Phytoestrogens Inhibit Growth and MAP Kinase Activity in Human Aortic Smooth Muscle Cells

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Abstract—Estrogens are known to induce cardioprotective effects by inhibiting smooth muscle cell (SMC) growth and neointima formation. However, the use of estrogens as cardioprotective agents is limited by carcinogenic effects in women and feminizing effects in men. If noncarcinogenic and nonfeminizing estrogenlike compounds, such as natural phytoestrogens, afford cardioprotection, this would provide a safe method for prevention of cardiovascular disease in both men and women. Therefore, we evaluated and compared in human aortic SMCs the effects of phytoestrogens (formononetin, genistein, biochanin A, daidzein, and equol) on 2.5% fetal calf serum–induced proliferation (³H-thymidine incorporation and cell number), collagen synthesis (³H-proline incorporation), and total protein synthesis (³H-leucine incorporation) and on PDGF-BB (25 ng/mL)–induced migration (modified Boydens chambers). Moreover, the effects of phytoestrogens on PDGF-BB (25 ng/mL)–induced mitogen-activated protein kinase (MAP kinase) activity in SMCs was also studied. Phytoestrogens inhibited proliferation, collagen and total protein synthesis, migration, and MAP kinase activity in a concentration-dependent manner and in the following order of potency: biochanin A > genistein > equol > daidzein > formononetin. In conclusion, our studies provide the first evidence that in human aortic SMCs phytoestrogens inhibit mitogen-induced proliferation, migration and extracellular matrix synthesis and inhibit/downregulate MAP kinase activity. Thus, phytoestrogens may confer protective effects on the cardiovascular system by inhibiting vascular remodeling and neointima formation and may be clinically useful as a safer substitute for feminizing estrogens in preventing cardiovascular disease in both women and men. *(Hypertension. 1999;33[part II]:177-182.)*

Key Words: estrogen ■ muscle, vascular, smooth ■ women, postmenopausal ■ cardiovascular disease ■ phytoestrogens ■ proliferation ■ migration

Estrogen (hormone) replacement therapy (HRT) markedly reduces the risk of cardiovascular disease in postmenopausal women.†,‡ However, the use of HRT as a cardioprotective strategy is greatly limited owing to carcinogenic effects of estrogens on the endometrium in women and feminizing effects in men. Hence, there is a strong interest in finding alternative estrogen-like agents that are noncarcinogenic and nonfeminizing, yet induce cardioprotective effects. In this regard tamoxifen and 4-hydroxytamoxifen, noncarcinogenic high affinity estrogen receptor ligands, have been shown to induce cardioprotective effects. More recently, interest has focused on phytoestrogens, which are natural dietary plant compounds with estrogenic activity. Phytoestrogens have been identified in the urine of humans, and epidemiological studies suggest that consumption of a phytoestrogen-rich diet, as seen in traditional Asiatic societies, is associated with a lower risk of breast and prostate cancer and cardiovascular disease.† Recent studies in nonhuman primates have shown that a phytoestrogen-rich diet prevents high fat diet–induced vaso-occlusive disorders. Moreover in vitro studies provide evidence that phytoestrogens inhibit growth of MCF-7 breast cancer cells and prevent angiogenesis. Together, these findings suggest that phytoestrogens could be a promising substitute for estrogen in preventing cardiovascular disease. However, the cellular and biochemical mechanisms by which phytoestrogens mediate their antivaso-occlusive and cardioprotective effects remain largely unknown.

Phytoestrogens are classified into 3 categories: isoflavones, coumestans, and lignans, and multiple phytoestrogens can originate from a single plant. In this regard soya beans and soya products are a rich source for the isoflavones genistein and daidzein, which are derived from precursors biochanin A and formononetin by the action of intestinal glucosidases.
Moreover, daidzein can be further metabolized to equol. As shown in Figure 1, all the phytoestrogens are heterocyclic phenols with a structure similar to endogenous 17β-estradiol. Inasmuch as most studies have used soya diets, we selected the isoflavones genistein, daidzein, formononetin, biochanin A, and equol to study the cellular and biochemical mechanisms by which phytoestrogens induce their antiatherosclerotic effects.

