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Extracellular Prostaglandin E_2 Upregulation Effect of the Methanol Extract of *Artemisia argyi*

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Abstract – Since 15-hydroxyprostaglandin dehydrogenase (15-PGDH) is the key metabolic enzyme of prostaglandin E_2 (PGE_2), inhibition of 15-PGDH is supposed to facilitate various physiological functions by increasing PGE_2. Methanol extract of *Artemisia argyi* (AAME) inhibited 15-PGDH (IC_{50}: 13.13 μg/mL) with relatively low cytotoxicity (IC_{50}: 415.00 μg/mL) and elevated extracellular PGE_2 levels in HaCaT cells. Real-time PCR analysis showed that AAME decreased significantly mRNA expression of PG transporter (PGT) in HaCaT cells. These results indicate that AAME could be applicable to functional materials as a 15-PGDH inhibitor and PGT expression inhibitor for the upregulation of extracellular PGE_2 level.
Keywords – *Artemisia argyi*, Prostaglandin E_2, 15-Hydroxyprostaglandin dehydrogenase, Cyclooxygenases 1/2, Multidrug resistance-associated protein 4, PG transporter

Introduction

The genus *Artemisia*, belonging to the Compositae family, comprises about 500 species all over the world. *Aremisia* plants are important medicinal sources for colic pain, diarrhea, osteoblastic bone formation, gastric ulcer, microbial infection, inflammatory diseases, malaria, hepatitis, cancer, and so on (Park, 2008; Ding *et al*., 2009; Park, 2009; Lee *et al*., 2010; Li *et al*., 2010; Lee *et al*., 2004; Choi *et al*., 2008). *A. argyi* is called 'Aeyup' in Korea, and classified as the same medicinal plants with *A. montana* and *A. princeps*.

Prostaglandin (PG) E_2, synthesized from arachidonic acid (AA) by cyclooxygenases (COX) and terminal prostaglandin E synthases (PGES), acts as both an inflammatory mediator and fibroblast modulator (Sandulache *et al*., 2007). PG production is also directly dependent on the availability of free arachidonic acid, which is released from membrane glycerophospholipid by the hydrolysis of fatty acid from its *sn-2* position by phospholipase A_2 (Balsinde *et al*., 1999). In addition, COX is a rate-limiting enzyme in the biosynthesis of PGs from AA, and exists in two isoforms (COX-1 and COX-2). The former is constitutively expressed in a wide variety of tissues, where it serves a homeostatic function. PGs are not stored in cells but are released into the cellular environment, where they exert autocrine or paracrine effect on neighboring cells. Synthesized PGE_2 is simply diffused and actively extruded by the multidrug resistance-associated protein 4 (MRP4) from the cells (Schuster, 2002). After acting via its PGE_2 receptor (EPR), pericellular PGE_2 is cleared via re-uptake by PG transporter (PGT) and then rapidly metabolized by cytosolic enzyme named NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Schuster, 1998; Anggard and Samuelsson, 1964). PGE_2 in turn modulates the activity of a wide variety of cells, including keratinocytes, dendritic cells, and fibroblasts via four EPR (Konger *et al*., 1998; Harizi *et al*., 2003; Kolodsick *et al*., 2003). PGE_2 has also been identified as an important mediator for gastric ulcer healing (Wallace, 2008; Araki *et al*., 2002), and dermal wound healing (Parekh *et al*., 2009; Wilgus *et al*., 2004).

In this study, we selected the methanol extract of *A. argyi* (AAME) as a potent 15-PGDH inhibitor after screening plant extracts and investigated the regulation

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and expression of COX-1, COX-2, MRP4 and PGT in fibroblast cell line (HaCaT) for the regulation of PGE_2 level.

Experimental

Plant materials and extraction – *A. argyi* was purchased from the Jeonnam Sengyak Nonghyup (Hwasoon, Korea) and voucher samples are preserved at Biothechnology Industrialization Center of Dongshin University. *A. argyi* was extracted with 99.6% methanol under reflux. The methanol extract was evaporated to dryness by using a rotary vacuum evaporator at 50 ºC to get crude extract.

