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Cyclooxygenase-2 Can Modulate ICAM-1 Expression in Aorta or Heart Tissues of Rats Treated with Synthetic Estrogen or Soy-isoflavones

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ABSTRACT **: The identification of COX-2 (cyclooxygenase-2) has led to potential novel insights on disease pathogenesis (atherosclerosis, cancer, Alzheimer's disease) and the regulation of normal organ function. The present *in vivo* study with estrogen or soy-isoflavones has provided evidence for the association between COX-2 and ICAM-1 (Intercellular adhersion molecule-1). In the system of mature female rats, soy-isoflavones exerted more pronounced effect on ICAM-1 inhibitory and COX-2 stimulatory effect than estrogen. In the system of ovariectomized estrogen deficient rats, the down-regulatory properties of soy-isoflavones on ICAM-1 was less evident, whereas estrogen exerted the inhibitory activity. These results demonstrate that COX-2 limits adhersion molecule expression on rat aorta cells and suggest that COX-2 may play a protective role in cardiovascular system in mature female rats. Soy-isoflavones appear to have beneficial effect on vascular systems through modulation of ICAM-1 and COX-2, and these molecules appeared to be closely associated.**

Key words : **Cyclooxygenase-2, ICAM-1, soy-isoflavones, estrogen**

Introduction

Cyclooxygenase (COX), the central enzyme in prostanoid biosynthesis, is involved in the first step of prostanoid synthesis from arachidonic acid (Hyslop and De Nucci 1993). COX-2 is not normally present in the endothelium of major vessels, but overexpressed in endothelium and smooth muscle of atherosclerotic lesions (Schonbeck *et al.*, 1999; Baker *et al.*, 1999). Furthermore, evidence indicates that COX-2 plays important roles in the pathogenesis of diseases such as colon carcinoma, Alzheimer's disease, heart failure, and hypertension (Golden and Abramson 1999). Selective inhibition of COX-2 decreases atherogenesis in apo-E deficient mice (Belton *et al.*, 2003), and abrogates angiogenesis and suppresses tumorigenesis in colon cancer system (Ghosh *et al.*, 2000). Control of the transcriptional level by proximal 5'-elements, E-box and cAMP response elements is related to ultimate expression of COX-2 (Kim and Fischer 1998). And the post-transcription control is also possible (Shao *et al.*, 2000). Also a distal peroxisomal proliferators response element (PPRE) was identified to be sensitive to non-steroidal anti-inflammatory drugs or certain fatty acids (Meade *et al.*, 1999).

Antioxidants, such as vitamin E and many flavonoids, possess free radical scavenging effects. Some flavonoids are known to inhibit the production of COX-2 in certain systems (Park and Surh 2004). Genistein is a polyphenolic flavonoid and a potent antioxidant and anti-inflammatory agent. Numerous previous studies have also indicated that genistein has anti-inflammatory effects, which appear to be partly responsible for its antioxidant properties (reviewed in Park and Surh 2004). Genistein as a PKC inhibitor blocks transcriptional activities of COX-2 in macrophages (Liang *et al.*, 1999). Mutoh *et al.* (2000) have shown that genistein suppresses the COX-2 promoter activities. In contrast, 17-beta-estrogen has shown to mediate antioxidative effects, but possesses COX-2 up-regulatory properties (Ghanam *et al*, 2000).

The reported studies to evaluate the relationship

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between COX-2 and ICAM-1 have revealed down-regulatory properties of COX-2 on ICAM-1 (Bishop-Bailey etal., 1998). Adhesion of cells to other cells or to matrix of extracellular origin is controlled by several kinds of cell surface adhesion molecules. This phenomenon is important event in numerous physiological and pathological settings such as growth and differentiation, wound healing, cell migration, immune responses, and tumor invasion (Springer 1994). The adhesion molecules significantly correlated with tumor stage and the development of metastases of gastric cancer, colorectal cancer and breast cancer (O'Hanlon *et al.*, 2002). The suppression of COX-2 by NSAIDS was reported to influence the increase in leukocyte adherence (Muscara *et al.*, 2000).

In the present study it was explored whether genistein in the form of soy-isoflavones would modulate COX-2 expression in comparison with estrogen in adult female rat aorta or heart tissues, and this modulation would effect the regulation of ICAM-1 in these tissues in order to establish a molecular link between ICAM-1 and COX-2 *in vivo* system.

