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Modulation of Apoptosis-related Signal Transduction by Celecoxib, a Selective COX-2 Inhibitor in Comparison with Estrogen in Perimenopausal Mammary Glands

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This investigation was intended to study the expression of cyclooxygenase-2 (COX-2) and mapkinase, and apoptosis related gene expression in normal mammary glands of perimenopausal female rats fed oral celecoxib, a selective COX-2 inhibitor and estrogen. The expression of pERK1/2 showed the similar patterns as COX-2 by the oral treatment of celecoxib and estrogen. It was found that celecoxib induced up-regulation of bcl-2 in mammary gland buds. The regulation of bax was decreased in celecoxib supplemented rats. The bcl-2/bax ratio was higher in celecoxib supplemented rats. However, bcl-2/bax ratio was highest in celecoxib group. The up-regulation of COX-2 was observed in celecoxib in mammary gland buds. The similar trend was not displayed with mapkinase expression. Compared to estrogen feeding, bcl-2 expression was upregulated in celecoxib and down-regulating effect was observed with bax expression. The up-regulation of bcl-2 was accompanied by the decreased expression of COX-2. The oral administration of celecoxib caused significant reduction in bcl-2/bax ratio compared to the control indicating that there might be more apoptotic activity in celecoxib treatment. However, estrogen, a known stimulator of cell proliferation also showed the apoptotic potential compared to control or celecoxib. The increased apoptotic potential by celecoxib or estrogen resulted from the different patterns in bcl-2 or bax regulation. The lowering of bcl-2/bax ratio by celecoxib was resulted from the increased expression of bas, while the reduction of bcl-2/bax observed with estrogen was from the decreased bcl-2 and the increased bax. These findings suggest that both bcl-2 and bax are involved in the apoptotic control of celecoxib, and bcl-2 is a significant factor in apoptotic control of estrogen.

Key Words: Celecoxib, COX-2, Molecular markers related to apoptosis, Perimenopausal female rat, Mammary gland buds

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INTRODUCTION

It has been shown through *in vitro* and *in vivo* studies that estrogen might play a central role in the promotion of breast cancer and possibly in the initiation process.[1,2] *In vitro*, estrogen exerts direct and indirect proliferative effects on breast cancer cells.[3] Estrogen produces alkylation of cellular molecules, and generates active potential carcinogenic metabolites such as catechol estrogens and 16 α-hydroxyestrone.[4~7] It has been also shown that there are strong correlations between estradiol and estrone levels in postmenopausal women and subsequent breast cancer risk.[8~10] Along with the significance of the involvement of estrogen in breast cancer development, the importance of the modulation of signal transduction pathways for chemoprevention of breast cancer is being recognized.[11] It has been observed that COX-2 expression is seen in almost all tumor sites.[12] Fifty-six percent of all breast tumors express COX-2 at moderate to high level.[13] In rodent breast cancer models, COX-2 is expressed in both carcinogen-induced and oncogene-induced mammary tumors.[14,15] Evidence is indicating that the selective COX-2 inhibitors protect against breast cancer. Preclinical studies have shown that celecoxib reduced tumor incidence and growth of 7,12-dimethylbenzanthracene-induced mammary tumors in rats.[16~18] In a mouse model, the COX-2 inhibitor SC236 was effective in inhibiting mammary tumor growth.[19] Recent epidemiological data also suggested that aspirin and nonsteroidal anti-inflammatory drugs reduce the risk of breast cancer. The dramatic reduction of breast cancer incidence in women was shown with the supplementation of over-the-counter non-steroidal anti-inflammatory drugs in the Women's Health Initiative study.[20] COX-2 inhibitors have been shown to decrease cell proliferation,[21,22] stimulate apoptosis,[23] and inhibit angiogenesis.[24]

Apoptosis is a form of geneticaly programmed cell death, which plays a key role in regulation of cellularity in a variety of tissue and cell types, and a mechanism by which tissue removes unwanted, aged or damaged cells. Abnormal regulation of apoptosis is related to many disorders including tumor promotion, autoimmune and immunodeficiency disease, 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.[25] Overexpression of bcl-2 enhances the survival of several cell types and prevents apoptosis induced by various chemotherapeutic drugs.[26] 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.[27] A number of cellular and animal model experiments indicate that COX-2 may play a role in apoptosis. The overexpression of COX-2 is shown to relate to 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.[28~30] 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.[30,31]

The purpose of this study was to examine the modulation of the expression of *bcl-2* and *bax*, whose gene products are known to in involved the regulation of apoptosis, by celecoxib, a selective COX-2 inhibitor in comparison with estrogen in perimenopausal female rats. Also the interaction between apoptosis-related protein expression and COX-2 expression was examined in mammary gland buds. The changes in apoptosis with supplementation of chemopreventive agents might provide the basis for the alteration of apoptosis related proteins in comparison with estrogen.

