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Effects of a Selective COX-2 Inhibitor Celecoxib and Soy-Isoflavones on Molecular Markers Related to Apoptosis, and COX-2 and Mapkinase Expression in Estrogen-Fed Rats*

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The present study examined the effects of cyclooxygenase-2 (COX-2) inhibitor celecoxib or soy-isoflavones in the presence of estrogen on apoptosis related gene expression, COX-2 and mapkinase in 48-week old female rats. Expressions of bcl-2 and bax proteins, which are known to be involved in the regulation of apoptosis, were investigated in mammary glands and heart tissues. The elevated expression of bcl-2 expression was observed in mammary glands of celecoxib supplemented rats as well as soy-isoflavones. The mammary glands bcl-2/bax ratio was found to be higher in celecoxib or soy-isoflavones supplemented rats. However, in heart tissues, expression of bcl-2 and bax was in the order of control, celecoxib and soy-isoflavones. The up-regulation of COX-2 was observed in celecoxib or soy-isoflavones in mammary glands. The similar trend was not displayed with the mapkinase expression. In heart tissues, the down-regulation of COX-2 as well as mapkinase was observed in celecoxib or soy-isoflavones supplemented rats. Soy-isoflavones and celecoxib both had a similar regulatory pattern of bcl-2, bax and COX-2 in mammary glands, and in heart tissues, only COX-2 exhibited a similar down-regulatory property. These findings revealed that in estrogen sufficient state, celecoxib and soy-isoflavones might not exhibit proapoptotic potential or COX-2 inhibition in normal mammary glands.

Key Words: Soy-isoflavones, Celecoxib, Molecular markers related to apoptosis, Mature estrogen-supplemented rat

INTRODUCTION

Genistein (4,5,7-trihydroxyisoflavone), the major phytoestrogen in soybeans and other kinds of legumes, has a structural similarity to the estrogens. The molecular distances between the two hydroxyl groups on the equol nucleus of genistein and that residing in 17β-estradiol are similar and the hydroxyl groups confer a strong binding affinity to the estrogen receptor.[1] There are multiple lines of compelling evidence from several epidemiological studies supporting a positive association between dietary soy consumption and the reduction of cancer risk. Asian women with high soy intake have a low incidence of breast cancer[2,3] and the protection by soy intake is lost following the adoption of a Western-based diet in second generations emigrating to the US.[4] Furthermore, urinary levels of phytoestrogens were lower in breast cancer cases when compared to the case-controls.[5,6] Despite the close association between soy product intakes and the risk of breast cancer in many epidemiological studies, the biochemical role of genistein for understanding and recommending its use as a chemopreventive agent has not been clarified.

The maintenance of normal tissues involves apoptosis, a regulated cell death process. Abnormal regulation of apoptosis is related to many disorders including tumor promotion, autoimmune and immunodeficiency diseases, and neurodegenerative pathologies. One of the major genes responsible for regulating apoptotic cell death is *bcl-2* that encodes a 26 kDa protein found in the nuclear envelope, parts of the endoplasmic reticulum, and the outer mitochondrial membrane. Bcl-2 family proteins play a role in regulating apoptosis.[7] Overexpression of bcl-2 enhances the survival of several cell types and prevents apoptosis induced by various chemotherapeutic drugs.[8] Whereas bax represents a pre-apoptotic member of the bcl-2 family, which controls important checkpoints during the apoptotic process. Overexpression of bax has been shown to accelerate the cell death.[9] Li *et al.* have suggested that the up-regulation of bax may be the molecular mechanism by which genistein induces apoptosis.[10] A number of cellular and animal model experiments have indicated that COX-2 may play a role in the apoptosis. The overexpression of COX-2 has shown

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to be associated with the inhibition of apoptosis and further the cause of tumorigenesis. It has been also observed that COX-2 is overexpressed in many tumors, and this up-regulation of COX-2 has been shown to promote cancer progression and recurrence.[11-13] In addition, the selective COX-2 inhibitor such as celecoxib or NS398 has been found to induce apoptosis, which may contribute to their antitumor effects.[14,15]

The purpose of this study was to examine the modulation of the expression of *bcl-2* and *bax*, whose gene products are known to be involved in the regulation of apoptosis, by soy-isoflavones in comparison with celecoxib, a selective COX-2 inhibitor in normal estrogen-fed female rats. Also, the interaction between apoptosis-related protein expression and COX-2 expression was examined in mammary glands and heart tissues in order to evaluate tissue differences. In the state of estrogen sufficiency, the regulatory pattern of the cell signal proteins by a selective COX-2 inhibitor celecoxib and soy-isoflavones might provide the background information of their chemopreventive potential.