Materials and methods

Chemicals

17β-Estradiol, cholesterol, L-cysteine, α-cellulose, choline bitartrate and tert-butylhydroquinone were purchased from Sigma Chemical (St. Louis, Mo). Other reagents were of chemical grades and purchased from commercial reagent suppliers. Soy-isoflavones for feeding mature female rats were obtained from Tokiwa Phytochemicals, Japan, and soy-isoflavones in the form of GCP (Genistein Concentrated Polysaccharide: Amino Up Co., Sapporo, Japan) were used for supplementing ovariectomized rats. Corn starch was supplied by Miwon Co, Seoul, Korea; casein was a product of The New Zealand Dairy Board (Wellington, New Zealand); soybean oil and lard were commercial brands.

Animals and feeding regimens

Female Sprague-Dawley rats, six month old, were fed a standard laboratory diet (Cheil Feed Co., Seoul, Korea) for one week. Using a randomized complete block design, rats were divided by initial body weight into three groups of twelve. Rats were housed individually in an environmentally-controlled animal laboratory with a 12-h light:dark cycle. For five weeks, rats were fed one of the three diet regimens (Table 1) and water *ad libitum*. The basal diet was the high fat and high cholesterol. Diets were stored at –40°C before use. Six-week old female rats were ovariectomized or sham-operated, and afterward fed a laboratory diet for three weeks in order to get the full response of ovariectomy (i.e. post-menopausal state). Rats were housed individually in an environmentally-controlled animal laboratory with a 12-h light:dark cycle. For eight weeks, rats were fed one of the five diet regimens (Table 2) and water *ad libitum*. Diets were stored at –40°C before use.

Table 1. The composition of the diet in experiment using mature female rats

Groups	
1. Control	No supplementation
2. Estrogen supplemented	17β-estradiol (1100 *u*g/kg)
3. Soy-isoflavones supplemented	Soy-isoflavones (10 g/kg)

The basal diet was high fat (120 g lard/kg diet) and high cholesterol (1 g/kg diet)[1)].

1) High-fat and high-cholesterol diet contains corn starch 438 g; sucrose 100 g; soybean oil 41 g; lard 120 g; cholesterol 1 g; casein, 200; L-cysteine, 3.0; α-cellulose, 50; choline bitartrate, 2.5; tert-butylhydroquinone, 0.014; AIN 93G salt mix[2)], 35.0; AIN 93G vitamin mix[3)] 10.0 g/kg.

2) AIN 93G salt mix (g/kg): calcium carbonate, 357.0; potassium phosphate monobasic, 196.0; potassium citrate, 70.78; sodium chloride, 74.0; potassium sulfate, 46.6; magnesium oxide, 24.4; ferric citrate, 6.08; zinc carbonate, 1.65; manganous carbonate, 0.63; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenate, 0.01025; ammonium paramolybdate, 0.00795; chromium potassium sulfate, 0.275; sodium meta-silicate, 1.45; powdered sucrose, 221.2268

3) AIN 93G vitamin mix (g/kg): nicotinic acid, 3.0; calcium pantothenate, 1.6; pyridoxine hydrochloride, 0.7; thiamin hydrochloride, 0.6; riboflavin, 0.6; D-biotin, 0.02; folic acid, 0.2; vitamin B_{12}, 0.025; α-tocopherol acetate, 15.0; retinyl acetate, 0.8; vitamin D_3, 0.25; vitamin K_1, 0.075; powdered sucrose, 974.655

Tissue collection

After the designated periods of feeding the animals, the rats were anesthesized with ethylether and either aorta (mature female rats) or heart tissues (ovariectomized rats) were collected, frozen in liquid nitrogen and stored at –80°C before use.

Western Blotting

A small portion of tissues was homogenized and lysed with ice-cold lysis buffer, including 50 mM Tris-

Table 2. The composition of the diet in experiment using ovariectomized rats

Groups	
1. Sham-operated Control	No supplementation
2. Ovariectomized Control	No supplementation
3. Ovariectomized Estrogen	17β-estradiol (200 *u*g/kg)
4. Sham-operated Soy-isoflavones	GCP (0.8 g/kg)
5. Ovariectomized Soy-isoflavones	GCP (0.8 g/kg)

The basal diet was high fat (120 g lard/kg diet) and high cholesterol (1 g/kg diet)[1].

