Preventive Effects of a Kampo Medicine, Orento on Inflammatory Responses in Lipopolysaccharide Treated Human Gingival Fibroblasts

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Received August 28, 2009; accepted December 17, 2009; published online January 8, 2010

In the present study, we investigated the effects of a Kampo medicine Orento (TJ-120) on the production of prostaglandin E₂ (PGE₂), interleukin (IL)-6 and IL-8 by human gingival fibroblasts (HGFs) treated with lipopolysaccharide from Porphyromonas gingivalis (PgLPS). HGFs proliferation was dose-dependently decreased with Orento at days 3 and 7. However, treatment with PgLPS (10 ng/ml), Orento (up to 1 mg/ml) and their combinations for 24 h did not affect the viability of HGFs. Orento suppressed PgLPS-induced PGE₂ production in a dose-dependent manner but did not alter basal PGE₂ level. In contrast, Orento did not alter PgLPS-induced IL-6 and IL-8 productions. These alterations by Orento were similar to those by a mitogen-activated protein kinase kinase (MAPKK/MEK) inhibitor, PD98059. A Orento showed no effect on cyclooxygenase (COX)-1 and COX-2 activities, and increased cytoplasmic phospholipase A₂ (cPLA₂) expression and increased PgLPS-induced COX-2 expression. Orento suppressed PgLPS-induced mobility retardation of cPLA₂ band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, that is cPLA₂ phosphorylation and its activation, while Orento alone did not alter cPLA₂ phosphorylation. Orento suppressed PgLPS-induced extracellular signal-regulated kinase (ERK) phosphorylation, which is known to lead to ERK activation and cPLA₂ phosphorylation. These results suggest that Orento decreased PGE₂ production by inhibition of cPLA₂ phosphorylation and its activation via inhibition of ERK phosphorylation, and also that Orento may be useful to improve gingival inflammation in periodontal disease.

Key words Orento; prostaglandin E₂; anti-inflammatory effect; human gingival fibroblast

Materials and Methods

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Materials and Methods

Reagents Powder of Orento was obtained from Tsumura & Co. (Tokyo, Japan), and its components were indicated in Table 1. Orento was suspended in Dulbecco’s modified Eagle’s medium (D-MEM, Sigma, St. Louis, MO, U.S.A.) containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 mg/ml streptomycin (culture medium) and was rotated at 4°C overnight. Then, the suspension was centrifuged and the supernatant was filtrated through 0.45 μm-pore membrane. LPS from Porphyromonas gingivalis 381 (PgLPS) was provided by Drs. Tatsuji Nishihara and Nobuhiro Hanada (National Institutes of Public Health, Wako, Japan). Phorbol 12-myristate 13-acetate (PMA) and PD98059 [mitogen-activated protein kinase kinase (MAPKK/MEK) inhibitor] were purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). Arachidonic acid solution (from porcine liver) was purchased from Sigma. The anti-
bodies against cyclooxygenase-2 (COX-2, sc-1745), cytoplasmic phospholipase A2 (cPLA2, sc-438), annexin I (sc-11387) and actin (sc-1616), which detects a broad range of actin isoforms, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and the antibodies against extracellular signal-regulated kinase (ERK; p44/42 MAP kinase antibody) and phosphorylated ERK [Phospho-p44/42 MAPK (Thr202/Tyr204) (E10)] monoclonal antibody were from Cell Signaling Technology (Danvers, MA, U.S.A.). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.).

**Cells** HGFs were maintained as described previously. HGFs were maintained in Dulbecco’s modified Eagle’s medium (D-MEM; Sigma, St. Louis, MO, U.S.A.) containing 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin and 100 µg/ml streptomycin, at 37 °C in a humidified atmosphere of 5% CO2. HGFs were used between 10th and 20th passages in the assays. This study was approved by the Ethical Committee of our institution. Informed consent was obtained from each subject for the collection of HGFs.

**Cell Viability** The numbers of cells were measured using WST-8 (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. In brief, the media were removed by aspiration and the cells were treated with 100 µl of mixture of WST-8 with culture medium for 2 h at 37 °C in CO2 incubator. Optical density was measured (measure wavelength at 450 nm and reference wavelength at 655 nm) using a microplate reader (Model 550; Bio-Rad, Hercules, CA, U.S.A.), and background value was subtracted from each value.

