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Regulation of cyclooxygenase-2 and mapkinases by isoflavones in ovariectomized and estrogen-supplemented mature female rats fed a high fat-high cholesterol diet

Jang-In Shin and Ock Jin Park[§]

Department of Food and Nutrition, Hannam University, 133 Ojeong-dong, Daedeok-gu, Daejeon 306-791, Korea

The effects of soy-isoflavones, which are phytoestrogens derived from plants with a flavonoid structure, on cyclooxygenase -2 (COX-2) expression, PGE2 production, and mapkinases expression, were investigated in experimentally-induced atherogenic rats by feeding a high fat-high cholesterol diet. Female Sprague-Dawley rats were bilaterally ovariectomized; sham-operated animals were used as controls. Three weeks later, the animals were randomized to the following treatments for an eight-week experimental period: 17β-estradiol (200μg/kg diet), low concentration of isoflavones (0.8g/kg diet), and high concentration of isoflavones (4.0g/kg diet). In the group supplemented with a high dose of isoflavones, COX-2 expression was down-regulated. This down-regulation was accompanied by a reduced expression of pERK1/2. In the second experiment using 48-week old female Sprague-Dawly rats, the effects of isoflavones and estrogen were compared in the basal estrogen-supplementation at the level of 600μg/kg diet. Isoflavones induced the marked down-regulation of COX-2 protein and the decrease in PGE_2 production in estrogen supplemented states and this was followed by the down-regulation of p38 among mapkinases. The two different mapkinases are involved in the down-regulation of COX-2 depending on estrogen-deficient and estrogen supplemented states. This kind of COX-2 down-regulation by isoflavones was not observed in the different tissue, mammary glands. Further investigations on the relationship between COX-2 and biological activities such as vasodilation by isoflavones in the absence or the presence of estrogen are required in vivo system of female rats.

Key word : Isoflavones · COX-2 expression · mapkinases · ovariectomized rats · mature estrogen-supplemented rats · prostaglandin production

INTRODUCTION

It is clearly recognized that the incidence of cardiovascular disease (CVD) increases substantially after menopause, most possibly due to the loss of estrogen protection[1]. The increased incidence of CVD in younger hysterectomized women supports this hypothesis. Also, a gender difference in the incidence of CVD persists until the onset of menopause[2]. These observations have suggested that estrogen may play a protective role in the prevention and treatment of CVD. Therefore, estrogen replacement therapy has frequently been used in post-menopausal women[3]. However, recently it has been found that hormone replacement therapy (HRT) has numerous problematic side effects, and, furthermore, may not have the expected protective effect. Estrogen administration has been found to be associated with increased risks of venous thrombosis. The Heart and Estrogen/progestin Replacement Study (HERS) showed that hormone replacement therapy increased the occurrence of myocardial infarction within 3-4 years of beginning treatment, influenced coagulation parameters and enhanced markers of inflammation in women having CVD[4]. From the Women's Health Initiative (WHI) clinical trials it was concluded that overall health risks exceeded benefits from use of combined estrogen and progestin among healthy postmenopausal US women[5].

Phytoestrogens are compounds found in a wide variety of plant foods which are known to exhibit estrogen-like activity, and more recently, have been reported to display both estrogenic and anti-estrogenic effects[6-7]. Population studies have suggested that consumption of a phytoestrogen-rich diet could be protective against cardiovascular diseases and relieve some of the estrogen-deficiency symptoms in postmenopausal women[8]. Clinical trials have shown that soy supplementation is related to a reduction in lipids and lipoproteins in hypercholesterolemic and nonhypercholesterolemic subjects.[9-12]

Cyclooxygenases (COXs) are key enzymes in the conversion of arachidonic acid into prostanoids, which are involved in apoptosis and inflammation. Two distinct COXs have been identified: COX-1 which is constitutively expressed and COX-2 which is induced in

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[§]To whom correspondence should be addressed.