1) High-fat and high-cholesterol diet contains corn starch 438 g; sucrose 100 g; soybean oil 41 g; lard 120 g; cholesterol 1g;casein, 200; L-cysteine, 3.0; α-cellulose, 50; choline bitartrate, 2.5; tert-butylhydroquinone, 0.014; AIN 93G salt mix[2], 35.0; AIN 93G vitamin mix[3] 10.0 g/kg.

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HCl, pH 7.4, 1% MP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NAF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Solubilized proteins were centrifuged at 14,000xg in a microfuge (4°C) for 10 min, and supernatant protein was acquired. The cell lysates were separated on SDS-PAGE and then electrotransfered onto a nitrocellulose membrane (Scheicher & Schuell, Dassel, Germany). Antibodies (COX-2, ICAM-1, pERK, pJNK, pp38 and β-actin) were purchased from Santa Cruz Biotechnology (Santa Cruz, Ca). The transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences, USA) according to the manufacture's procedure.

Statistical analysis

Statistical significance between groups was determined by one-way analysis of variance (ANOVA) with Duncan's multiple range model and data are presented as means ± SD. Values of $P < .05$ were considered significant.

Results and Discussion

The effect of estrogen or soy-isoflavones on expressions of ICAM-1, COX-2 and mapkinases was monitored in the system of mature female rats or ovariectomized post-menopausal state rats. As shown in Fig. 1, the treatment of mature female rats with estrogen or soy-isoflavones significantly decreased expression of ICAM-1. Whereas, the stimulating effect on COX-2 by soy-isoflavones as well as estrogen was observed. Expression of ICAM-1 and VACM-1 is necessary for leukocyte adhesion to the endothelial surface, a process central to the pathogenesis of atherosclerosis. ICAM-1, an 80-114 kDa inducible surface glycoprotein belonging to the immunoglobulin superfamily, is involved in as wide range of inflammatory and immune responses (Saunton *et al.*, 1988). In the system of HUVEC, genistein, an active component of soy-isoflavones was shown to inhibit the secretion of ICAM-1 in a dose-dependent manner (May *et al.*, 1996). It is known that protein tyrosine kinase-mediated phosphorylation events are important for the regulation of adhesion molecule expression (Rimbach *et al.*, 2004). Estrogen was less potent than soy-isoflavones at inhibiting ICAM-1 expression in this study. Also the COX-2 stimulatory effect was greater with soy-isoflavones than estrogen. Recent studies demonstrated that estrogen, through activation of the estrogen receptor a, activates COX-2 (FitzGerald *et al.*, 2003). Estrogens are implicated in the aberrant cell proliferation observed in breast and reproductive-tract cancers (Hayashi *et al.*, 2003), and COX-2 is found to exist in many invasive tumors and in the genesis of breast cancer (Soslow *et al.*, 2000). Therefore, estrogens appear to elicit both beneficial and harmful effects in a tissue-dependent manner. However, genistein may confer beneficial effects in both the protection of atherosclerotic progress and the inhibition of genesis of breast cancer through COX-2 modulation. For example, in breast cancer cell lines, genistein was shown to be a potent inhibitor of COX-2, and in human endothelial cells genistein increased prostacyclin production through estrogen receptor-dependent mechanism, which involved the enhancement of COX-2 protein and activity (Hermenegildo *et al.*, 2005). Expression of COX-2 appears to be highly regulated by a number of mitogen-activated protein kinases (MAPK) and transcription factors (Xie and Herschman 1996; Cobb and Goldsmith 1995). The results of MAPK