**Cytokine Measurement by Enzyme-Linked Immunosorbent Assay (ELISA)** HGFs (10000 cells/well) were seeded in 96-well plates (AGC Techno Glass Co., Chiba, Japan) and incubated in serum-containing medium at 37 °C overnight. Then, the cells were treated with various concentrations of Orento (0, 0.01, 0.1, 1 mg/ml) in the absence or presence of PgLPS (10 ng/ml) for 24 h (200 µl per each well). In some experiments, 10 µM of arachidonic acid was added simultaneously. The numbers of cells were measured using WST-8. The concentrations of IL-6, IL-8 and PGE2 in the culture supernatants were measured by ELISA according to the manufacturer’s instructions (IL-6 and IL-8, Biosource International Inc., Camarillo, CA, U.S.A.; PGE2, Cayman Chemical, Ann Anbor, MI, U.S.A.), and were adjusted by the number of remaining cells.

**Measurement of Cyclooxygenase Activity** The effects of Orento on the activities of COX-1 and COX-2 were analyzed using COX inhibitor screening assay (Cayman Chemical, Ann Anbor, MI, U.S.A.) according to the manufacturer’s instructions. COXs activities were evaluated by the measurement of prostaglandin produced from arachidonic acid by COX-1 or COX-2 in duplicate. These values were normalized to a relative value of 100% for the cells without both PgLPS and Orento treatment.

**Western Blotting** HGFs were cultured in 60 mm dish and treated with the combinations of PgLPS and Orento. After 24 h, cells were washed twice with phosphate buffered saline, transferred into microcentrifuge tubes, and centrifuged at 6000 g for 5 min at 4 °C. Supernatants were aspirated and cell 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 ethylene glycol bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 5 µg/ml leupeptin and 1 µg/ml pepstatin) for 30 min at 4 °C. Then, samples were centrifuged at 12000 g for 15 min at 4 °C and supernatants were collected. The protein concentration was measured using BCA Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL, U.S.A.).

The samples (10 µg proteins) were fractionated in polyacrylamide gel under reducing conditions and transferred onto polyvinylidene difluoride (PVDF) membrane (Hybond-P; GE Healthcare, Uppsala, Sweden). The membranes were blocked with 5% ovalbumin (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature and incubated with primary antibody for 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 ECL kit (GE Healthcare).

**Statistical Analysis** Data are presented as means±standard deviation (S.D.). Differences between control group and experimental groups were evaluated by Dunnett method (Table 2). Differences between groups were evaluated by the pairwise comparison test corrected with Holm method (4 null hypotheses, without PgLPS vs. with PgLPS; 3 + 3 null hypotheses without Orento vs. with 0.01, 0.1 and 1 mg/ml of Orento in the absence or presence of PgLPS; total 10 null hypotheses; Figs. 1B, C and 2). All computations were performed with the statistical program R and Dunnett method was performed using the package ‘multcomp’. Values with \( p<0.05 \) were considered as significantly different.

**RESULTS**

**The Effect of Orento on HGFs Proliferation** We ex-
examined the effect of Orento on HGFs proliferation. In the absence of presence of PgLPS, Orento suppressed HGFs proliferation in the dose-dependent manner (Figs. 1A, B). In particular, HGFs treated with 1 mg/ml of Orento were almost dead at day 7. Moreover, HGFs proliferation was slightly suppressed by PgLPS treatment (Fig. 1B). These results indicate that 1 mg/ml of Orento shows a cytotoxicity.

The Effect of Orento on PGE$_2$, IL-6 and IL-8 Production

We examined whether Orento affects the productions of PGE$_2$ and inflammatory cytokines (IL-6 and IL-8) by HGFs. Because Orento affects cell viability, the concentrations of PGE$_2$, IL-6 and IL-8 were needed for adjustment by the results of WST-8. When HGFs were treated with PgLPS and Orento for 24 h, the viability of HGFs were hardly affected (Fig. 1C). These results indicate that 1 mg/ml of Orento shows a cytotoxicity.

