Dioscorea japonica extract down-regulates prostaglandin E\(_2\) synthetic pathway and induces apoptosis in lung cancer cells

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Prostaglandin E\(_2\) plays a role in a range of pathophysiological responses, including inflammation, carcinogenesis and so on. Prostaglandin E\(_2\) is synthesized from arachidonic acid through the COX and PGES (cPGES)-1, (5) mPGES-2, (6,7) and cytosolic PGES (cPGES)-8 (8). Among these, mPGES-1 belongs to the membrane-associated proteins involved in eicosanoid and glutathione metabolism and is usually up-regulated in accordance with COX-2 induction. In several types of cancer cells, both COX-2 and mPGES-1 are highly expressed, and the consequent excessive production of PGES worsens the symptoms. (9–10)

COX-2 has been considered an attractive target for PGES inhibition. Indeed, selective COX-2 inhibitors, as well as other traditional non-steroidal anti-inflammatory drugs (NSAIDs), are used to provide symptomatic relief to patients with chronic diseases. (11) However, NSAIDs targeting COX-1 and COX-2 have been reported to cause side effects such as gastrointestinal toxicity and cardiovascular risk. In light of this, inhibition of mPGES-1, which acts downstream of COX-2, represents an attractive target for selective inhibition of PGES production, with a potentially safer profile than COX-2 inhibitors. Thus, identification of natural substances from foods that have safe and functional effects on the regulation of PGES production is essential. Accordingly, in this study, we focused on the functional effect of Dioscorea japonica (D. japonica) on the regulation of the expression of COX-2 and mPGES-1.

D. japonica, a wild yam, is a relative of the Dioscoreaceae family native to Japan. Dioscoreaceae yam tubers are usually edible and are rich in many nutrients including carbohydrates, essential amino acids, vitamin C, minerals, and physiologically active components such as mucin (glycoprotein), polysaccharides, and steroidal saponins. (12–15) It is well known that D. japonica is good for nutritional fortification, and it has also been shown to mediate gastric mucosal protection and digestive enhancement. However, the other physiological effects of D. japonica have not been completely elucidated.

The aim of this study was to explore the novel functional effects of D. japonica on the regulation of the PGES synthesis pathway. We also examined the possibility that D. japonica exerts anti-tumoral effects in lung cancer cells via down-regulation of PGES synthesizing enzymes.

Materials and Methods

Preparation of D. japonica powder and extract.

D. japonica was obtained from Autoraimu Yoshio Ltd. (Niimi, Japan). D. japonica was pared away the outer skin and was dried under 40°C. The ground D. japonica was prepared to uniform

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powders through 250 μm mesh. The powders were eluted with 20–80% ethanol (v/v, EtOH), and the extracts were immediately dried-up with nitrogen gas and were completely filling with the gas to protect oxidation. They were stored at −80°C until using for the experiments. When the *D. japonica* extract (DJE) was added to the medium for cell culture, the dried DJE was re-dissolved in DMSO as a vehicle. The final concentration of DMSO was 0.1% in the medium for control cells and DJE-treated cells.

**Cell culture.** Human non-small cell lung carcinoma cell line A549 (ATCC CCL-185) and human colon carcinoma cell line Caco-2 (ECACC 86010202) were obtained from RIKEN BioResource Center (Tsukuba, Japan). The cells were cultured in Dulbeco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were treated with 0–100 μg/ml DJE in dimethyl sulfoxide, 0–10 μM dioxigenin (Sigma, St. Louis, MO) in dimethyl sulfoxide or were added only vehicle as a control.

**RNA purification and quantitative RT-coupled real-time PCR.** A549 or Caco-2 cells after the treatment for 24 h were collected and the total RNA was isolated using the acid guanidinium thiocyanate procedure and analyzed for gene expression by real-time quantitative RT-PCR (iQ5 real-time PCR system, Bio-Rad, Hercules, CA). After converting total RNA to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), quantitative PCR was performed using Eva Green-Comparative Ct method (Bio-Rad). The following primer pairs were used for amplification of COX-2, mPGES-1, B-cell chronic lymphocytic leukemia/lymphoma 2 (Bcl-2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH); 5′-TGC-ATTCTTGGCCACGACT-3′ (COX-2 forward), 5′-AAAGGCCG-CAGTTAACCTGT-3′ (COX-2 reverse), 5′-TGATCACACCCACAGTTGAC-3′ (mPGES-1 forward), 5′-TGGATGACGCTGTA-3′ (mPGES-1 reverse), 5′-TGGATGACTGAG-3′ (COX-2 forward), 5′-TGATGATGCTCAGTTTACGCTGT-3′ (COX-2 reverse), 5′-AAAGGCT-3′ (Bcl-2 forward), 5′-TACCTGAA-3′ (Bcl-2 reverse), 5′-GCTGCTCAG-3′ (GAPDH forward), and 5′-TGATCACACCCACAGTTGAC-3′ (GAPDH reverse). The cycling conditions were 30 s at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for 10 s. Expression levels of COX-2, mPGES-1 and Bcl-2 mRNAs were normalized to those of GAPDH mRNA. The relative expression levels were shown against control and were mean ± SD of 5 separate experiments.