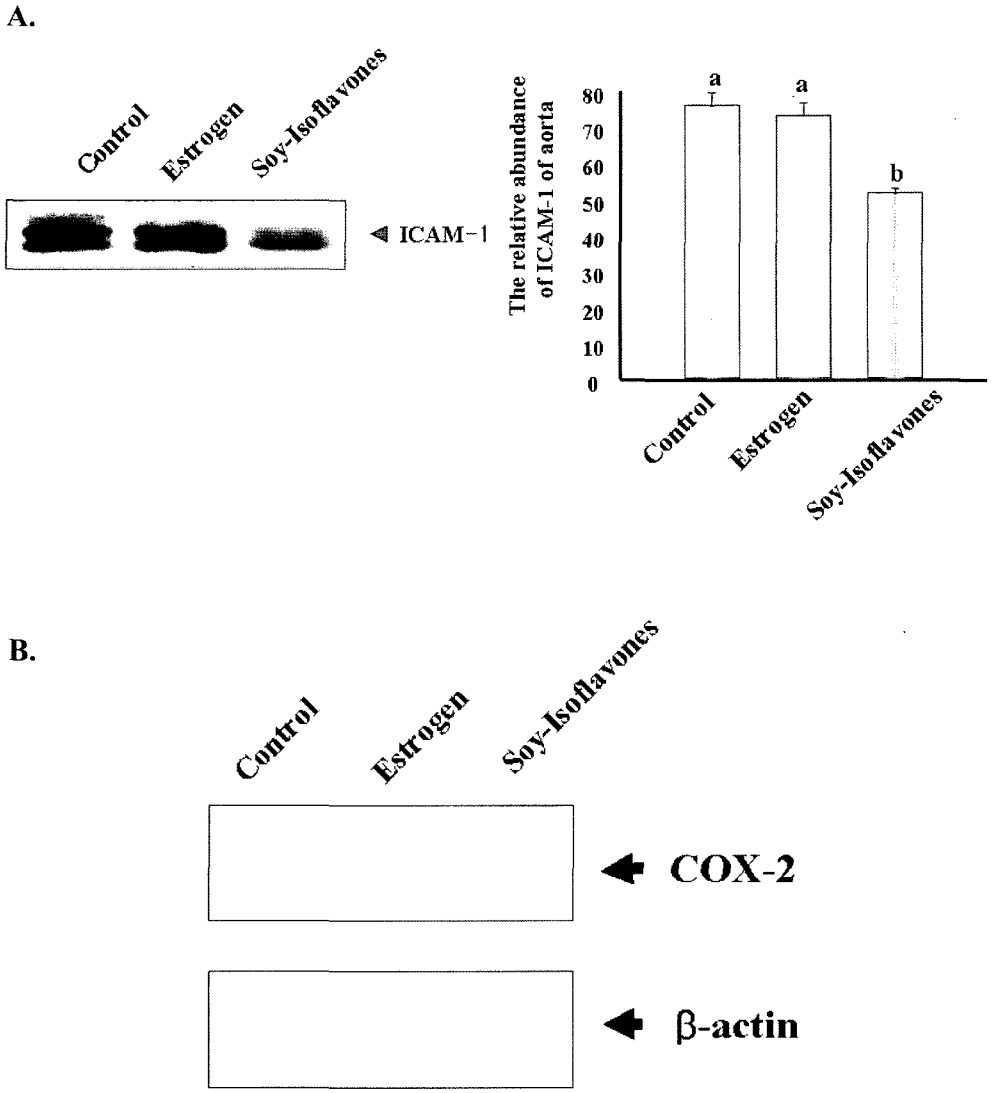

Fig. 1. Expression of ICAM-1 (A) and COX-2 (B) after estrogen or soy-isoflavones treatments in mature female rats. Rats were treated with estradiol or soy-isoflavones for four weeks. Tissues were collected and lysates were prepared, subjected to electrophoresis on 7% SDS-PAGE, Western- blotted, and visualized with ECL detection kit described in Materials and Methods. Lane 1. Control; lane 2. 17 β-estradiol treated and lane 3. soy-isoflavones-treated respectively as described in Materials and Methods.

regulation by estrogen or soy-isoflavones showed that ERK was related to COX-2 in soy-isoflavones treated animals, and p38 to COX-2 in estrogen treatment (Fig. 2A and Fig. 2B). In the system of ovariectomized estrogen deficient female rats, the up-regulation of ICAM-1 was found with ovariectomized groups of control and soy-isoflavones treated animals (Fig. 3A). In this system, supplementation of estrogen to ovariectomized rats did not stimulate ICAM-1. This shows clearly that in post-menopausal state there is an increased tendency of ICAM compared to sham-operated states, and this up-regulation cannot be reversed by the genistein, but by estrogen. COX-2 expression was down-regulated in ovariectomized control, sham-operated and ovariectomized soy-isoflavones groups compared to sham-operated control and estrogen treated groups (Fig. 3B). There were conflicting results of MAPK expression in ovariectomized rats (Fig. 4A and 4B). The stimulatory pattern of JNK was the opposite of COX-2. These results of