MATERIALS AND METHODS

1. Chemicals

17β-estradiol, cholesterol, L-cysteine, α-cellulose, choline bitartrate and tert-butylhydroquinone were purchased from Sigma Chemical, St. Louis, USA. Soy isoflavones were the mixture of genistein, daidzein and glycetin (Tokiwa Phytochemical Co, Japan). Celecoxib was in the form of Celebrex (Korea Searle Co.). Other reagents were all chemical-grade and purchased from commercial reagent suppliers. 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.

2. Animals and Feeding Regimens

Female Sprague-Dawley rats (Daehan Animal Co), forty-eight weeks old, were fed a standard laboratory diet (manufactured by Cheil Feed Co., Korea) for one week. Using a randomized complete block design, rats were divided by initial body weight into three groups of nine. Rats were housed individually in an environmentally-controlled animal laboratory with a 12-h light:dark cycle. The experimental groups were estrogen control (control), celecoxib+estrogen, and soy-isoflavones+estrogen, and three groups of rats were fed one of the three dietary regimens (Table 1) for four weeks. During this period, rats were fed the diets and water *ad libitum*. Diets were stored at -40 ℃ before use.

Table 1. The treatment groups and the composition of diets.[1] The basal diet was high fat (120 g lard/kg diet) and high cholesterol 1 g/kg diet[2]

	Groups
Estrogen Control	17β-estradiol (500 mg/kg)
Celecoxib supplemented	celecoxib (500 mg/kg)+17β-estradiol (500mg/kg)
Soy-isoflavones supplemented	soy-isoflavones (10 g/kg)+17β-estradiol (500mg/kg)

1) Each group of nine rats were fed orally high fat · high cholesterol diet containing indicating amount of either 17β-estradiol celecoxib+17β-estradiol or soy-isoflavones+17β-estradiol per kg diet.
2) High-fat and high-cholesterol diet contains Corn starch 438 g; sucrose 100 g; soybean oil 41 g; lard 120g; cholesterol 1g;casein,200g; L-cysteine, 3.0 g; α-cellulose, 50 g; choline bitartrate, 2.5g; tert-butylhydroquinone, 0.014g; AIN 93G salt mix[3], 35.0 g; AIN 93G vitamin mix[4] 10.0g/kg.
3) 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
4) 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, 0.075; powdered sucrose, 974.655

3. Tissue Preparation

Rats were fasted for 14 hr before the end of the experiment and anesthetized with ether. Mammary gland tissues were collected from 4 mammary buds and frozen with liquid nitrogen, and stored at -80 ℃ until the analyzed. Rats were then dissected and the portion of heart tissues was isolated and frozen with liquid nitrogen, and stored at -80 ℃ until analyzed.

4. Western Blotting

Collected tissues were lysed in ice-cold 120 ml lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.4, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, protease inhibitor cocktail tablet (Boehringer Mannheim, Mannheim, Germany)) for 40 min. Lysates were centrifuged at 14,800x g for 30 min, and aliquots of supernatant containing 30 mg protein were boiled in SDS sample loading buffer for 5 min before electrophoresis on 12% SDS-polyacrylamide gel. After 3 h transfer of SDS-polyacrylamide gel to PVDF membrane (Amersham Life Sciences, Arlington Heights, IL), the blots were blocked with 5% fat-free dry milk-PBST buffer Phosphate-buffered saline (PBS) containing 0.1% Tween-20) for 2 h at room temperature and then washed in PBST buffer. The membranes were incubated for 1 h at room temperature with 1:1000 dilution of goat bcl-2, bax, COX-2 or ERK polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. Blots were rinsed with PBST, incubated with 1:5000 dilution of anti-gout-horseradish peroxidase conjugated-secondary antibody and then washed again three times in PBST

buffer for 5 min. The transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences, USA) according to the manufacture's procedure.

5. Statistical Analyses

ANOVA was performed to determine whether there were significant differences among the groups ($p<0.05$). When ANOVA indicated any significant difference among the means, the Duncan follow-up multiple comparison test was used to determine which means were significantly different.