MATERIALS AND METHODS

1) Chemicals

17 β-estradiol, cholesterol, L-cysteine, α-cellulose, choline bitartrate and tert-butylhydroquinone were purchased from Sigma Chemical, St. Louis, USA. Celecoxib is the product 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, forty-eight weeks old, were fed a standard laboratory diet (manufactured by 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 nine. Rats were housed individually in an environmentally-controlled animal laboratory with a 12-h light:dark cycle. For four weeks, rats were fed one of the three diet regimens (Table 1) and water *ad libitum*. Diets were stored at $-40^{\circ}C$ before use.

3) Tissue Preparation

Rats were fasted for 14 hr before the end of the experiment and anaesthetized with ether. Mammary gland tissues were collected from 4 mammary buds and frozen with liquid nitrogen, and stored at $-80^{\circ}C$ until analysis.

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 aliqouts 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

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

Groups	
1. Control	Basal diet
2. Celecoxib supplemented:	celecoxib (500 mg/kg)
3. Estrogen supplemented:	17 β-estradiol (500 μg/kg)

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 g; L-cysteine, 3.0 g; α-cellulose, 50 g; choline bitartrate, 2.5 g; tert-butylhydroquinone, 0.014 g; AIN 93 G salt mix[2)], 35.0 g; AIN 93 G vitamin mix[3)] 10.0 g/kg.

2) AIN 93 G 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 93 G 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

were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences, USA) according to the manufacture's procedure.

5) Measurement of estradiol and leptin

Plasma estradiol was measured by γ-counter (Cobra 5010 II, Packard, USA) using coat-A count estradiol kit (DPC, Diagnostic Products, Co.) and leptin concenrations were measured by by γ-counter (Cobra 5010 Quantum, Packard, USA) using human leptin RIA kit (Finco research, Inc., USA).

6) 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

Fig. 1 shows the estrogen concentrations of rats grouped 1. control, 2. celecoxib supplemented and 3. estrogen supplemented. Compared to the control, the estrogen group showed the higher estrogen level, and celecoxib showing the in-between values from control and estrogen. The leptin concentrations were compared, and there were no significant differences in their concentrations (Fig. 2). The effects of celecoxib in comparison with estrogen on expressions of COX-2 and molecular markers related to apoptosis in animal model system, bcl-2 and bax expression, the ratio of bcl-2 and bax were examined. In the system of perimenopausal rat mammary gland buds, celecoxib treatment resulted in the down-regulation of COX-2, and the further down-regulation was noticed with estrogen treatment (Fig. 3). The similar trend of pERK1/2 regulation was observed (Fig. 3). Celecoxib induced no changes in bcl-2, but increased bax protein (Fig. 4) compared to control. There were the marked dexpression of bcl-2 expression by estrogen (Fig. 4). The expression of bax showed the increasing trend in the order of control, celecoxib and estrogen (Fig. 4). The significant differences in bcl-2-bax-ratio was observed showing control the highest value, the next

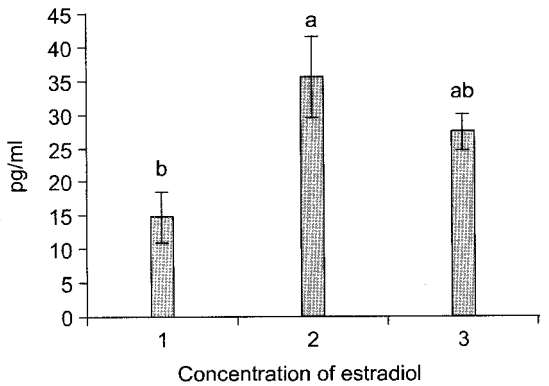

Fig. 1. Concentrations of plasma estradiol after celecoxib or estrogen treatments in perimenopausal female rats. Rats were treated with basal diet, celecoxib or estrogen supplemented diets for four weeks. Lane 1. Control; lane 2. celecoxib treated and lane 3. estrogen treated respectively as described in Materials and Methods. Values with different letters in the graph are significantly different ($p < 0.05$).