Smooth muscle cells (SMCs) contribute to pathological structural changes within the vessel wall by migrating from the media into the intima, proliferating, and depositing extracellular matrix (ECM) proteins such as collagen. Thus the media into the intima, proliferating, and depositing structural changes within the vessel wall by migrating from the media into the intima, and deposition of ECM. Therefore, the isoflavones genistein, daidzein, formononetin, biochanin A, and equol to study the cellular and biochemical mechanisms by which phytoestrogens induce their antiatherosclerotic effects.

Methods

All tissue culture media, reagents, and wares were purchased from Gibco Laboratories. Fetal calf serum (FCS) was obtained from HyClone Laboratories Inc. 17β-Estradiol, genistein, biochanin A, daidzein, myelin basic protein, triton X-100, β-glycerophosphate, EGTA, diithiothreitol, Na3VO4, aprotinin, pepstatin, leupeptin, and benzamidine were purchased from Sigma Chemical Co. Formononetin and equol were procured from Extrasynthese. Estrogen receptor antagonist ICI 182,780 was a gift from Tocris (Bristol, UK). 4-Hydroxytamoxifen was purchased from Research Biochemicals International. [3H]-thymidine (specific activity 11.8 Ci/mmol) was purchased from ICN Biomedicals and [3H]-estradiol (specific activity 72 Ci/mmol) from NEN. L-[3H]-Proline (specific activity 23 Ci/mmol), L-[3H]-leucine (specific activity 151 Ci/mmol), and γ32P-ATP (specific activity 3 Ci/mmol) were purchased from Amersham. All other chemicals used were of tissue culture or best grade available.

Arterial SMCs cultured from adult thoracic aortas were obtained from 3 female and 3 male normal donor heart transplants. The cells were cultured by the explant method and cultured as described by us previously. SMC purity was characterized by immunofluorescence staining with smooth muscle specific anti-smooth muscle–α-actin monoclonal antibodies and by morphologic criteria specific for SMC as described previously. SMCs in the third through fifth passages were used for all the studies.

[3H]-thymidine incorporation and cell number studies were conducted to investigate the effects of phytoestrogens on mitogen-induced DNA synthesis and cell proliferation, respectively. SMCs were plated at a density of 1×104 cells/well in 24 well tissue culture dishes and allowed to grow to subconfluence in DMEM/F12 (phenol red free) containing 10% fetal calf serum (steroid-free and delipidated) under standard tissue culture conditions. The cells were then growth-arrested by feeding DMEM (phenol red free) containing 0.4% albumin for 48 hours. For DNA synthesis, growth was initiated by treating growth-arrested cells for 20 hours with DMEM containing 2.5% FCS and containing or lacking phytoestrogens, with or without the estrogen receptor antagonists ICI 182,780 and 4-hydroxytamoxifen. After 20 hours of incubation the treatments were repeated with freshly prepared solutions but supplemented with [3H]-thymidine for an additional 4 hours. The experiments were terminated by washing the cells twice with Dulbecco’s phosphate buffered saline and twice with ice-cold trichloroacetic acid (10%). The precipitate was solubilized in 500 μL of 0.3 N NaOH and 0.1% sodium dodecysulfate after incubation at 50°C for 2 hours. Aliquots from 4 wells for each treatment with 10 mL scintillation fluid were counted in a liquid scintillation counter. For cell number experiments SMCs were allowed to attach overnight, growth-arrested for 48 hours, and subsequently treated every 24 hours for 4 days; on day 5 the cells were dislodged and counted on a Coulter counter.

[3H]-proline and [3H]-leucine incorporation studies were done to investigate the effects of phytoestrogens on FCS-induced collagen and total protein synthesis, respectively. Confluent monolayers of SMCs were made quiescent by feeding complete culture medium containing 0.4% bovine serum albumin (BSA; Sigma) for 48 hours. Collagen and protein synthesis were initiated by treating growth-arrested SMCs with medium supplemented with 2.5% FCS and containing or lacking the different phytoestrogens. For collagen synthesis, the cells were treated for 36 hours in the presence of [3H]-proline, whereas for total protein synthesis, after 20 hours of treatment the cells were pulsed for 5 hours with [3H]-leucine. The experiments were terminated by washing the cells twice with PBS and twice with ice-cold TCA (10%) and the precipitate solubilized and counted on a liquid scintillation counter. Each experiment was conducted in triplicate and with separate cultures. To ensure that the inhibitory effects of the experimental agents on collagen and protein synthesis were not due to changes in cell number, the experiments were conducted in confluent monolayers of cells in which changes in cell number were precluded. Additionally, cell counting was performed in cells treated in parallel to the cells used for the collagen/protein synthesis studies, and data were normalized to cell number.