RESULTS

To investigate the effect of celecoxib and soy-isoflavones in the presence of estrogen on the expressions of molecular markers related to apoptosis in animal model system, bcl-2 and bax expression, and the ratio of bcl-2 and bax were monitored. In the system of estrogen supplemented rat mammary glands, celecoxib or soy-isoflavones induced the up-regulation of bcl-2 and the down-regulation of bax protein (Fig. 1) when com- pared to control rats. The up-regulation of bax was ob- served in the estrogen control group mammary glands, and the bcl-2-bax-ratio showed a decreased value in estrogen control animals when compared to celecoxib or soy-isoflavones. In heart tissues, the expressions of bcl-2 and bax showed the increasing trend in the order of control, celecoxib and soy-isoflavones (Fig. 2). The highest bcl-2-bax-ratio was observed in celecoxib treated animals (Fig. 2). It was tested whether the regulation of bcl-2 or bax was accompanied with the COX-2 modulation, and it was shown that celecoxib or soy-isoflavones treatment induced the COX-2 expression (Fig. 3). One of the

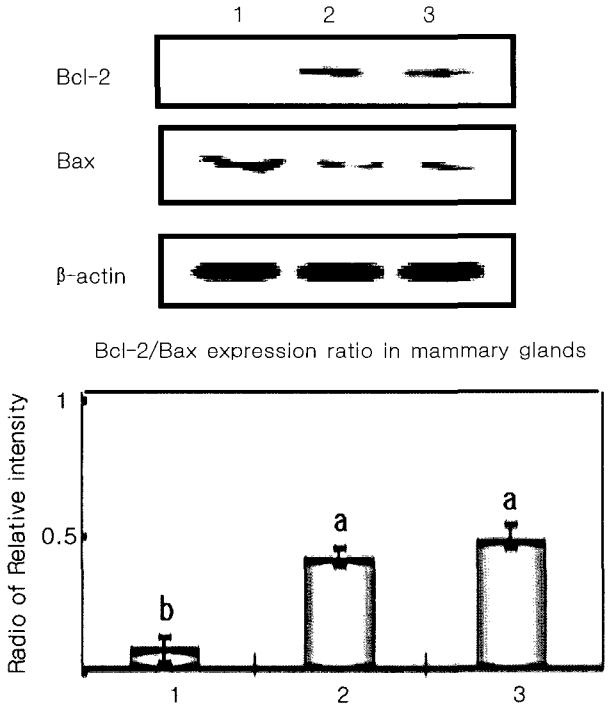

Fig. 1 Expression of bcl-2 and bax proteins (top) and bcl-2/bax ratio (bottom) of mammary tissues after estrogen or estrogen along with celecoxib or soy-isoflavones treatments in female rats. Rats were treated with estradiol, celecoxib + estradiol and soy-isoflavones +estradiol for four weeks. Mammary 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. celecoxib treated and lane 3. soy-isoflavones treated respectively as described in Materials and Methods. Bcl-2 and bax ratio was expressed as ratio of relative intensity of the each band. Values with different letters in the graph are significantly different ($p<0.05$)