**Western blotting.** Collected A549 cells (5 × 10⁶ cells) after the treatment with or without DJE for 48 h were sonicated in 100 μl of 10 mM Tris-HCl (pH 7.6) and then were centrifuged at 10,000 × g. The supernatants (30 μg protein/well) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were transferred electrophoretically to a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). After treatment with block reagent (Roche, Penzberg, Germany), the membrane was incubated with rabbit anti-COX-2 antibody (1:1000, Immuno-Biological Laboratories Co., Ltd., Gunma, Japan), rabbit anti-mPGES-1 antibody (1:1000, Cayman Chemical Co. Ann Arbor, MI) or rabbit anti-β-actin antibody (1:1000, Cell Signaling Technology, Boston, MA). The immunoreactive proteins were visualized using BM chemiluminescence Western blotting kit (Roche). The density of each protein bands was analyzed with Fujiﬁlm Multi Gauge ver. 3.0 software (Fujiﬁlm, Tokyo, Japan). Protein concentration was determined using a BCA protein assay kit (Thermo Scientiﬁc Inc., Rockford, IL).

**COX activity assay.** COX activity was analyzed as described previous report.(16) The lysates were incubated with 100 mM Tris–HCl buffer (pH 7.4) containing 25 mM linoleic acid, and the products were analyzed by reversed-phase HPLC using a COSMOSIL 5C18-MS-II column (5 mm particle, 4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan) with a solvent system of methanol/water/acetic acid (80:20:0.01, v/v). The produced 9- and 13-hydroxyoctadecadienoic acids (HODEs) which chromatographed on reversed-phase HPLC as a single peak were quantified by the comparison of the area of the peak with that of an internal standard, 15-hydroxyeicosatetraenoic acid (HETE).

**Enzyme immunoassay.** After the treatment with or without DJE by 50% ethanol (100 μg/ml) for 48 h, arachidonic acid (10 μM) was added to the culture medium of A549 cells. After incubation for 30 min, PGE₂ concentration in the medium was measured by enzyme immunoassay using PGE₂-Enzyme Immunoassay Kit (Cayman Chemical Co.) as instructed by the manufacturer.

**Immunocytochemistry.** The intracellular localization of transcription factor nuclear factor-κB (NF-κB) was analyzed by fluorescence immunocytochemistry. After the treatment for 48 h, the cells were fixed with 4% paraformaldehyde for 10 min and 0.5% ethanol for 2 min. After blocking with Block Ace (Dainippon Seiyaku, Osaka, Japan), the cells were incubated with anti-NF-κB p65 (C22B4) rabbit antibody (Cell Signaling Technology), and then were incubated with Cyanine (Cy) 3-labeled donkey anti-rabbit IgG (1:400, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The sections were counter stained with 4',6-diamidino-2-phenylindole (DAPI). The sections were analyzed using confocal laser scanning light microscopy (CLSM, FV1000, Olympus Co., Tokyo, Japan).

**Promoter assay.** COX-2 promoter assay was carried out as described previously.(17) COX-2/Cluc plasmid with COX-2 promoter region and Cypridina luciferase (Cluc) as a reporter gene was transfected to A549 cells. After the transfection, A549 cells were treated with 0.1 mM 12-O-tetradecanoylphorbol 13-acetate (TPA) and with or without DJE for 48 h, and the luciferase activity in the culture medium was measured using BioLux™ Cypridina Luciferase Assay Kit (New England BioLabs Inc., MA). A549 cells without the treatment of TPA and DJE was used as a control.

**TUNEL assay.** After treatment of with or without DJE for 48 h, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was carried out for identification of the extent of DNA fragmentation in A549 cells, using Peroxidase end labeling (TUNEL) assay was carried out for identification of the extent of DNA fragmentation in A549 cells, using Peroxidase in Situ Apoptosis Detection Kit (EMD Millipore, Billerica, MA) as instructed by the manufacturer. TUNEL positive reactivity was observed with a light microscope (ECLIPSE 80i; Nikon Co., Tokyo, Japan).