various tissues[13]. Previous studies have reported that cyclooxygenase modulates vascular contractions in vitro, by endothelium-dependent and independent mec hanisms[14-15]. Also there is evidence for the localized induction of COX-2 in atherosclerotic plaques in an animal model of atherosclerosis[16]. It has been suggested that the induction of COX-2 in atheroscerotic lesions may play a role in altering localized prostanoid production and propagating these events, which lead to diseased progression by favoring cell adhesion and proliferation[17]. Indomethacin, the non-selective cyclo-oxygenase inhibitor, has been shown to improve endothelium-dependent relaxations in mesenteric arteries from spontaneously hypertensive rats (SHR), presumably by blocking the production of vasoconstrictor prosta-glandins[18].

On the other hand, aortic relaxation induced by 17β -estradiol in cholesterol-fed rabbits was observed, and the involvement of the COX-2 in this protective effect on hypercholesterolemic rabbit aorta has been suggested[19].

The purpose of this study was to examine the regulation of COX-2 and up-stream mapkinases, and PGE_2 concentrations, by soy isoflavones in ovariecto-mized estrogen deficient female rats and mature estrogen-supplemented female rats fed a high fat-high cholesterol diet.

MATERIALS AND METHODS

Chemicals. 17β-estradiol, cholesterol, L-cysteine, α -cellulose, choline bitartrate and tert-butylhydroquinone were purchased from Sigma Chemical, St. Louis, USA. Soy isoflavone was in the form of Genistein Concen-trated Polysaccharides (GCP; Amino Up Co., Sapporo, Japan) and other isoflavones were from Tokiwa Phytochemical Co. Other reagents were all chemical grade, and were 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); and soybean oil and lard were commercial brands.

ANIMALS AND FEEDING REGIMENS

Experiment A: Seventy-two female Sprague-Dawley rats, eight weeks old, were fed a standard laboratory diet (manufactured by Cheil Feed Co., Seoul, Korea) for one week. Rats were ovariectomized or sham-operated, and afterward fed a laboratory diet for three weeks in order to obtain the full response of the ovariectomy (i.e. post-menopausal state). Rats were housed individually in an environmentally-controlled animal laboratory with a 12-hour light/dark cycle. For eight weeks, rats were fed one of the six diet regimens (Table 1) and water was given ad libitum. Diets were stored at −40℃ before use.

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

Groups	High-fat and high -cholesterol diet[1]
1. Sham-operated Control	No supplementation
2. Sham-operated Isoflavones Control	Isoflavones (GCP 0.8g/kg)
3. Ovariectomized Control	No supplementation
4. Ovariectomized Estrogen	17β - estradiol (200g/kg)
5. Ovariectomized Low Isoflavones	Isoflavones(GCP 0.8g/kg)
6. Ovariectomized High Isoflavones	Isoflavones(GCP 4.0g/kg)

1) High-fat and high-cholesterol diet contains Corn starch 438 g; sucrose 100g; soybean oil 41g; lard 120g; 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] 10.0g/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, 0.075; powdered sucrose, 974.655

Table 2. The groups of rats and the composition of the experimental diet(Experiment B) The basal diet was high fat(120g lard/kg diet) and high cholesterol(1g/kg) diet

Groups	High-fat and high-cholesterol diet[1]
1. Control	No supplementation
2. Estrogen Control	17β - estradiol(600μg/kg)
3. Estrogen Supplementation	17β - estradiol(600μg/kg) + 17β - estradiol (600ug/kg)
4. Isoflavones Supplementation	17β - estradiol(600μg/kg) + Soy Isoflavones (10.0g/kg)

1) High-fat and high-cholesterol diet contains Corn starch 438 g; sucrose 100 g; soybean oil 41g; lard 120g; 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] 10.0g/kg.
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Experiment B: 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 four 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 four diet regimens (Table 2) and water was given *ad libitum*. Diets were stored at −400℃ before use.