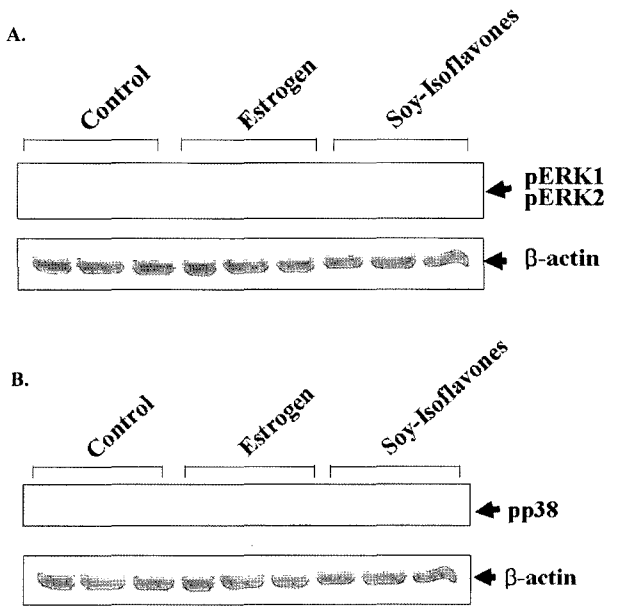

Fig. 2. Expression of pERK (A) and pp38 (B) after estrogen or soy-isoflavones treatments in mature female rats. Rats were treated with estradiol or soy-isoflavones for four weeks. Tissues were collected and lysates were prepared, subjected to electrophoresis on 7% SDS-PAGE, Western- blotted, and visualized with ECL detection kit described in Materials and Methods. Lane 1, 2, 3. Control; lane 4,5,6. 17 β-estradiol treated and lane 7,8,9. soy-isoflavones-treated respectively as described in Materials and Methods.

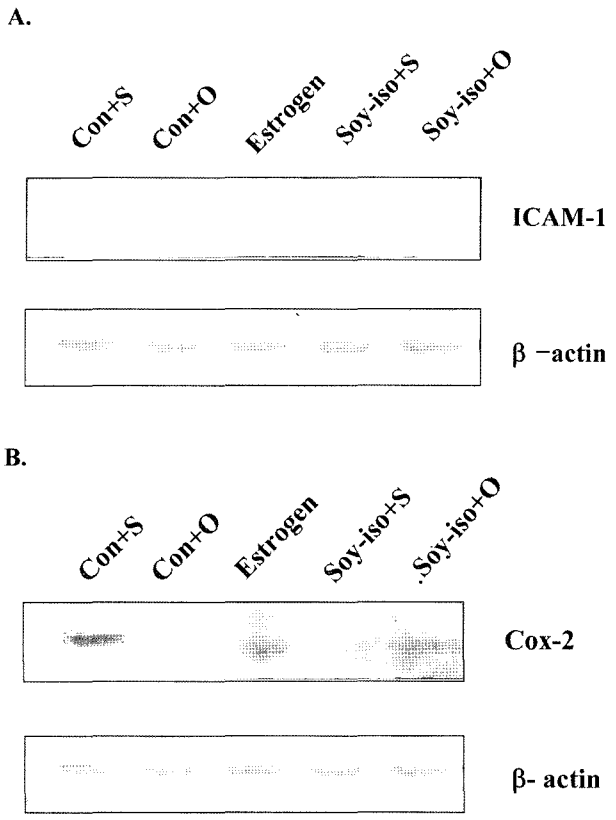

Fig. 3. Expression of ICAM-1 (A) and COX-2 (B) after estrogen or soy-isoflavones treatments in ovariectomized female rats. Rats were treated with soy-isoflavones, estrogen for eight weeks. Tissues were collected and lysates were prepared, subjected to electrophoresis on 7% SDS-PAGE, Western- blotted, and visualized with ECL detection kit described in Materials and Methods. Lane 1. Untreated control (sham); lane 2. Control (ovariectomized); lane 3. 17 β-estradiol treated (ovariectomized); lane 4. Soy-isoflavones (sham); lane 5. Soy-isoflavones (ovariectomized) as described in Materials and Methods.