Instruments and reagent – PGE_2, NAD^+, NADH, gluthathione-sepharose 4B, dithiothreitol (DTT), sodium dodecylsulfate (SDS), EDTA, reduced glutathione, COX-1 inhibitor (SC 560), COX-2 inhibitor (Celecoxib), mitomycin and rest of essential chemicals and reagents were purchased from Sigma (St. Louis, MO, USA). The GST gene fusion pGEX-2T expression vector was purchased from Pharmacia Crop. (New Jersey, USA). The cDNA of human 15-PGDH was cloned from a human placenta cDNA library, as described previously [36]. The UV spectra were obtained using a UV-VIS spectrophotometer (SHIMADZU, Japan). PGE_2 enzyme immunoassay kit was purchased from Thermo Scientific (Rockford, IL, USA). Real-time PCR was performed with the Light Cycler 2.0 Instrument (Roche, Mannheim, Germany).

Expression and purification of 15-PGDH – 15-PGDH cDNA plasmid containing *BamHI* and *EcoRI* sites of the pGEX-2T expression vector was used to transform *Escherichia coli* BL-21 lysS. The cells were grown in 500 mL medium containing 50 μg/mL ampicillin at 37 ºC and 220 rpm until the OD_{600} reached 0.6. Isopropyl B-D-thiogalactoside (1 M stock solution) of 500 μL was added and the cells were allowed to grow for 12 hours at 25 ºC . Then the cells were harvested by centrifugation at 4000 × g for 30 minutes at 4 ºC. The cell pellets were resuspended in 20 mL cold cell Lysis buffer (1 × PBS buffer pH 7.4 containing 1 mM EDTA and 0.1 mM DTT) and sonicated (4 × 10 s at 4 ºC). The disrupted cells were centrifuged at 4,000 × g for 20 minutes at 4 ºC. The supernatant was applied slowly to a gluththione-sepharose 4B column, which was equilibrated at 4 ºC with a lysis buffer. The column was washed with lysis buffer until OD_{280} reached below 0.005. The 15-PGDH was eluted from the glutathione-sepharose 4B column by incubation at room temperature for 5 minutes with the elution buffer (50 mM Tris-HCl pH 8.0 containing 10 mM reduced glutathione, 1 mM EDTA and 0.1 mM DTT). The concentration of enzyme was determined and the purity of the 15-PGDH was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

15-PGDH assay – Assay for the activity of the 15-PGDH inhibitors was performed using a fluorescence spectrophotometer by measuring the formation of NADH at 468 nm following excitation at 340 nm. Tris-HCl buffer (50 mM, pH 7.5) containing 0.1 mM DTT, 0.25 mM NAD^+, purified enzyme (10 μg), 21 μM PGE_2 and various concentrations of AAME (total 2 mL) were added to each cell. Each concentration was assayed in triplicate. The absorbance at 340 nm after incubation with AAME was determined from a standard curve of various concentrations of NADH at 340 nm.

Cell culture – HaCaT cells, a human keratinocyte cell line, were cultured in Dulbecco's modified Eagle's media (DMEM). The cultured media were supplemented with 10% heat inactivated fetal bovine serum (Sigma) and 100 μg/mL penicillin, in 5% CO_2 at 37 ºC.

Cell viability assay – Cell viability was determined by the MTT assay. HaCaT cells (1×10^4) cells were seeded in 96 well plates per 90 μL of DMEM medium. After the overnight of incubation, AAME was treated for 72 hours followed by 4 hours of incubation with 10 μL of MTT (5 mg/mL stock solution). Then medium was removed and followed by addition of 150 μL of DMSO to dissolve formazin. Absorbance was measured at 540 nm using an ELISA microplate reader (Perkin-Elmer, Gly., USA).

Determination of PGE_2 release – HaCaT cells was seeded (5×10^5 cells/well) onto 6-well culture plates in DMEM medium containing fetal bovine serum and antibiotic for overnight in 5% CO_2 incubator at 37 ºC. AAME of different concentrations were treated and media was collected after 12 hours of sample treatment. PGE_2 levels were determined by PGE_2 enzyme immunoassay kits according to manufacturer's protocol.