**Statistics.** Data were statistically evaluated by Student’s t test (Fig. 2B and 4) and ANOVA with Bonferonni’s (Fig. 2A, 3B, 6A)
or Dunnett’s (Fig. 1 and 7) post-hoc test at significance level of $p<0.05$ or 0.01.

**Results**

**Dose-dependent effect of DJE on the expression of COX-2 and mPGES-1 in A549 cells.** COX-2 and mPGES-1 are constitutively and highly expressed in human lung carcinoma A549 cells. A549 cells were treated with DJE by 50% ethanol at 0.1–100 μg/ml concentration, and mRNA expression of COX-2 and mPGES-1 in the cells was analyzed by real-time PCR. DJE suppressed the expression of COX-2 and mPGES-1 in a dose-dependent manner (Fig. 1). Treatment with 100 μg/ml DJE significantly decreased mRNA levels of COX-2 and mPGES-1 to 27% and 46% compared with the control, respectively. Therefore, this concentration of DJE (100 μg/ml) was used for the following experiments. Addition of DJE made no significant difference on cell proliferation in the experimental period.

**Suppression of the expression of COX-2 and mPGES-1 by DJE.** Among the water-soluble extract of *D. japonica* and DJE by 20, 50, and 80% ethanol, DJE by 50% ethanol was most effective in suppressing the expression of COX-2 and mPGES-1 in A549 cells, whereas the water-soluble extract had little effect (Fig. 2A). Further, in human colon carcinoma model Caco-2 cells, treatment with DJE led to the suppression of COX-2 and mPGES-1 mRNAs to approximately 67% compared with the control (Fig. 2B).

After treatment with DJE by 50% ethanol for 48 h, COX-2 and mPGES-1 protein levels were decreased to 1.37 and 0.82, respectively, compared with the control (2.67 and 5.58) in A549 cells (Fig. 3).

**Changes in COX activity and PGE$_2$ production by DJE treatment.** COX activity was assayed by quantifying the metabolism of linoleic acid to HODEs using A549 and Caco-2 cells lysates as enzyme sources, as described in the Materials and Methods section. COX catalyzes the metabolism of arachidonic acid to PGH$_2$ and linoleic acid to 9- and 13-HODEs.$^{(18)}$ In the present study, linoleic acid was used as the substrate because it presents several advantages over arachidonic acid. HODEs metabolized from linoleic acid were stable and these are quantified by the typical reversed-phase HPLC analysis for low-molecular-weight lipids, although PGH$_2$ synthesized from arachidonic acid is highly unstable and is immediately and non-enzymatically metabolized to several PGs. After treatment with DJE for 48 h, COX activity was decreased to 46% (39 pmol/min/mg protein) and 50% (27 pmol/min/mg protein) when compared with each control in A549 cells and Caco-2 cells, respectively (Fig. 4A).

PGE$_2$ production was quantified by enzyme immunoassay. Released PGE$_2$ in the culture medium of A549 cells was also significantly decreased to 31% (647 pg/ml) by DJE treatment (Fig. 4B).

**Translocation of NF-κB and suppression of COX-2 promoter activity by DJE.** After treatment with DJE by 50% ethanol (100 μg/ml) for 48 h, localization of the transcription factor NF-κB was analyzed by immunocytochemistry (Fig. 5A). In some pathological conditions such as cancer and inflammation, NF-κB is translocated from the cytosol to the nucleus and plays a key role in regulating COX-2 transcriptional activity. NF-κB immunoreactivity merged with the nuclear counterstain DAPI in
control A549 cells (Fig. 5A top panels). The findings indicate that NF-κB is constitutively localized in the nucleus in A549 cancer cells, which exhibit high levels of COX-2. In contrast, DJE treatment induced the translocation of NF-κB from the nucleus to the cytosol (Fig. 5A bottom panels). Additionally, the COX-2 promoter activity was measured using COX-2-Cluc plasmid with Cypridina luciferase reporter gene (Fig. 5B). TPA (0.1 μM), a COX-2 inducer, increased luciferase activity by approximately 4-fold, and addition of DJE significantly decreased TPA-induced luciferase activity to a level similar to that of the control.

**Suppression of Bcl-2 mRNA expression and induction of apoptosis by DJE.** mRNA expression of the anti-apoptotic factor Bcl-2 was suppressed after treatment with DJE for 24 h (Fig. 6A). Compared with the control, DJE by 50% ethanol significantly decreased Bcl-2 mRNA levels to 18%.

Further, in situ detection of apoptotic cells with TUNEL method revealed a large number of brown-colored positive A549 cells after DJE treatment, although colored cells were hardly observed in the control cells (Fig. 6B).