Plasma Collection, Tissue Preparation and PGE_2 Determination. Rats were fasted for 14 hours before the end of the experiment and anaesthetized with ether. Mammary gland tissues were collected from 4 mammary buds, frozen with liquid nitrogen, and stored at -800℃ until they were analysed. Rats were then dissected and the blood was drawn from the portal vein into a heparinized syringe. Blood was allowed to settle for 3 hours at room temperature and centrifuged at 2,800 rpm for 15 minutes to obtain plasma, before being stored at -80℃ until analysis. A portion of thoracic aorta was isolated and frozen with liquid nitrogen, and stored at -80℃ until analysis. PGE_2 was determined with an enzyme-immunoassay kit (Ammersham Pharmacia Biotech., UK).

Western Blotting. Collected tissues were lysed in 120 ml of ice-cold 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, and a protease inhibitor cocktail tablet (Boehringer Mannheim, Mannheim, Germany)] for 40 min. Lysates were centrifuged at 14,800 x g for 30 minutes, and aliquots of the supernatant containing 30 mg protein were boiled in SDS sample loading buffer for 5 minutes before electrophoresis on 12% SDS-polyacrylamide gel. Three hours after the transfer of SDS-polyacrylamide gel to a 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 hours at room temperature and then washed in PBS-Tween (PBST) buffer. The membranes were incubated for 2 hour at room temperature with a 1:1000 dilution of goat COX-2, pERK , pJNK, p38, and pp38 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were rinsed with PBST, incubated with a 1:5000 dilution of anti-goat-horseradish peroxidase conjugated-secondary antibody and then washed again three times in PBST buffer each time for 5 minutes. The transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences, USA) according to the manufacture's procedure.

Statistical analyses. Data analysis, estimation of means and standard error of means for each of the groups, was carried out with the SAS package[20]. ANOVA was performed to determine whether there were significant ($p < 0.05$) differences among the groups. 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 whether isoflavones could down-regulate COX-2 and therefore decrease the production of PGE_2, COX-2 expression and the plasma concentrations of PGE_2 were monitored in estrogen deficient and estrogen supplemented rats.

Experiment A: In ovariectomized estrogen-deficient female rats, COX-2 expression was down-regulated by supplementation with a high concentration of isoflavones compared to the control group, and the groups supplemented with estrogen or with a low concentration of isoflavones (Figure 1). It is well known that mitogen-activated protein kinase (MAPK) signaling pathways control COX-2 induction in a number of cell types. This down-regulation of COX-2 was accompanied by the reduced expression of pERK1/2. pJNK and phospho-p38 expressions were not changed by the different COX-2 expression induced by isoflavones or estrogen.

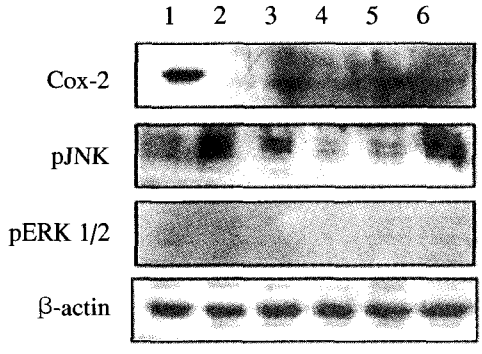

Fig 1. COX-2, pJNK and pERK1/2 expression in the rat aorta isolated from 1. sham-operated control, 2. sham-operated isoflavones control, 3. overariec-tomized control, 4. ovariectomized estrogen supplemented, 5. ovariectomized low isoflavones supple-mented and 6. ovariectomized high isoflavones supplemented.

Experiment B: In estrogen-supplemented, mature, rats, isoflavones induced the marked down-regulation of COX-2 protein (Figure 2), compared to the estrogen non-supplemented control rats or basal estrogen-supplemented rats. It was tested whether pERK1/2 was involved in COX-2 down-regulation, and the results showed that pERK1/2 was not changed with the down-regulation of COX-2. Instead, p38 expression was

correlated with COX-2 protein levels, indicating that the p38 MARK pathway might be involved in the isoflavones -induced down regulation of COX-2.