Quantitative real-time PCR – Total cellular RNA was isolated from HaCaT cells using TRI reagent (RNAiso Plus, Takara) according to manufacturer's protocol. cDNA for each RNA sample was synthesized in 20 μL reactions using the SupeScript First Strand synthesis system for reverse transcription-PCR (Invitrogen) following manufacturer's protocol. PCR reaction contained 4 μl of 1 : 5 diluted cDNA, 4 mM $MgCl_2$ 10 pmole of each primer and 4 μl of Fast Starter Mix buffer (dNTPs, SYBR Green dye and Tag polymerase). Primers used for real-time PCR were as follows: human PGT forward, 5'-GGATGCTGTTTGGAGGAATCCTCA-3' and reverse, 5'-GCACGATCCTGTCTTTGCTGAA-3'; MRP4 forward, 5'-AACCTCTAACCGACATTCCTG-3' and reverse, 5'-

Table 1. 15-PGDH inhibitory activity and cytotoxicity of AAME in HaCaT cells

15-PGDH inhibition (ED_{50})	13.13 μg/mL
Cytotoxicity (IC_{50})	415.00 μg/mL

Table 2. Intracellular and extracellular PGE_2 levels after the treatment of AAME in HaCaT cells

	Intracellular (pg/g)	Extracellular (pg/mL)
Control	1.89 ± 0.20	393.67 ± 12.46
AAME	0.39 ± 0.24**	459.57 ± 34.07*

Values are mean ± SD (n = 4). * $p < 0.05$. ** $p < 0.005$.

TCAACATATTACAGCCACCATC-3'; COX-1 forward 5'-CCTCATGTTTGCCTTCTTTGC-3' and reverse 5'-GGCGGGTACATTTCTCCATC-3'; COX -2 forward, 5'-GATCTACCCTCCTCAA-3' and reverse 5'-GAACAAC TGCTCATCAC-3' and β-actin forward 5'-GACTATGA CTTAGTTGCGTTA-3' and reverse 5'-GTTGAACTCTC TACAT ACTTCCG-3'.

Statistical analysis – The results were expressed as the mean ± SD and statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA).

Results and Discussion

15-PGDH inhibitory activity and cytotoxicity – PGE_2 has been known as an important mediator of healing of oral and gastric ulcer, scalp hair, eyelashes, bone formation, and dermal wound healing and so on. Since 15-PGDH is the key metabolic enzyme of PGE_2, inhibition of 15-PGDH is supposed to facilitate previous biological effects by increasing PGE_2. Therefore, inhibitors of 15-PGDH will be valuable for the disease therapy requiring PGE_2 elevation. It is reported that 15-PGDH is inhibited by a variety of pharmacological agents including NSAIDs such as indomethacin, anti-paltelet aggregatory drugs such as panaxynol (Fujimoto *et al*., 1998), anti-allergic drugs such as flavonoid baicalein (Iijima *et al*., 1980), and so on.

Therefore, we have screened plant extracts for potential 15-PGDH inhibitory activity. The effective dose for 50% inhibition (ED_{50}) was used for the expression of 15-PGDH inhibitory activity. We found that methanol extract of *A. argyi* (AAME) contained potent 15-PGDH inhibitor ED_{50} value, 13.13 μg/mL (Table 1). In favor of pharmaceutical application, cytotoxicity is very important. The cytotoxicity of AAME was determined by MTT assay. IC_{50} (concentration for 50% of survival) of AAME in HaCaT cells appeared was 415.00 μg/mL.

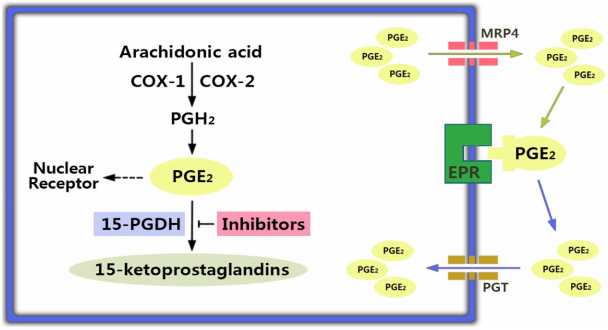

Fig. 1. The effect of AAME (15-PGDH inhibitor) on COX-1, COX-2, MRP4 and PGT which regulate the fate of PGE_2.