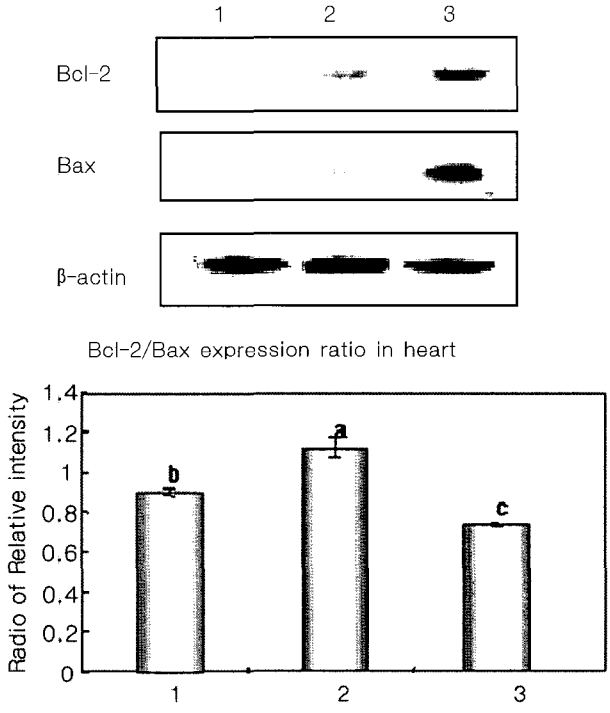

Fig. 2 Expression of bcl-2 and bax proteins (top) and bcl-2/bax ratio (bottom) of heart tissues after estrogen or estrogen along with celecoxib or soy-isoflavones treatments in female rats. Rats were treated with estradiol, ccelecoxib + estradiol and soy-isoflavones +estradiol for four weeks. Heart tissues 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. celecoxib treated and lane 3. soy-isoflavones treated respectively as described in Materials and Methods. Bcl-2 and bax ratio was expressed as ratio of relative intensity of the each band. Values with different letters in the graph are significantly different ($p<0.05$)

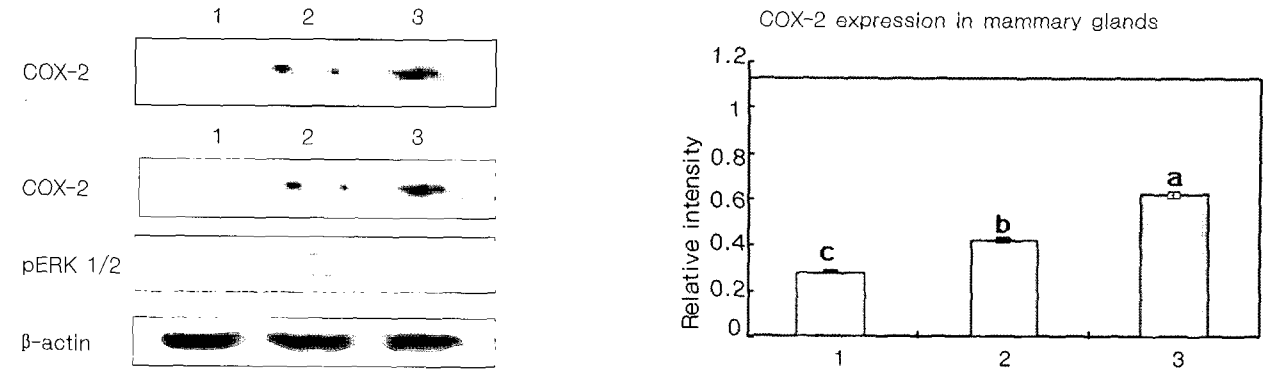

Fig. 3 Expression of COX-2 (top) and pERK1/2 (bottom) of mammary tissues after estrogen or estrogen along with celecoxib or soy-isoflavones treatments in female rats. Rats were treated with estradiol, celecoxib+estradiol and soy-isoflavones+estradiol for four weeks. Mammary tissues were collected and 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. celecoxib treated and lane 3. soy-isoflavones treated respectively as described in Materials and Methods. COX-2 expression was graphed as relative intensity of the each band. Values with different letters in the graph are significantly different ($p<0.05$)