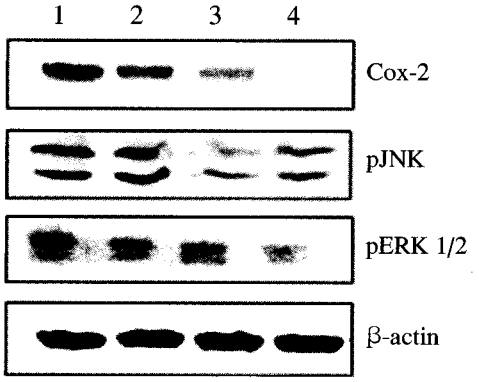

Fig 2. COX-2, pERK1/2 and p38 expressions in the rat aorta isolated from 1. control, 2. estrogen control (600μg), 3. estrogen supplementation (1200μg) and, 4. isoflavones supplementation

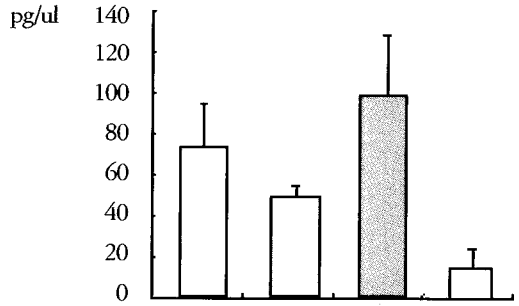

Fig 3. PGE_2 concentrations in plasma from rats isolated from 1. control, 2. estrogen control (600μg), 3. estrogen supplementation (1200μg) and 4. isoflavones supplementation shown mean and standard error of the mean; number 4 is significantly different from 1, 2, 3 at $p < 0.05$ by ANOVA test

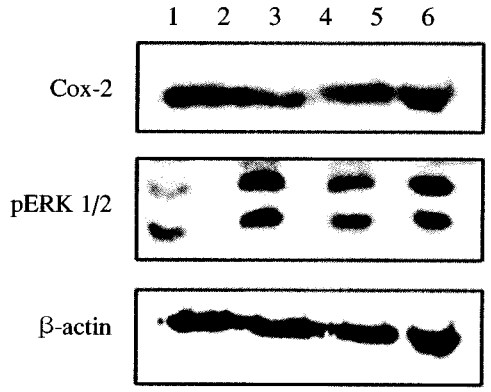

Fig 4. COX-2, pERK expressions of mammary glands of rats from 1. control, 2. estrogen control (600μg), 3. estrogen supplementation (1200μg) and 4. isoflavones supplementation

Treatment with isoflavones resulted in a marked decrease in PGE_2 production (Figure 3). In order to determine if isoflavones could affect other organs besides the aorta, the mammary gland COX-2 expression was examined. Isoflavone supplementation did not influence COX-2 expression and pERK1/2 expression in mammary glands (Figure 4).

DISCUSSION

In this study, the effects of isoflavones on COX-2 expression in rat aorta were examined in ovariectomized rats and basal estrogen-supplemented rats in comparison with estrogen. Isoflavones are naturally occurring plant-derived estrogen-like compounds. The biologically-active isoflavones originating from soybean products are converted by intestinal bacteria into genistein, daidzein and glycetin, which are hormone-like compounds with weak antioxidative and estrogenic activity[21]. These plant-derived estrogens appear to exert both estrogenic and antiestrogenic effects on metabolism, depending on their biological concentration, endogenous estrogen levels and the individual's properties such as sex and menopausal status[22-23]. The antiestrogenic activity may be partially explained by competition with endogenous 17β-estradiol for estrogen receptors[24]. Depending upon the concentrations of phytoestrogen the isoflavones might show estrogen agonist or antagonist actions.[25-26] Also, the interaction with two kinds of estrogen receptors (alpha and beta) appeared to play a crucial role in generating the complex responses of phytoestrogens.[27-30] Diets containing soy products have been shown to reduce CVD risk factors in non-human primates[31]. Recent work has shown the potential of genistein to enhance the dilator response in atherosclerotic arteries[32-33].