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HGFs without any treatment produced low level of PGE$_2$. When HGFs were treated with PgLPS, HGFs produced significant level of PGE$_2$. Orento suppressed PgLPS-induced PGE$_2$ production in a dose-dependent manner (Fig. 2A). However, Orento had little effect on PGE$_2$ production in the absence of PgLPS.

In the absence of PgLPS, Orento did not affect IL-6 and IL-8 production (Figs. 2B, C). When HGFs were treated with 10 ng/ml of PgLPS, HGFs produced large amount of IL-6 and IL-8. Orento did not affect PgLPS-induced IL-6 and IL-8 productions (Figs. 2B, C). These results indicate that Orento suppressed PgLPS-induced production PGE$_2$ by HGFs but shows little effect on IL-6 and IL-8 productions.

The Effects of Orento on COX Activities

Because PGE$_2$ production was regulated by COXs and suppressed by acid NSAIDs such as aspirin and diclofenac sodium, which inhibit COXs activities, we examined whether Orento inhibits COX-1 and COX-2 activities. However, up to 1 mg/ml of Orento did not affect both COX-1 and COX-2 activities (Table 2).

The Effects of Orento on Molecular Expressions in Arachidonic Acid Cascade

We examined whether Orento affects the molecular expression in arachidonic acid cascade. COX-2 was not detected in the absence of PgLPS, and induced by PgLPS treatment. PgLPS-induced COX-2 expression was increased with up to 0.1 mg/ml of Orento and slightly decreased with 1 mg/ml of Orento (Fig. 3). cPLA$_2$ is the most upstream enzyme in arachidonic acid cascade and releases arachidonic acid from plasma membrane. Orento increased cPLA$_2$ expression in a dose-dependent manner in the absence or presence of PgLPS (Fig. 3). In contrast, other PLA$_2$ isoforms, calcium-independent PLA$_2$ (iPLA$_2$) and secretory PLA$_2$ (sPLA$_2$), were not detected (data not shown), indicating that cPLA$_2$ is a major isoform which release arachidonic acid in HGFs.

Annexin 1, also named as lipocortin 1, is the anti-inflammatory mediator produced by glucocorticoids and inhibits the cPLA$_2$ activity. However, both PgLPS and Orento showed no effect on annexin 1 expression (Fig. 3).

The Effects of Orento on cPLA$_2$, Phosphorylation and ERK Phosphorylation

cPLA$_2$ activity were regulated by its phosphorylation at Ser505 and its phosphorylation causes mobility retardation on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Then,
We examined whether Orento affects cPLA2 phosphorylation. First, we examined PgLPS-induced cPLA2 phosphorylation in the absence or presence of Orento (1 mg/ml). cPLA2 was phosphorylated at 0.5 and 1 h after PgLPS treatment with or without of Orento. At 2 h after treatment, cPLA2 was phosphorylated with PgLPS alone but was not phosphorylated with the combination of PgLPS and Orento (Fig. 4A). These results indicated that Orento suppressed PgLPS-induced cPLA2 phosphorylation at 2 h after treatment (Fig. 4A).

cPLA2 is reported to be phosphorylation and activated by extracellular signal-regulated kinase (ERK) directly.17 Previously, we examined the effect of an ERK inhibitor, PD98059, on PgLPS-induced IL-8 production by HGFs and obtained the results similar to Orento.20 Then, we again examined the effect of PD98059 on PGE2, IL-6 and IL-8 productions (Table 3). PD98059 did not affect a basal level of PGE2 but decreased PgLPS-induced PGE2 production. Basal levels of IL-6 and IL-8 productions were increased by PD98059. PD98059 slightly increased PgLPS-induced IL-6 production but not IL-8. These results indicate that the effects of Orento on PGE2, IL-6 and IL-8 productions are overall similar to those of PD98059, and prompted us to examine whether Orento suppresses PgLPS-induced ERK phosphorylation. ERK phosphorylation was enhanced 0.5 h after PgLPS treatment and thereafter was attenuated, and 1 mg/ml of Orento suppressed PgLPS-induced ERK phosphorylation at 0.5 to 2 h (Fig. 4B).