Modified Boydens chambers (Neuro Probe Inc) were used to evaluate the effects of phytoestrogens on PDGF-BB-induced SMC migration and as previously described. Briefly, confluent monolayers of SMCs were growth-arrested by feeding with DMEM supplemented with 0.4% albumin for 48 hours. Growth-arrested SMCs were trypsinized, washed, and suspended at a concentration of 1×106 cells/mL in fresh DMEM containing 0.4% albumin and containing or lacking 0.1 to 25 μmol/L phytoestrogens. After incubation the SMCs (50 000 cells per 50 μL; 6000 cells per mm2) were...
layered on the top chamber, and DMEM containing the respective treatments plus PDGF-BB (25 ng/mL) was added to the lower chamber. After 6 hours of incubation, the monolayers were vigorously washed with PBS, and the migrated cells were fixed with 0.4% paraformaldehyde and stained with Diff-Quick stain (Baxter Scientific Corp.). The migrated cells on the lower surface were counted manually under ×200 high-power field (HPF).

For MAP kinase studies, SMCs grown to confluence in 35-mm culture dishes were made quiescent by feeding DMEM containing 0.4% BSA for 48 hours. Growth-arrested SMCs pretreated for 60 minutes or 24 hours, with or without phytoestrogens, in presence of 0.4% BSA, were stimulated with PDGF-BB (25 ng/mL) for 7 minutes. After stimulation, the cells were washed with ice-cold PBS and the cytosolic fraction extracted by the method of Bornfeldt et al. MAP kinase activity in the cytosolic fractions, diluted to a concentration of 1 mg protein/mL, was assayed by quantitating phosphorylation of myelin basic protein (MBP) in the presence of γ-32P-ATP. To calculate the MAP kinase activity, samples incubated in the absence of MBP were subtracted from the same samples incubated in presence of MBP.

For binding studies, cytosolic fractions were extracted from SMCs grown to confluence in 75 cm² flasks and as described in detail by Wang et al. Briefly, 0.5 mL aliquots of the cytosolic extracts (1 mg protein per 0.5 mL) were pretreated with or without phytoestrogens, 17β-estradiol, ICI 182,780, or 4-hydroxytamoxifen were incubated for 8 hours at 4°C with 1H-17β-estradiol. After incubation of the cells, 0.1 mL of dextran-coated charcoal was added and the assay mixture incubated on ice for 30 minutes, with gentle mixing every 5 minutes. The charcoal was subsequently separated by centrifuging the assay mixture at 6000 rpm for 10 minutes; 500 μL aliquots of the supernatant was transferred into scintillation vials and counted after adding 10 mL of scintillation fluid. To calculate bound-to-free ratio of 1H-17β-estradiol, total counts were measured in cytosols not treated with dextran-charcoal.

All growth experiments were performed in triplicate or quadruplicate with aortic SMCs cultured from 3 separate female donors and 3 separate male donors (n=3 females and n=3 males). Data are presented as mean±SEM. Statistical analysis was performed using ANOVA, paired or unpaired Student’s t test, or Fisher’s least significant difference test as appropriate. A value of P<0.05 was considered statistically significant.

