acid cascade. The IC₅₀ values of these components for the inhibition of COX-2 activity are on the order of μ M, 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 μ M order (Lantz et al., 2007). Among ginger extracts, 10 μ M 10-gingerol, 6-shogaol, 8-shogaol, and 10-shogaol reduced cPLA₂ activity to approximately 50 % (Nievergelt et al., 2011). Although the concentrations of these components in shokyo and kankyo are lower than 10 μ M, shokyo and kankyo may inhibit cPLA₂ 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 LTB₄ 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.

Next, we will discuss about protective effect on gastric ulcer. In general, ginger reduced PGE₂ production in macrophages and inflammatory site. In contrast, orally administered cuttlebone complex (CBC), which includes fresh ginger roots, demonstrated a protective potentiality against indomethacin-induced gastric ulcer in rats via increment of the indomethacin-declined PGE₂ levels in the stomach (Chien et al., 2015). Therefore, ginger has opposite effects between inflammatory cells and gastric mucosal cells. Moreover, ginger powder protected aspirin-induced gastric ulcers in rat (Wang et al., 2011). These results suggest that ginger has both anti-inflammatory effect and protective effect on gastric ulcers in contrast to acid NSAIDs.

There are several limitations in this study. We evaluated only cPLA₂ among PLA₂ isotypes. Therefore, in the future, the evaluation of the effects of shokyo and kankyo on iPLA₂ and sPLA₂ — expressions and activities — will be needed. Moreover, the evaluation of the anti-inflammatory effects of shokyo and kankyo on the inflammatory diseases such as periodontal disease or stomatitis by animal models will be needed.

CONCLUSION

We demonstrated that shokyo and kankyo, which are water-extracted fractions of ginger, reduced LPS-induced PGE₂ production in RAW264.7 cells. Their mechanisms of action were suggested to via the inhibition of cPLA₂ 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 nM
kankyo	4.87 µM	97.4 nM

Chromatography profiles are shown in Supplemental Figure 3

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 PGE₂ 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 PGE₂ were measured by ELISA, adjusted by cell number, and expressed as 100 % at LPS alone (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 PGE₂ 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. (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 PGE₂ 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 cPLA₂, annexin 1, and COX-2 expression. RAW264.7 cells were treated with a combination of LPS (0 or 100 ng/ml) and medium, each herb (100 µg/ml), or dexamethasone (100 nM) for 8 h, and protein levels were examined by Western blotting. med: medium, s: shokyo, k: kankyo, and Dex: dexamethasone. The band densities were normalized against LPS alone and actin. The values were indicated below each band.

Figure 4

Effects of shokyo and kankyo on the intracellular signal transduction. (A) Effects of herbs on NF-κB p65 translocation into nucleus. RAW264.7 cells were treated with each herb (100 µg/ml), or BAY 11-7082 (10 µM) for 2 h, and further treated with LPS (100 ng/ml), and 6-shogaol or BAY

11-7082 for 30 min. The cellular localization of p65 was determined by immunofluorescence analysis. Nuclei of the corresponding cells were visualized with DAPI, and observed at $400 \times$ magnification. The bar represents $20 \mu\text{m}$. (B, C) Effects of herbs on NF- κ B p65 and ERK phosphorylation. RAW264.7 cells were treated with each herb ($100 \mu\text{g}/\text{ml}$), BAY 11-7082 ($10 \mu\text{M}$), or PD98059 ($20 \mu\text{M}$) for 1 h, and further treated with LPS ($100 \text{ ng}/\text{ml}$) and each herb, BAY 11-7082, or PD98059 for 15 min. p65 and phosphorylated p65 levels (B), and ERK and phosphorylated ERK (pERK) levels (C) were examined by Western blotting. The upper band indicates ERK1 (p44 MAPK) and the lower band indicates ERK2 (p42 MAPK) in (C). med: medium, s: shokyo, k: kankyo, BAY: BAY 11-7082, and PD: PD98059. The band densities were normalized against LPS alone, and p65 or ERK. The values were indicated below each band.