Next, we examined cPLA2 phosphorylation at 2 h after PgLPS and Orento treatment. Without PgLPS treatment, cPLA2 was not phosphorylated (Fig. 4C). When HGFs were treated with both PgLPS and 0.1 or 1 mg/ml of Orento, cPLA2 was not phosphorylated (Fig. 4C). These results indicate that Orento dose-dependently suppresses PgLPS-induced cPLA2 phosphorylation in HGFs.

The Effects of Orento on the Downstream Molecules of Arachidonic Acid Cascade Finally, we confirmed whether the suppression of PGE2 production by Orento is mediated on the downstream molecules of arachidonic acid cascade. For this purpose, exogenous arachidonic acid was added. Exogenous arachidonic acid increased PgLPS-induced PGE2 level. Moreover, when HGFs were treated with both PgLPS and Orento, arachidonic acid increased PGE2 level comparable to that treated with both PgLPS and arachidonic acid (Table 4). These results indicate that Orento did not affect the downstream molecules of arachidonic acid cascade.

DISCUSSION

In the present study, we examined the effect of Orento in PgLPS-treated HGFs. We showed that Orento suppressed PgLPS-induced PGE2 production, but not IL-6 and IL-8, by HGFs as well as Shosaikoito.31 It is widely known that PGE2 leads to inflammatory responses such as vasodilation, enhanced vascular permeability and pain generation. Acid NSAIDs show anti-inflammatory effect by suppression of
PGE₂ production even though they did not affect IL-6 and IL-8 productions. The findings that Orento suppresses PgLPS-induced PGE₂ production to a basal level suggest that Orento also has anti-inflammatory effects in periodontal disease and that its effects are mainly mediated by suppression of PGE₂, but not IL-6 and IL-8, production.

In the present study, we focused on the effect of Orento on cPLA₂ activity and demonstrated that Orento suppresses PgLPS-induced cPLA₂ phosphorylation (namely, activation). Considering that Orento did not affect COXs activities, that Orento did not suppress COX-2 and cPLA₂ expression, did not increase annexin 1 expression, and that the suppression of PGE₂ production by Orento was canceled by the addition of exogenous arachidonic acid, it is suggested that the action mechanism of Orento is suppression of arachidonic acid release through the suppression of PgLPS-induced cPLA₂ activation but not downstream of arachidonic acid cascade, which includes microsomal prostaglandin E synthase-1 (mPGES-1) converting PGH₂ to PGE₂.

The mechanism of Orento is thought to be different from those of generally used anti-inflammatory drugs, acid NSAIDs and steroidal anti-inflammatory drugs (SAIDs). Acid NSAIDs inhibit COXs activities, while Orento showed no inhibitory effect on both COX-1 and COX-2 activities (Table 2). SAIDs suppress cPLA₂ COX-2 and inflammatory cytokines (such as IL-6 and IL-8) and induce annexin 1, which inhibits cPLA₂ activity. In fact, dexamethasone suppressed PgLPS-induced IL-6 and IL-8 productions as well as PGE₂ production (data not shown). However, Orento showed little or no effect on the expression of these molecules (Fig. 3). These results suggest that Orento has a novel anti-inflammatory mechanism.