It was investigated whether the protective effects of isoflavone supplementation might be involved in the alteration of COX-2 production. To test this hypothesis, the effects of isoflavones were compared with 17β -estradiol in bilaterally-ovariectomized estrogen deficient female rats and the basal concentrations of 17-estradiol-supplemented mature female rats. The results suggested that different mechanisms might be involved in the vasoprotective effects of estrogen and isoflavones, via prostaglandin production. In ovariectomized endothelial dysfunctional female rats, the group supplemented with a high concentration of isoflavones down-regulated COX-2 expression compared to groups supplemented with estrogen. COX-2 down-regulation by isoflavones was also observed with estrogen-supplemented mature female rats. A higher supplementation of estrogen, at the total concentration of 1,200 ug/kg, did not have the same effect as isoflavones. It is not clear that isoflavones and estrogen do not show the similar effects on COX-2 expression. It is more likely

that high concentrations of isoflavones might have different vasodilatory effects via prostanoids. Isoflavones in these systems might show a possible vasoprotective effect through different mechanism as estrogen not involving prostanoid. Cellular mechanisms underlying the decreased expression of COX-2, and the reduced production of PGE_2 by isoflavones compared to estrogen, are unclear at present but these might be related to the ability of isoflavones to suppress protein tyrosine kinases[34]; as a consequence, the activation of NF-kB[35] would be decreased, since isoflavones are known as inhibitors of protein tyrosine kinases, and NF-kB is one of the response elements on the promoters of the COX-2 gene[36]. Actually, Mutoh et al. observed the ability of genistein to suppress COX-2 promoter activity[37].

Expression of COX-2 appears to be highly regulated by a number of mitogen-activated protein kinases and transcription factors[38-39]. Several extracellular stimuli elicit a broad spectrum of biological responses through activation of mapkinase cascades[40]. There are three major superfamilies of mapkinases in several cell types: ERK, JNK and p[38-41]. Two different mapkinases seem to be involved in the down-regulation of COX-2 expression in ovariectomized rats and estrogen supplemented rats. In ovariectomized rats, pERK1/2 was shown to be correlated with COX-2 expression. In contrast, p38 appeared to be related to COX-2 expression in estrogen supplemented rats. It is not clear why there might be two different mapkinases involved in these two systems. However, there is a possibility that the presence of estrogen might have a distinctive role in eliciting upstream transduction.

COX-2 regulatory responses by isoflavones appeared to be restricted to the aorta, since the mammary gland examination did not show any differences in these responses.

This study has shown that isoflavone supplementation induces COX-2 down-regulation in ovariectomized estrogen deficient rats and estrogen supplemented rats. Whether this is the underlying mechanism of cardioprotection requires further study. Mapkinase expressions related to the regulation of COX-2 were also examined, and ERK1/2 and p38 appeared to be involved in this regulation for ovariectomized rats and estrogen sufficient rats, respectively.