Results

Treatment with 2.5% FCS stimulated DNA synthesis by approximately 10-fold (P<0.001 versus 0.4% BSA), and these effects were similar in male and female SMCs (data not shown). FCS also induced 1H-proline and 1H-leucine incorporation in female SMCs by approximately 8-fold (P<0.001 versus 0.4% BSA; data not shown). In female SMCs, biochanin A, genistein, equol, daidzein, and formononetin inhibited FCS-induced 1H-thymidine incorporation in a concentration-dependent manner (Figure 2). The lowest concentrations that significantly inhibited FCS-induced DNA synthesis in female SMCs were 0.1 μmol/L each of biochanin A, genistein, and equol and 10 μmol/L each of daidzein and formononetin (Figure 2). A 50% decrease in FCS-induced DNA synthesis in female SMCs was observed at 25, 50, 60, >100, and >100 μmol/L, respectively. FCS-induced proliferation (cell number) of growth-arrested SMCs by approximately 9-fold (data not shown). Biochanin A, genistein, daidzein, and formononetin inhibited FCS-induced increases in cell number in a concentration-dependent manner (Figure 4). The lowest concentrations of biochanin A, genistein, equol, daidzein, and formononetin that significantly inhibited FCS-induced increases in cell number after 4 days of treatment were 0.1, 0.1, 1, 25, and 25 μmol/L, respectively. Trypan blue exclusion tests indicated no loss in viability of cells treated with the various concentrations of phytoestrogens (data not shown).

Figure 2. Inhibition of 1H-thymidine incorporation in female (A) and male (B) SMCs by phytoestrogens. Results are expressed as percentage of control, and values represent mean±SEM from 3 separate experiments, each conducted in quadruplicate and with cells cultured from aortas of 3 females and 3 males. *P<0.05 versus control (cells treated with 2.5% FCS alone).

Figure 3. Inhibition of 1H-proline (A) and 1H-leucine (B) incorporation in female SMCs by phytoestrogens. Results are expressed as percentage of control, and values represent mean±SEM from 3 separate experiments, each conducted in quadruplicate and with cells cultured from aortas of 3 females. *P<0.05 versus control (cells treated with 2.5% FCS alone).

Biochanin A, genistein, equol, daidzein, and formononetin also inhibited FCS-induced 1H-proline and 1H-leucine incorporation (Figure 3A and 3B) in SMCs, in the following order of potency: biochanin A>genistein>equol>daidzein=formononetin. A 50% decrease in 1H-proline incorporation in female SMCs by biochanin A, genistein, equol, daidzein, and formononetin was observed at 25, 50, 60, >100, and >100 μmol/L, respectively. FCS-induced proliferation (cell number) of growth-arrested SMCs by approximately 9-fold (data not shown). Biochanin A, equol, daidzein, and formononetin inhibited FCS-induced increases in cell number in a concentration-dependent manner (Figure 4). The lowest concentrations of biochanin A, genistein, equol, daidzein, and formononetin that significantly inhibited FCS-induced increases in cell number after 4 days of treatment were 0.1, 0.1, 1, 25, and 25 μmol/L, respectively. Trypan blue exclusion tests indicated no loss in viability of cells treated with the various concentrations of phytoestrogens (data not shown).
Compared with untreated controls, treatment of growth-arrested female SMCs with PDGF-BB (25 ng/mL) induced SMC migration from 7 to 68 cells per HPF (P, 0.05 versus SMCs treated with 0.4% albumin; data not shown). In SMCs pretreated with 1 to 25 μmol/L of biochanin A, genistein, equol, daidzein, and formononetin, the stimulatory effects of PDGF-BB were inhibited in a concentration-dependent manner. The lowest concentrations at which the phytoestrogens significantly inhibited SMC migration were 0.1, 0.1, 1, 10, and 10 μmol/L biochanin A, genistein, equol, daidzein, and formononetin, respectively (Figure 4B).

The inhibitory effects of biochanin A, genistein, equol, daidzein, and formononetin FCS-induced DNA synthesis, proline incorporation, and leucine incorporation were not blocked but rather were enhanced by 4-hydroxytamoxifen (1 μmol/L; Figure 5). However, pretreatment with ICI 182,780 (10 μmol/L), significantly reversed the inhibitory effects of low (1 μmol/L) but not high (>10 μmol/L) concentrations of all phytoestrogens on DNA synthesis and cell number (Figure 5).

Incubation of cytosolic extracts from female SMCs with 
17β-estradiol resulted in a significant amount of 
17β-estradiol binding in the charcoal extracted fractions. Moreover, addition of cold 17β-estradiol (10^-9-10^-5 mol/L) as well as the estrogen receptor antagonists, ICI 182,780 (10^-9 mol/L) and 4-hydroxytamoxifen (10^-9 mol/L), inhibited 17β-estradiol binding. In cytosolic fractions pretreated with 10^-9 to 10^-5 mol/L, biochanin A, genistein, equol, daidzein, and formononetin, 17β-estradiol binding was also inhibited in a concentration-dependent manner (Figure 6). The potency of various phytoestrogens in inhibiting 17β-estradiol binding was in the following order: biochanin A>genistein>equol>daidzein=formononetin.