Figure 5

Effects of 6-shogaol on LPS-induced PGE_2 production, and the arachidonic acid cascade. (A) Effects of 6-shogaol on LPS-induced PGE_2 . RAW264.7 cells were treated with LPS ($100 \text{ ng}/\text{ml}$) and 6-shogaol (0 to 1000 nM) for 24 h. Concentrations of PGE_2 were measured by ELISA, adjusted by cell number, and expressed as per 10000 cells (mean \pm 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 in shokyo and kankyo 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 $100 \text{ ng}/\text{ml}$) and 6-shogaol (0, 0.1, 1, or $10 \mu\text{M}$), or dexamethasone (100 nM) for 8 h, and protein levels were examined by Western blotting. The band densities were normalized against LPS alone and actin. The values were indicated below each band.

Figure 6

Effects of 6-shogaol on the intracellular signal transduction. (A) Effects of 6-shogaol on NF- κ B p65 translocation into nucleus. RAW264.7 cells were treated with 6-shogaol (0, 0.1, 1, or $10 \mu\text{M}$), or BAY 11-7082 ($10 \mu\text{M}$) for 2 h, and further treated with LPS ($100 \text{ ng}/\text{ml}$), and 6-shogaol or BAY 11-7082 for 30 min. The cellular localization of p65 was determined by immunofluorescence analysis. Nuclei of the corresponding cells were visualized with DAPI, and observed at $400 \times$ magnification. The bar represents $20 \mu\text{m}$. (B, C) Effects of 6-shogaol on NF- κ B p65 and ERK phosphorylation. RAW264.7 cells were treated with 6-shogaol (0, 0.1, 1, or $10 \mu\text{M}$), BAY 11-7082 ($10 \mu\text{M}$), or PD98059 ($20 \mu\text{M}$) for 1 h and further treated with LPS ($100 \text{ ng}/\text{ml}$), and 6-shogaol, BAY 11-7082, or PD98059 for 15 min. p65 and phosphorylated p65 levels (B), and ERK and phosphorylated ERK (pERK) levels (C) were examined by Western blotting. BAY: BAY 11-7082, and PD: PD98059. The band densities were normalized against LPS alone, and p65 or ERK. The values were indicated below each band.