Literature Cited

1) Barrett-Connor E, Bush TL. Estrogen and the coronary heart disease in women. *J Am Med Assoc* 265:1861-1867, 1991
2) Knopp RH, Zhu X, Bonet B. Effects of estrogens in lipoprotein metabolism and cardiovascular disease in women. *Atherosclerosis* 110:S83-S91, 1994
3) Stampfer MJ, Colditz GA. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence. *Prev Med* 20:47-63, 1991
4) Hully S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff. Randomized trial of estrogen plus protestin for secondary prevention on coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) research Group. *J Am Med Assoc* 280:605-613, 1998
5) Rossouw JE, Anderson GL, Prentice RL, LaCorix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *J Am Med Assoc* 288:321-333, 2002
6) Braden A, Hart N, Lamberton J. The estrogenic activity and metabolism of certain isoflavones in sheep. *Aust J Agric Res* 18:335-348, 1976
7) Setchell KDR. Phytoestrogens: the biochemistry, physiology and implications for human health of soy isoflavones. *Am J Clin Nutr* 68:S1333-S1346, 1998
8) Setchell KDR. Soy isoflavones-benefits and risks from nature's selective estrogen receptor modulators (SERMs). *J Am Coll Nutr* 20:S345-S362, 2001
9) Anderson JW, Johnstone BM, Cook-Newell ME. Meta-analysis of the effects of soy protein intake on serum lipids. *N Eng J Med* 333:276-282, 1995
10) Baum J, Teng H, Erdman JW, Weigel RM, Klein BP, Persky VW, Freels S, Uurya P, Bakhit RM, Ramos E, Shay NF, Potter SM. Long-term intake of soy protein improves blood lipid profiles and increases mononuclear cell low-density-lipoprotein receptor messenger RNA in hypercholesterolemic, postmenopausal women. *Am J Clin Nutr* 68:545-551, 1998
11) Potter SM, Bakhit RM, Essex-Sorlie DL, Weingartner KE, Chapman KM. Depression of plasma cholesterol in men by consumption of baked products containing soy protein. *Am J Clin Nutr* 58:501-506, 1993
12) Tikkanen MJ, Adlercreutz H. Dietary soy-derived isoflavone phytoestrogens: Could they have a role in coronary heart disease prevention? *Biochem Pharm* 60:1-5, 2000
13) Hyslop S, De Nucci G. Prostaglandin biosynthesis in the microcirculation: regulation by endothelial and non-endothelial factors. *Leukotrienes Essent Fatty acids* 49:723-760, 1993
14) Ge T, Hughes H, Junquero DC, Wu KK, Vanhoutte PM, Boulanger CM. Endothelium-dependent contractions are associated with both augmented expression of prostaglandin H synthase-1 and hypersensitivity to prostaglandin H_2 in the SHR aorta. *Cir Res* 76:1003-1010, 1993
15) Dyer SM, Taylor DA, Bexis S, Hime NJ, Frewin DB, Head RJ. Identification of a non-endothelial cell thromboxane-like constrictor response and its interaction with the renin-angiotensin system in the aorta of spontaneously hypertensive rats. *J Vasc Res* 31:5-60, 1994
16) Wong E, Huang J, Tagari P, Riendear D. Effects of COX-2 inhibitors on aortic prostacyclin production in cholesterol-fed rabbits. *Atherosclerosis* 157:393-402, 2001
17) Baker CS, Hall RJ, Evans TJ. Cyclooxygenase-2 is widely expressed

in atherosclerotic lesions affecting native and transplanted human coronary arteries and colocalizes with inducible nitric oxide synthase and nitrotyrosine particularly in macrophages. *Arterioscler Thromb Vasc Biol* 19:646-655, 1999