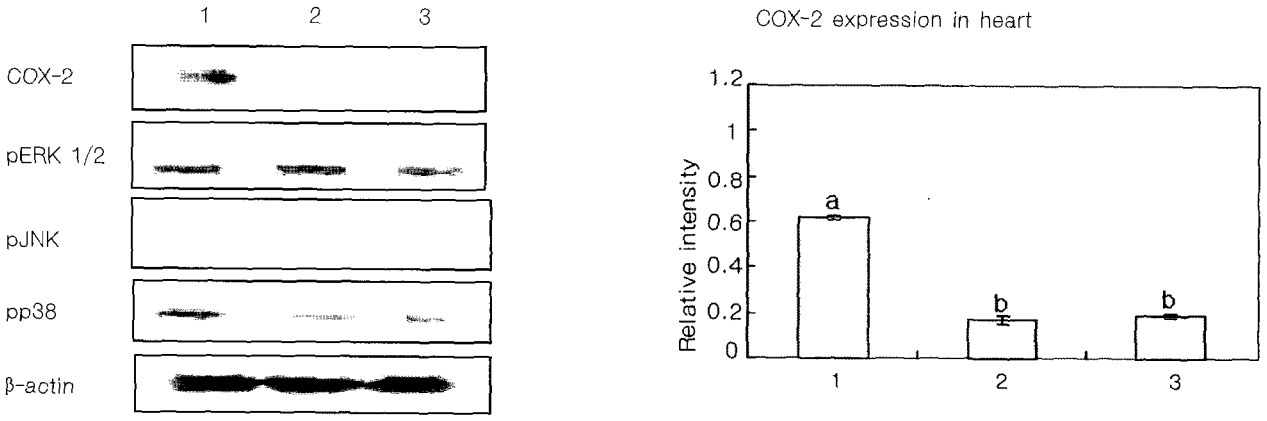

Fig. 4 Expression of COX-2 (top), and pERK1/2, pJNK and pp38 (bottom) of heart tissues after estrogen or estrogen along with celecoxib or soy-isoflavones treatments in female rats. Rats were treated with estradiol, celecoxib+estradiol and soy-isoflavones+estradiol for four week. Heart tissues were collected 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. celecoxib treated and lane 3. soy-isoflavones treated respectively as described in Materials and Methods. COX-2 expression was graphed as relative intensity of the each band. Values with different letters in the graph are significantly different ($p<0.05$)

mapkinases pERK was not stimulated by the up-regulation of COX-2. This mapkinase expression was up-regulated in celecoxib treated rats, but down-regulated in soy-isoflavones treated animals (Fig. 3). Heart tissues treated with either celecoxib or soy-isoflavones showed a marked decrease in the COX-2 expression with a more prominent effect in the COX-2 inhibitor treatment (Fig. 4). Among three mapkinases tested, pp38 exhibited the similar pattern of expression with COX-2 (Fig. 4).

DISCUSSION

In this study, the effects of celecoxib and soy-isoflavones on cell-signaling protein expression related to apoptosis such as bcl-2 and bax, and COX-2 and mapkinase were examined in comparison with the estrogen supplemented control. The rats used in this study were 48-week old representing the perimenopausal state, and estrogen was supplemented in the control group as well as celecoxib and estrogen groups in order to mimic the state of hormonal replacement therapy. It has been shown

that estrogen produces the alkylation of cellular molecules, and generates active potential carcinogenic metabolites such as catechol estrogens and 16-β-hydroxyestrone.[16-19] It has been also observed that there are strong correlations between estradiol and estrone levels in postmenopausal women and subsequent breast cancer risk.[20-22] Genistein, the active component of soy-isoflavones is a naturally occurring plant-derived compound. This biologically active type is originated from soybean products through the conversion by intestinal bacteria, and known as a hormone-like compound with antioxidative and estrogen receptor binding activities.[23] 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 gender and menopausal status.[24,25] Initially, the interference at the level of estrogen receptor was suggested as a major role of genistein in the inhibition of tumor-promoting effect of estrogens.[26,27] At the concentration lower than 10 micromol/L, the growth of MCF-7 cell, an estrogen receptor positive cancer cell line, was stimulated by genistein, however, genistein did not stimulate the growth of estrogen receptor-negative breast cancer cell. The antiestrogenic activity may be partially explained by the competition with endogenous 17β-estradiol for estrogen receptors.[27] Depending upon the concentrations of phytoestrogen, they might show either estrogen agonist or antagonist actions.[28,29] Also, the interaction with two kinds of estrogen receptors (alpha and beta) appeared to play a crucial role in exhibiting the complex responses of phytoestrogens.[30-33] The recent mechanism of genistein on chemopreventive actions has been emerged in the field of signal transduction regulatory proteins.[34,35]