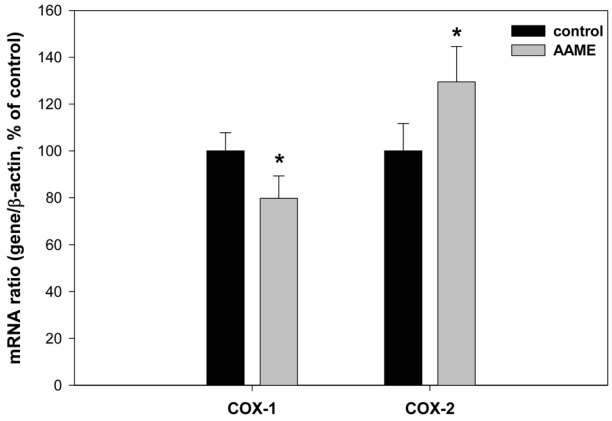

Fig. 2. Effect of AAME on mRNA expression of COX-1 and COX-2. Values are mean ± SD (n = 4). * $p < 0.05$.

Determination of extracellular and intracellular PGE_2 levels – It is necessary to test whether 15-PGDH inhibitor could increase PGE_2 in biological system or not. As shown in Table 2, AAME increased extracellular PGE_2 levels 16.74% and decreased intracellular PGE_2 levels 79.37% in HaCaT cells. Relatively low concentration of intracellular PGE_2 as compared with that of extracellular one after treatment with AAME suggests that other factors would be determinants in PGE_2 levels.

COX-1, COX-2, MRP4 and PGT expression – COX pathway increased PGE_2 level by inducing the synthesis of PGE_2 from AA in biological system. PGE_2 can cross through the membrane by simple diffusion or via a prostaglandin efflux transporter, such as MRP4. After acting via its EPR, pericellular PGE_2 is cleared via re-uptake by PGT and then rapidly metabolized by cytosolic 15-PGDH (Fig. 1). Thus, PGE_2 levels would be functions of expression levels of these genes. Therefore, we treated HaCaT cells with AAME and checked the regulation of COX-1, COX-2, MRP4 and PGT. Real-time PCR assay showed that AAME decreased expression of COX-1,

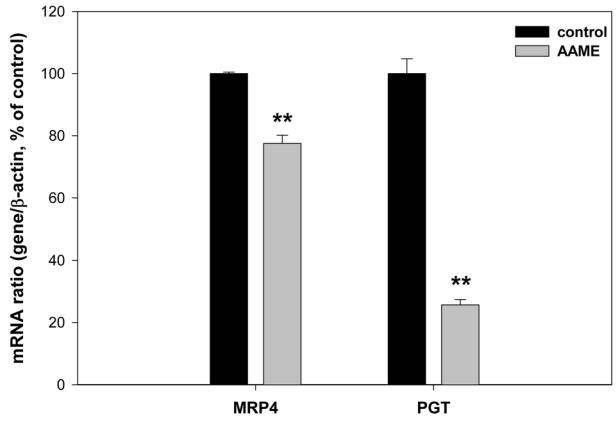

Fig. 3. Effect of AAME on mRNA expression of MRP4 and PGT. Values are mean ± SD (n = 4). ** $p < 0.005$.

MRP 4 and PGT (Fig 2, 3). Generally, PGE_2 elevation is mediated by COX-2 in pathological conditions (Takehara *et al*., 2006; Chan *et al*., 2007). In Table 2 and Fig. 2, intracellular PGE_2 level decreased despite increasing of COX-2 expression. It seems to be that decreasing COX-1 expression and obstruction of PGE_2 re-uptake by conspicuous decreasing of PGT expression caused a low intracellular PGE_2 level. In conclusion, low intracellular PGE_2 and high extracellular PGE_2 concentration after the treatment of AAME may be due to the inhibition of PGT expression as compared with that of MRP4. These results indicate that AAME could be applicable to functional materials for as a 15-PGDH inhibitor and PGT expression inhibitor.

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