18) Luscher TF, Aarhus LL, Vanhoutte PM. Indomethacin improves the impaired endothelium-dependent relaxations in small mesenteric arteries of the spontaneously hypertensive rats. *Am J Hypertens* 3:55-58, 1990
19) Ghanam K, Lavagna C, Burgaud J-L, Javellaud J, Ea-Kim L, Oudart N. Involvement of cyclooxygenase 2 in the protective effect of 17 β-estradiol in hypercholesterolemic rabbit aorta. *Biochem Biophy Resear Comm* 275:696-703, 2000
20) SAS Institute, Inc. SAS/STAT *User's guide,* Cary NC: SAS Institute, Inc, USA, 1995
21) Thom DM, Gardner CD, Haskell WL. Potentail health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence. *J Clin Endocrinol Metab* 83 : 2223-2235, 1998
22) Adlercreutz H, Mazur W. Phyto-estrogens and western diseases. *Ann Med* 29:95-120, 1997
23) Knight DC, Eden JA. A review of the clinical effects of phytoestrogens. *Obstet Gynecol* 87:897-904, 1996
24) Martin PM, Horwitz KB, Ryan DS, McGuire WL. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. *Endocrinol* 103:1860-1867, 1978
25) Wang TTY, Sathyamoorthy N, Phang JM. Molecular effects of genistein on estrogen receptor mediated pathways. *Carcinogenesis* 17:271-275, 1996
26) Allred CD, Allred KF. Ju YH. Virant SM, Helferich WG. Soy diets containing varying amounts of genistein stimulate growth of estrogen-dependent (MCF-7) tumors in a dose-dependent manner. *Cancer Res* 61:45-50, 2001
27) Barkhem T, Carlsson B, Nilsson Y, Enmark E, Fustafsson J, Nilsson S. Differential response of estrogen receptor α and estrogen receptor β to partial estrogen agonists/antagonists. *Mole Pharm* 54:105-112, 1998
28) Breinholt V, Hossaini A, Svendsen G, Brouwer C, Nielsen SE. Estrogenic activity of flavonoids in mice. The importance of estrogen receptor distribution, metabolism and bioavailability. Food *Chem Toxicol* 38:555-564, 2000
29) Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson J. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinol* 139:4252-4263, 1998
30) Routledge EJ, White R, Parker MG, Sumpter JP. Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor(ER) αand ERβ. *J Biol Chem* 275:35968-35993, 2000
31) Anthony MS, Clarkson TB, Highes CL, Morgan TM, Burke GL. Soybean isoflavones, improve cardiovascular risk factors without affecting the reproductive system of peripubertal rhesus monkeys. *J Nutr* 126:43-50, 1996
32) Honore EK, Williams JK, Anthony MS, Clarkson TB. Soy isoflavones enhance coronary vascular reactivity in atherosclerotic female macaques. *Fertil Steril* 67:148-154, 1997
33) Squadrito F, Altavilla D, Squadrito G, Saitta A, Cucinotta D, Minutuli L, Deodato B, Ferlito M, Campo GM, Bova A, Caputi AP. Genistein supplementation and estrogen replacement therapy improve endothelial dysfunction induced by ovariectomy in rats. *Cadiovascular Research* 45:454-462, 2000
34) Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S-I, Itoh N, Shibuya M, Fukkami Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 262:5592-5595, 1987
35) Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* 9:484-496, 1995
36) Barnes PJ, Karin M. Nuclear factor-kB-a pivotal transcription factor in chronic inflammatory diseases. *N Eng J Med* 336:1066-1071, 1997
37) Mutoh M, Takahashi M,Fukuda K, Matsushima-Hibiya Y, Mutoh H, Sugimura T, Wakabayashi K. Suppression of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells by chemopreventive agents with a resorcin-type structure. *Carcinogenesis* 21:959-963
38) Xie W, Herschman HR. Transcriptional regulation of prostaglandin synthase 2 gene expression by platelet-derived growth factor and serum. *J Biol Chem* 271:31742-31748, 1996
39) Guan Z Buckman SY, Miller BW, Springer LD, Morrison AR. Interleukin-1 beta-induced cyclooxygenase-2 expression requires activation of both c-Jun NH2-terminal kinase and p38 MARK signal pathways in rat renal mesangial cells. *J Biol Chem* 273:28670-28676, 1998
40) Whitmarsh AJ, Sharrocks AD, Davis RJ. Integration of MAP kinase signal transduction pathways at the serum response element. *Science* 269:403-407, 1995
41) Cobb MH, Goldsmith EJ. How MAP kinases are regulated. *J Biol Chem* 270:14843-14846, 1995