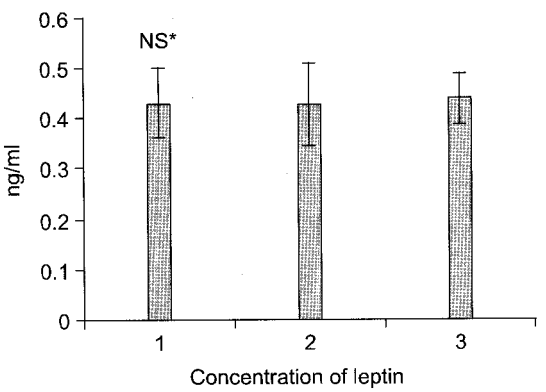

Fig. 2. Concentrations of plasma leptin after celecoxib or estrogen treatments in perimenopausal female rats. Rats were treated with basal diet, celecoxib or estrogen supplemented diets for four weeks. Lane 1. Control; lane 2. celecoxib treated and lane 3. estrogen treated respectively as described in Materials and Methods.
*Not significantly different.

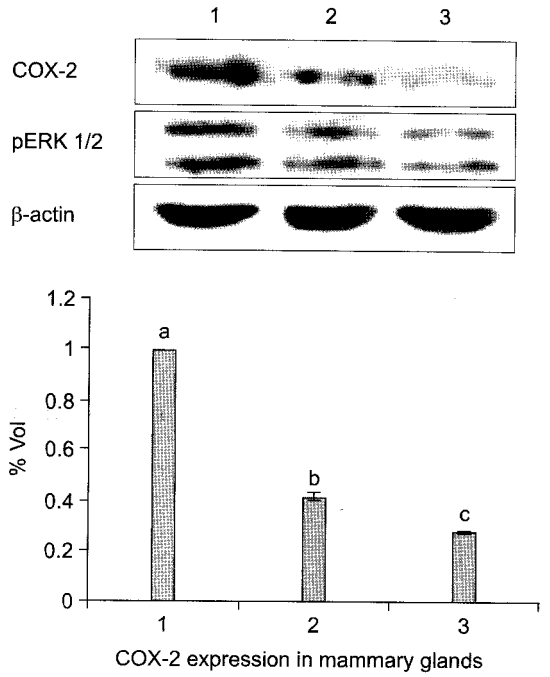

Fig. 3. Expression of COX-2 (top) and pERK1/2 (bottom) after celecoxib or estrogen treatments in perimenopausal female rats. Rats were treated with basal diet, celecoxib or estrogen supplemented diets for four weeks. Mammary tissues were collected and lysates were prepared, subjected to electrophoresis 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. estrogen treated respectively as described in Materials and Methods. Values with different letters in the graph are significantly different ($p<0.05$).

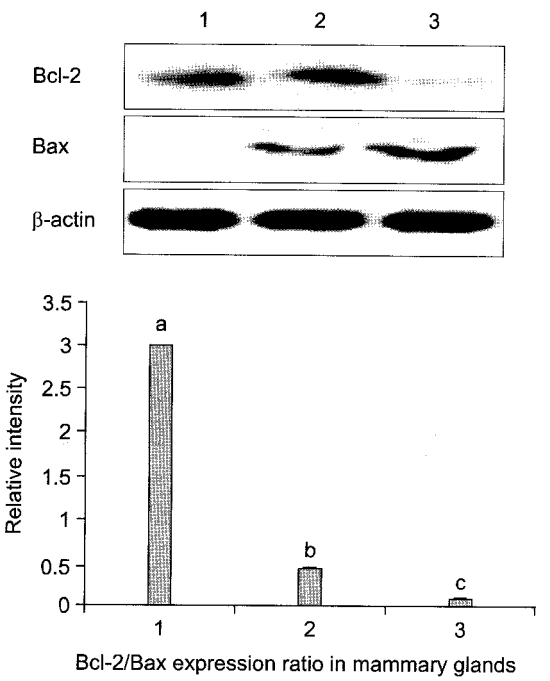

Fig. 4. Expression of bcl-2 and bax (top) and bcl-2 and bax ratio (bottom) after celecoxib or estrogen treatments in perimenopausal female rats. Rats were treated with basal diet, celecoxib or estrogen supplemented diets for four weeks. Mammary tissues were collected and lysates were prepared, subjected 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. estrogen treated respectively as described in Materials and Methods. Values with different letters in the graph are significantly different ($p<0.05$).

as celecoxib and the lowest was estrogen (Fig. 4).