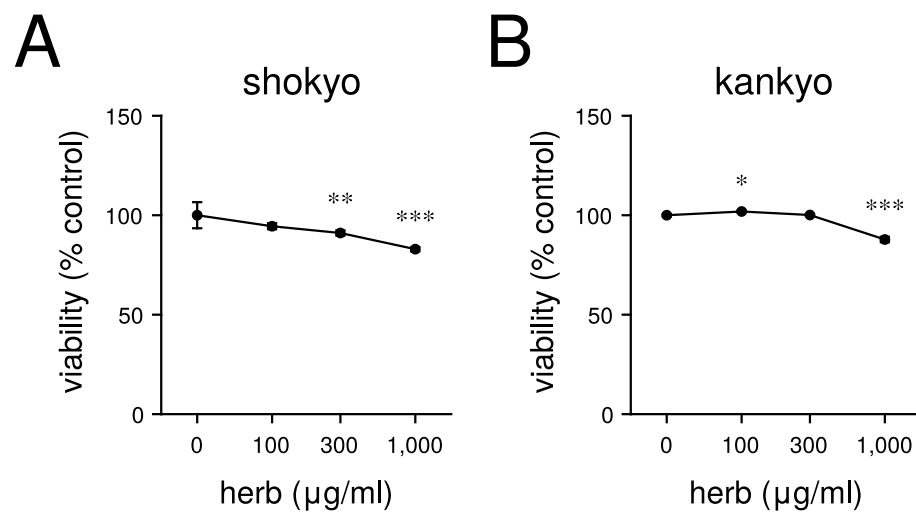


Figure 1. Cytotoxicity of shokyo and kankyo