The arachidonic acid release by cPLA₂ is regulated by two mechanisms. The first is Ser505 phosphorylation and the second is cPLA₂ localization to membrane or phospholipid vesicles elicited by increased intracellular Ca²⁺ concentration. Because Orento showed little effect on a basal level of PGE₂, the mechanism of Orento is suggested to suppress cPLA₂ phosphorylation (Fig. 4C) but not to inhibit cPLA₂ activity itself. Next, we discuss whether the suppression of cPLA₂ phosphorylation can explain for the alteration of PGE₂ production by Orento. (i) When HGFs were not treated or treated with only Orento, because cPLA₂ was not phosphorylated (cPLA₂ activity is basal level) and COX-2 was not expressed (Fig. 3), HGFs slightly release arachidonic acid and produce a small amount of PGE₂ mediated by COX-1. (ii) When HGFs were treated with only PgLPS or both PgLPS and low dose of Orento, both cPLA₂ phosphorylation (cPLA₂ activation) and COX-2 expression were observed (Figs. 3, 4C), resulting in production of large amount of PGE₂. (iii) When HGFs were treated with both PgLPS and high dose of Orento, COX-2 expression, but not cPLA₂ phosphorylation, was observed (Figs. 3, 4C). PgLPS-induced PGE₂ productions with 0.1 or 1 mg/ml of Orento were similar, but both were higher than those with only Orento (Fig. 2A). These results suggest that COX-2 produce PGE₂ from arachidonic acid released by cPLA₂ with basal level of activity, and therefore may explain the finding that Orento failed to suppress PGE₂ production to the level without PgLPS. Moreover, it is reported that phosphorylation of cPLA₂ increases its activity by 2- to 3-fold. This increased activity ratio is similar to the ratio of PGE₂ level with PgLPS to that without PgLPS (Fig. 2A). From these findings, we consider that the suppression of cPLA₂ phosphorylation by Orento explains for the alteration of PGE₂ production.

We also demonstrated that the alteration of PGE₂ IL-6 and IL-8 levels by Orento are quite similar to those by PD98059 (Fig. 2, Table 3). Moreover, ERK phosphorylates and activates cPLA₂. Indeed, Orento suppressed PgLPS-induced ERK phosphorylation (Fig. 4B). These results suggest that the effect of Orento on cPLA₂ phosphorylation is mediated by the modulation of ERK activation. Which components in Orento phosphorylate ERK and activate cPLA₂. In our previous report, Shosaikoto did not affect basal levels of IL-6 and IL-8, suggesting that Shosaikoto contains no components which inhibit ERK phosphorylation. Shosaikoto are composed of Saiko, Hange, Ogon, Taiso, Ninjin, Kanzo and Shokyo. Therefore, the components that are contained in only Orento (Oren, Kankyō and/or Keihi) may suppress cPLA₂ phosphorylation and PGE₂ production.

In contrast, it is reported that LPS failed to increase intracellular Ca²⁺ concentration in rat liver macrophage for at least 24 h. These results suggest that LPS does not translocate cPLA₂ to membrane or phospholipid vesicles and, therefore, that Orento may not be involved in translocation of cPLA₂.

To date, there is no drug that suppresses cPLA₂ activity among various anti-inflammatory drugs used generally. Therefore, Orento is an anti-inflammatory drug possessing a mechanism different from conventional drugs. Recently, the concentration of leptin, which is mainly synthesized in adipocytes and regulates weight control, is negatively correlated with periodontal disease progression. Moreover, leptin is reported to suppress PgLPS-induced cPLA₂ activation in salivary gland acinar cells and Helicobacter pylori LPS-induced cPLA₂ activation in gastric mucosal cells. However, leptin is also reported to increase COX-2 expression and PGE₂ production in human endometrial cancer cells. Therefore, leptin may be difficult to use as an anti-inflammatory drug.

Many studies demonstrated that NSAIDs administration prevents gingival inflammation (reviewed in ref. 2). And several clinical studies indicated that the concentration of PGE₂ in gingival crevicular fluid (GCF) is increased in periodontal disease and is decreased by oral administration or mouse wash of NSAIDs. Considering the facts that both NSAIDs and Orento suppress PGE₂ production, it is possible that administration of Orento also decreases PGE₂ concentration in GCF and results in the improvement of gingival inflammation. Therefore, Orento may be useful for the improvement of gingival inflammation in periodontal disease. Importantly, Orento did not alter COX-1 activity and basal level of PGE₂. Because PGE₂ produced by COX-1 protects gastric mucosa, these results suggest that Orento may have minimal gastrointestinal dysfunction.

In summary, we demonstrated that Orento suppresses PgLPS-induced ERK phosphorylation and following cPLA₂ phosphorylation and results in PGE₂ production by HGFs. Orento may be useful for the improvement of inflammation in periodontal disease.

**Acknowledgments** We thank Drs. Tatsuji Nishihara and...
REFERENCES AND NOTES