To test the effect of celecoxib and soy-isoflavones on apoptosis related molecular markers in the presence of estrogen, 48-week old female rats were supplemented with estrogen alone or the combination of estrogen and celecoxib or estrogen and soy-isoflavones. The up-regulated state in the antiapoptotic protein bcl-2 expression was observed in the combination of estrogen and celecoxib or soy-isoflavones. The down-regulation of bcl-2 by estrogen could be reversed by the co-treatment with celecoxib or soy-isoflavones. In this study, bcl-2 lowering or bax-elevating effect of soy-isoflavones was not observed, and the highest bcl-2/bax ratio was found in soy-isoflavones supplemented rats. This observation can be explained by the differences in normal or cancerous mammary cells as well as the relatively young age of rats used. Furthermore, in the developmental stage of maturation of rats, estrogen and soy-isoflavones may show synergistic effects in the regulation of bcl-2 and bax. Another possible explanation might be the dose of soy-isoflavones used. It was observed that the effect of soy-isoflavones on bcl-2 expression was dose-dependent, showing a stimulated expression at the low concentration and decreased expression with higher level of soy-isoflavones (unpublished result). Also, in genistein-treated MCF-7 cells, bcl-2 phosphorylation was increased at 24 to 48 h, while bax expression was not elevated during the same period.[36] The concentration dependency of bcl-2 expression has been observed in genistein-treated MCF-7 cells.[37] At the threshold concentration of genistein capable of inducing apoptosis, bcl-2 expression was not elevated.[37] However, at the higher concentrations, both bcl-2 and bax were up-regulated. Considering that estrogens are potent mitogens in the mammary gland playing a pivotal role in the development and progression of mammary carcinoma,[38] the supplementation of estrogen with soy-isoflavones at the premenopausal state may have more detrimental effect. Tissue differences in the regulation of bcl-2 and bax were clearly shown in this study. The stimulation of both bcl-2 and bax was observed in the soy-isoflavones treatment. The observation of the differed response of bcl-2 and bax by mammary glands and heart requires further investigation to give full explanation.

The arachidonate cascade generates a series of lipid mediators to regulate various biological events such as cell proliferation, differentiation and inflammation through COX pathways.[39-41] In female rats, oral supplementation of estrogen, estrogen+soy-isoflavones and estrogen +celecoxib showed different COX-2 expressions in mammary glands. Celecoxib in the presence of estrogen down-regulated the COX-2 expression when compared to estrogen+ soy-isoflavones. The clear down-regulation of COX-2 by either celecoxib or soy-isoflavones was observed in heart tissues. Previous studies have reported that cyclooxygenase modulates vascular contractions *in vitro*, by endothelium-dependent and independent mechanisms.[42,43] Also, there is evidence of localized induction of COX-2 in atherosclerotic plaques in an animal model of atherosclerosis.[44] It has been suggested that the induction of COX-2 in atherosclerotic lesions may play a role in altering the localized prostanoid production and propagating the events, which lead to disease progression by favoring cell adhesion and proliferation.[45] Indomethacin, anonselective cyclooxygenase inhibitor, has been shown to improve endothelium-dependent relaxations in mesenteric arteries from spontaneously hypertensive rats (SHR), presumably by blocking the production of vasoconstrictor prostaglandins.[46] On the other hand, the aortic relaxation induction by 17β-estradiol in cholesterol-fed rabbits was observed and the involvement of the COX-2 in this protective effect on hypercholesterolemic rabbits aorta has been suggested.[47]

This study has shown that both bcl-2 and bax were

regulated by soy-isoflavones or celecoxib in mammary glands of normal female rats on the co-treatment with estrogen. In this normal tissue, the proapoptotic potential of soy-isoflavones in the presence of estrogen was not evident from the pattern of bcl-2 and bax expressions. The observed differences in the activity of soy-isoflavones to estrogen alone in these signal transduction proteins require further investigation. Since the dose of soy-isoflavones or physiological status such as estrogen sufficiency and deficiency are turned out to be important factors from the previous study, the practical application of soy-isoflavones in the prevention of degenerative diseases or post-menopausal syndromes warrants in depth study. The up-regulation of bcl-2 was accompanied by the increased expression of COX-2 in soy-isoflavones or celecoxib treated mammary glands. The relevance of up-regulation of COX-2 to certain mammary tumorigenesis applied in this normal system is not clear at present. It appears that there exists a possibility of the agonostic effect of soy-isoflavones to estrogen in the COX-2 regulation in mammary glands. The noticeable tissue differences in bcl-2, bax, and COX-2 regulation were observed between mammary glands and heart. In heart tissues, the marked up-regulation of bcl-2 and bax and the down-regulation of COX-2 were observed in both soy-isoflavones and celecoxib treatments. In the previous study, high concentrations of both soy-isoflavones and estrogen have down-regulated the COX-2 expression in estrogen-deficient animals. Cellular mechanisms underlying the increased expression of COX-2 in mammary glands and the decreased expression of COX-2 by soy-isoflavones in heart are not clear at present. The marked depression of COX-2 expression by celecoxib was more evident in heart tissue than mammary glands.

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