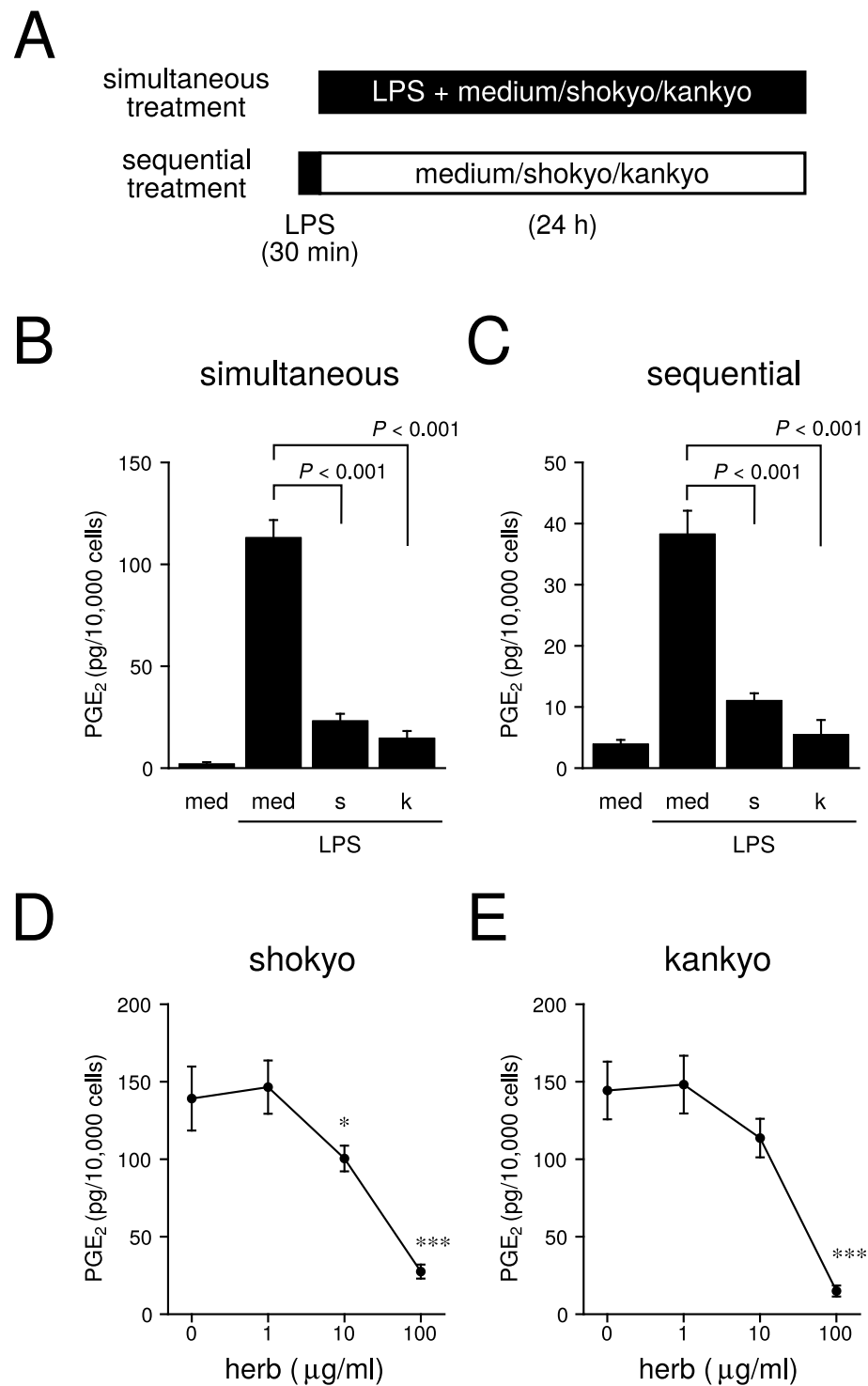


Figure 2. Effects of shokyo and kankyo on PGE₂ production