DISCUSSION

The effects of celecoxib or estrogen on cell-signaling protein expression related to apoptosis such as bcl-2 and bax as well as COX-2 were investigated. The peri-menopausal female rats were supplemented with celecoxib or estrogen. It is known that the arachidonate cascade generates a series of lipid mediators to regulate various biological events such as cell proliferation, differentiation and inflammation through COX pathways.[32~34] In perimenopausal female rats, oral supplementation of celecoxib or estrogen showed different COX-2 expressions in mammary gland buds. Celecoxib clearly down-regulated COX-2 expression, and further down-regulation was observed with estrogen supplementation. COX-2 expression is a critical part of inflammation and plays a major role in defending against exogenous stimuli,[35] whereas its over-expression causes cells to exhibit tumor phenotypical changes.[36] Several studies indicated that COX-2 expression is associated with parameters of aggressive breast cancer.[12,30,36] Based on the basic research with

animals clinical trials, a selective COX-2 inhibitor, celecoxib was evaluated on the prevention and treatment of breast cancer. Epideiologic evidence suggests the incidence of breast, colon, and lung cancers is inversely related to the use of aspirin and nonsteroidal anti-inflammatory drugs, which are nonspecific inhibitors of COX-2 or COX-1.[20] In this study, the COX-2 down-regulatory effect of celecoxib was observed with normal mammary gland buds. Nonetheless, estrogen, a known potent mitogen for breast cancer cells[31] did not showed COX-2 stimulatory effect. Rather estrogen was found to be a highly down-regulating agent of mammary gland buds.

The up-regulated state in antiapoptotic protein bcl-2 expression was observed with celecoxib, and in contrast there was no up-regulation of bcl-2 by estrogen. Considering that estrogens are potent mitogen in the mammary gland playing a pivotal role in the development and progression of mammary carcinoma[31], and celecoxib is one of the known breast cancer development inhibitors,[18] these results may be perplexing. The study of Basu *et al.* has shown that the oral administration of celecoxib caused increased tumor cell apoptosis which was associated with decreased expression of bcl-2 and increased expression of bax.[37] In MCF-7 mammary tumor cells lines, 17β-estradiol inhibits apoptosis by inducing bcl-2 expression. [38] In this study 17β-estradiol induced bcl-2 up-regulation was not observed unlike in pathologic conditions. In this normal tissue, the proapoptotic potential of celecoxib and estrogen was evident from the pattern of bcl-2 and bax expression. The reason for the differences in apototic protein expression of celecoxib and estrogen in normal or cancerous states require further investigation. The up-regulation of bcl-2 was accompanied by the decreased expression of COX-2 in celecoxib treated mammary gland buds. In the previous study, the high concentrations of estrogen down-regulated COX-2 in estrogen deficient animals.[39] Cellular mechanisms underlying the increased expression of bcl-2 by celecoxib and the marked depression of bcl-2 by estrogen are not clear at present. Furthermore, the stimulation of bax by celecoxib and the prominent stimulation of bax by estrogen requires explanation.

In summary, although complete understanding of the molecular mechanism by which celecoxib and estrogen control apoptosis in normal condition of mammary gland buds needs in depth investigation, it was observed that COX-2 expression was clearly down-regulated by celecoxib and estrogen similarly in mammary gland buds, and bcl-2/bax ratio were significantly reduced by celecoxib and estrogen compared to the control. The lowering of bcl-2/bax by celecoxib and estrogen resulted from the different patterns in bcl-2 and bax expression. The lowering of bcl-2/bax by celecoxib was mainly from the increased expression of bax with unchanged bcl-2, whereas estrogen reduced bcl-2/bax ratio by modulation of both bcl-2 and bax.

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