**Effect of diosgenin on the expression of COX-2 and mPGES-1 in A549 cells.** Diosgenin is a major steroidal saponin constituent and is found more abundantly in *D. japonica* compared with other food substances. Diosgenin suppressed COX-2 expression in A549 cells in a dose-dependent manner (0–10 μM), and 10 μM diosgenin reduced COX-2 expression to 46% compared with the control (Fig. 7). In contrast, diosgenin did not have a significant effect on the expression of mPGES-1.

**Discussion**

Of all cancers, lung cancer has the highest mortality, and the 5-year survival rate is very low. Therefore, new approaches for the management and prevention of lung cancer are highly essential. Increased expression of COX-2 is commonly observed in lung tumors as well as in many other tumors. Similarly, the expression of inducible mPGES-1, preferentially coupled with COX-2, for PGE2 synthesis is enhanced in tumors, NS-398, a type of NSAID, is an effective and selective COX-2 inhibitor and has been found to induce apoptosis in cancer cells. However, long-term use of NSAIDs can cause life-threatening side effects, including gastrointestinal injury and renal pathology. In our current study, we attempted to investigate the preventive effects of natural food products on lung cancer by targeting the PGE2 synthesis pathway. We demonstrated that DJE suppressed the expression of COX-2 and mPGES-1 and exerted anti-carcinogenic effects in human lung carcinoma A549 cells.

Our data show that DJE suppressed mRNA and protein expressions of COX-2 and mPGES-1 and consequently decreased COX activity and PGE2 production. Furthermore, DJE induced the translocation of NF-κB, the typical transcription factor of COX-2, from the nucleus to the cytosol in A549 cells (Fig. 5A). This finding indicated that DJE regulates COX-2 signal transduction pathway mediating COX-2 induction. Consistent with this hypothesis, the transcriptional activity of COX-2 was significantly decreased by DJE, as shown using an expression plasmid containing COX-2 promoter with a Cluc reporter gene (Fig. 5B). In lung cancer cells, inhibition of COX-2 by trichostatin A, a histone deacetylase inhibitor, or celecoxib, a NSAID that acts as a selective COX-2 inhibitor, correlates with a decrease in PGE2 production. Such inhibitors suppress tumor growth with decreasing the levels of the anti-apoptotic factor Bcl-2. Our results also showed that DJE suppressed the expression of COX-2 and mPGES-1, which are key enzymes for PGE2 production, and induced apoptosis with suppression of the anti-apoptotic factor Bcl-2 in cancer cells.

Several phytochemicals from other natural products, such as resveratrol, humulon, and chrysin, have been shown to suppress the expression of COX-2. However, the suppressive effect on mPGES-1 expression by phytochemicals other than sulforaphane has not been reported. Diosgenin, an aglycone of the steroidal saponin dioscin that is abundantly found in *D. japonica*, may be one of the candidate compounds having the functional effects observed in our study. There are some reports that support our results that diosgenin may have suppressive effects on the signal transduction pathway mediating COX-2 induction. Diosgenin inhibits Akt signaling and its downstream targets, NF-κB and Bcl-2, in human breast cancer cells. In addition, diosgenin has been shown to induce apoptosis in HepG2 and HEL cells. In a mouse model for colon cancer, diosgenin was shown to decrease gene expression of inflammatory cytokines such as IL-1β and IL-12β and to prevent colon carcinogenesis. In the present study, the effect of diosgenin (0–10 μM) on the expression of COX-2 and mPGES-1 in A549 cells was investigated. Diosgenin suppressed COX-2 expression in a dose-dependent manner, and COX-2 expression was decreased to 47% by 10 μM diosgenin (Fig. 6). However, the effect of diosgenin on COX-2 expression was lower than that of DJE by 50%
ethanol (100 μg/ml DJE), which decreased COX-2 expression to 27% (Fig. 2A). On the other hand, diosgenin did not have a significant effect on mPGES-1 expression. This suggests that multiple components in DJE mediate its ability to inhibit the expression of COX-2 and mPGES-1, leading to anti-inflammation and anti-carcinogenesis, suggesting there may be a novel functional component in DJE that has yet to be identified.

In conclusion, based on the present results, we propose that *D. japonica* results in the suppression of COX-2 and mPGES-1 expression and ultimately inhibits the production of PGE2. Thus, it may prove valuable in controlling inflammatory and metabolic processes as well as carcinogenesis. Our results may be important in understanding the preventive effects of *D. japonica* against the lifestyle-related disease conditions that are mediated by PGE2, although additional *in vivo* studies are required to address the physiological significance of our findings.

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Abbreviations

Bcl-2  B-cell chronic lymphocytic leukemia/lymphoma 2
Cluc  Cypridina luciferase

References


Conflict of Interest

No potential conflicts of interest were disclosed.