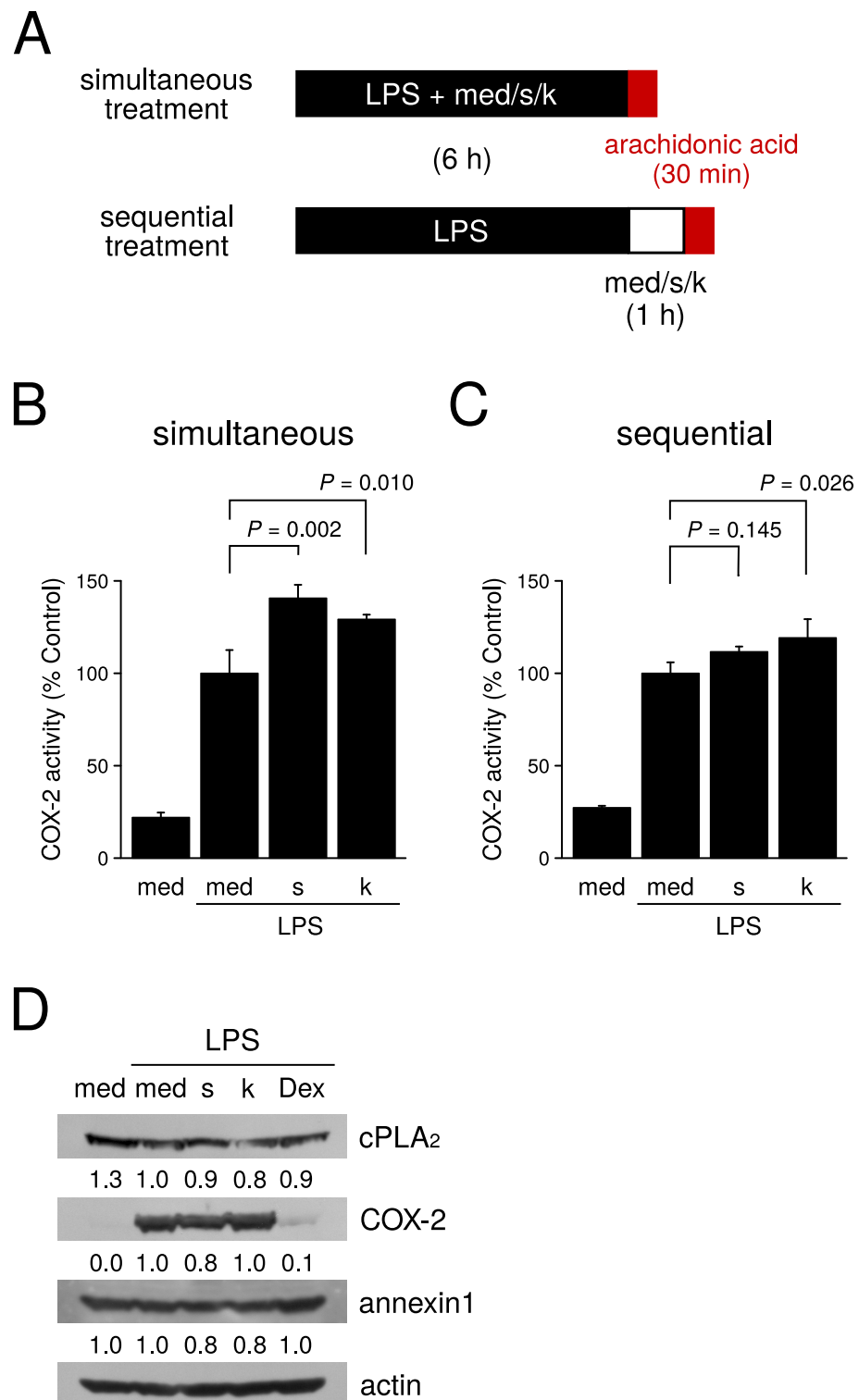
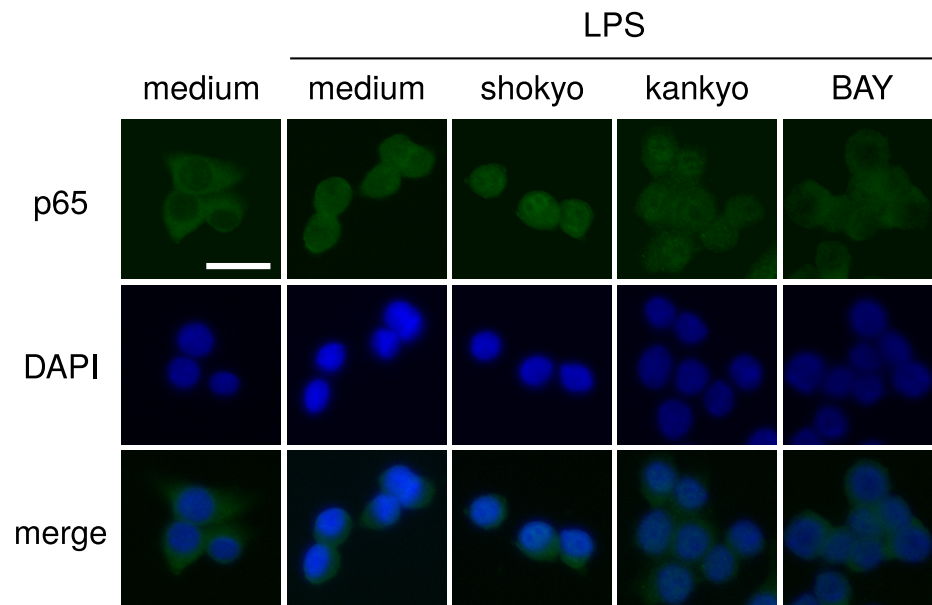
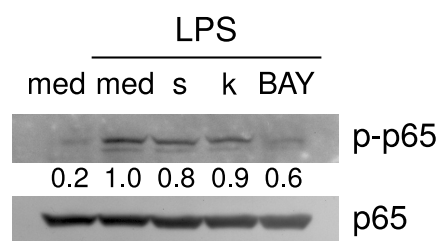