ICAM-1 regulation showed that the connection between ICAM and COX-2 or mapkinases *in vivo* system might be complicated and influenced by factors such as the presence of endogenous and exogenous estrogen and the type of phytoestrogen. Genistein, the active component of soy-isoflavonoid, is a naturally occurring plant-derived compound. This biologically active type is originated from soybean products by conversion with intestinal bacteria, and known to be a hormone-like compound with antioxidative and estrogen receptor binding activities (Tom *et al.*, 1998). This plant-derived estrogen including other products such as daidzein and glycetin appear to exert both estrogenic and antiestrogenic effects on metabolism, depending on their biological concentration, endogenous estrogen levels and individual properties such as sex and menopausal status (Knight and Eden 1996;Adlercreutz and Mazur 1997).

To test the effect of soy-isoflavones or genistein on COX-2, ICAM-1 and mapkainses, 24-month old female rats were supplemented with estrogen alone or with soy-isoflavononones. The up-regulated state in ICMA-1 expression was observed with soy-isoflavones accompanied by the elevation of COX-2 expression. In the experiment using ovariectomized rats, the down-regulatory effect of soy-isoflavones on ICAM-1 expression was less evident, whereas estrogen exerted the inhibitory activity of ICAM-1 expression. Soy-isoflavones were shown to exert beneficial effects in vascular systems through modulation of ICAM-1 and COX-2, and these molecules appeared to be closely associated. However, this association can be varied by the physiological state of estrogen sufficiency or deficiency. A possible mechanism by which COX-2 affects ICAM-1 is not clear from this study. It has been shown that the down-regulation of ICAM-1 expression was achieved

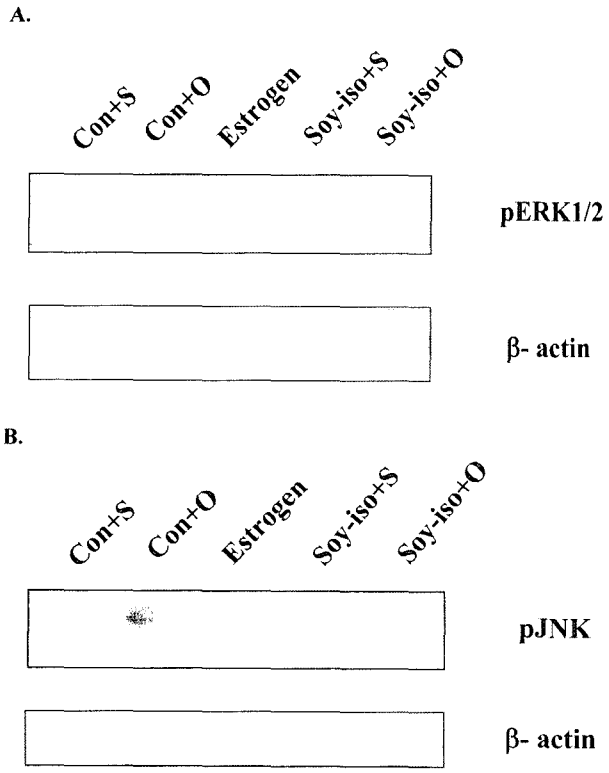

Fig. 4. Expression of pERK (A) and pJNK (B) after estrogen or soy-isoflavones treatments in ovariectomized female rats. Rats were treated with soy-isoflavones, estrogen for eight weeks. Tissues were collected and lysates were prepared, subjected to electrophoresis on 7% SDS-PAGE, Western- blotted, and visualized with ECL detection kit described in Materials and Methods. Lane 1. Untreated control (sham); lane 2. Control (ovariectomized); lane 3. 17 β-estradiol treated (ovariectomized); lane 4. Soy-isoflavones (sham); lane 5. Soy-isoflavones (ovariectomized) as described in Materials and Methods.

via EP2/EP4 receptors by prostaglandin E_2 in human fibroblast cells (Noguch *et al.*, 2001).

Since the dose of genistein or physiological status such as estrogen sufficiency and deficiency are turned out to be important factors from this study, the practical application of soy-isoflavones in the prevention of degenerative diseases or post-menopausal syndromes warrants in-depth study on these factors.

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