Figure 3. Effects of shokyo and kankyo on the arachidonic acid cascade

A



B



C

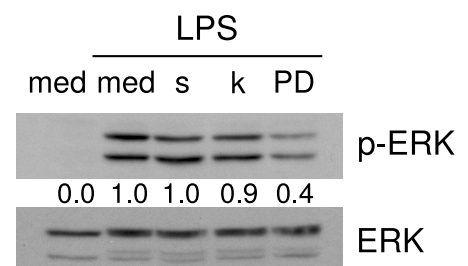


Figure 4. Effects of shokyo and kankyo on the intracellular signal transduction

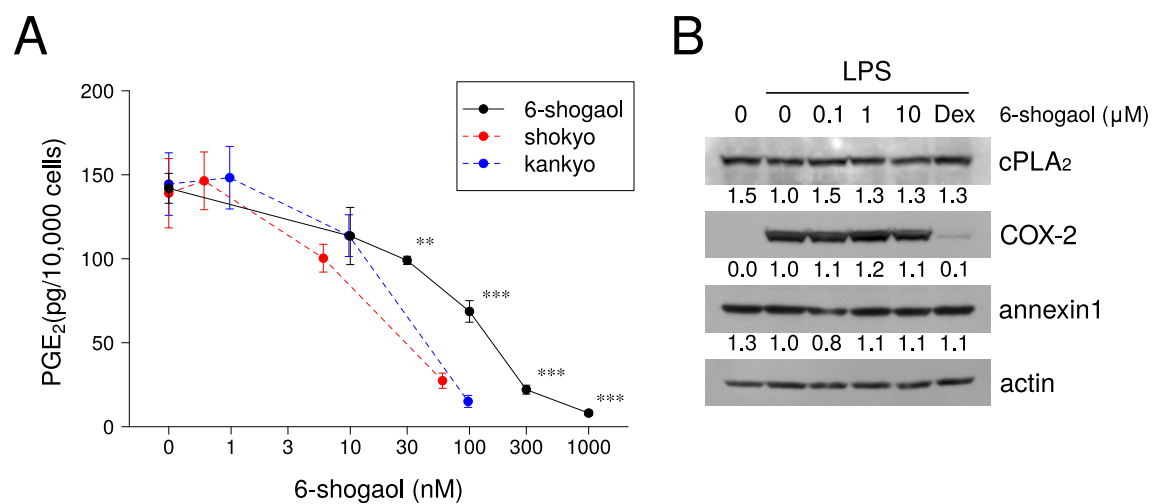
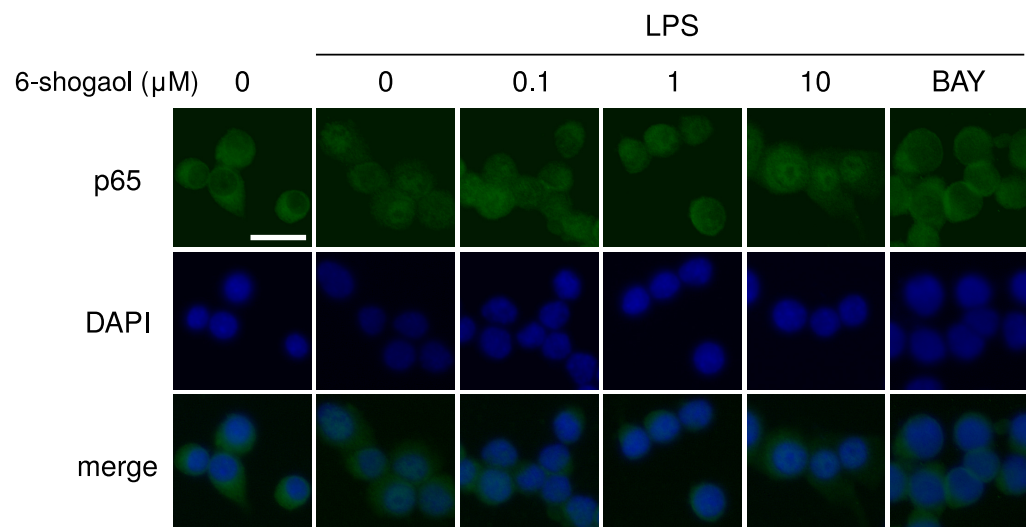
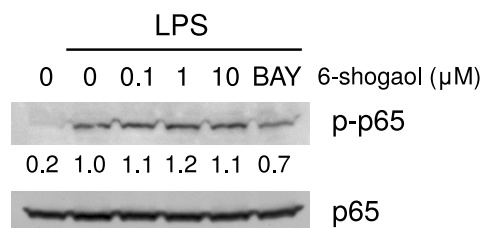


Figure 5. Effects of 6-shogaol on LPS-induced PGE₂ production, and the arachidonic acid cascade

A



B



C

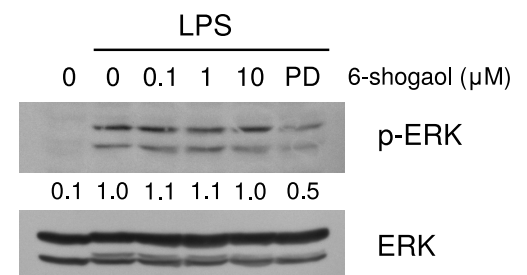


Figure 6. Effects of 6-shogaol on the intracellular signal transduction