Treatment of growth-arrested female SMCs with PDGF-BB (25 ng/mL) increased MAP kinase activity from 0.1 pmol min^-1 mg^-1 protein (0.4% albumin) to 7.2 pmol min^-1 mg^-1 protein. In SMCs pretreated with 1 to 25 μmol/L genistein, biochanin A, or daidzein, the stimulatory effects of PDGF-BB were inhibited in a concentration-dependent manner (Figure 7). The lowest concentration of biochanin A, genistein, and daidzein that significantly inhibited PDGF-BB-induced MAP kinase activity were 1, 1, and 10 μmol/L, respectively.

Discussion

The results of the present study demonstrate that phytoestrogens inhibit FCS-induced DNA synthesis, proliferation, collagen synthesis and total protein synthesis as well as PDGF-BB–induced SMC migration, all of which are behaviors of SMCs critical to the processes of neointima formation in
hypothesis that phytoestrogens inhibit mitogen-induced SMC growth by inhibiting MAP kinase activity. Our observation that genistein, biochanin A, and daidzein inhibit PDGF-BB-induced activity of MAP kinase, which is a key pathway by which multiple mitogens induce their mitogenic effects on SMCs. Together, our findings provide the first evidence that in human aortic SMCs natural dietary phytoestrogens inhibit mitogen-induced DNA synthesis, cell proliferation, extracellular matrix synthesis, and migration. Moreover, the inhibitory effects of 17β-estradiol are mediated via intracellular mechanisms that may not involve the participation of estrogen receptors and possibly involve direct inhibition of MAP kinase.

Our observation that phytoestrogens in human aortic SMCs inhibit mitogen-induced DNA synthesis, cell proliferation, and cell migration as well as extracellular matrix synthesis provides evidence that the cardioprotective effects of phytoestrogens may in part be mediated via inhibition of mitogen-induced SMC growth. Moreover, the fact that phytoestrogens had similar antimitogenic effects on male and female SMCs suggests that the direct effects of these hormones on SMC growth in vitro are not sexually dimorphic. Similar to phytoestrogens, 17β-estradiol has been shown to inhibit growth of male and female SMCs in vitro. However, in vivo, 17β-estradiol has dimorphic effects, and these effects are not due to the intrinsic property of the vascular SMCs, but rather due to androgenic factors produced by gonads in males.

The binding studies provide evidence for the presence of estrogen receptors on human aortic SMCs and demonstrate that phytoestrogens bind to these receptors. However, the inhibitory effects of phytoestrogens on cell growth were enhanced in the presence 4-hydroxytamoxifen, an estrogen receptor ligand with partial agonistic properties. Moreover, ICI 182,780, a specific estrogen receptor antagonist, only partially blocked the inhibitory effects of phytoestrogens. This suggests that the inhibitory effects of phytoestrogens are only in part estrogen receptor–mediated. In this regard, it recent research has demonstrated that phytoestrogens inhibit growth in estrogen receptor positive and estrogen receptor negative cell lines. Moreover, other estrogen-like compounds, for example, the estrogen receptor antagonists tamoxifen and 4-hydroxytamoxifen, are also known to inhibit SMC growth and induce cardioprotective effects. Additionally, several lines of evidence suggest that the inhibitory effects of 17β-estradiol on SMC growth are non–receptor mediated. Together, these findings demonstrate that phytoestrogens inhibit growth of vascular SMCs by a novel mechanism that is estrogen receptor–independent.

One common signaling pathway that is activated when mitogenic growth factors engage their receptors is the MAP kinase pathway. It has been demonstrated that bFGF-, PDGF-, and Ang II-induced migration and proliferation of vascular SMC are inhibited by the MEK inhibitor PD 98059. Moreover, 17β-estradiol has been shown to inhibit SMC growth by inhibiting MAP kinase activity. Our observation that genistein, biochanin A, and daidzein inhibited PDGF-BB–induced SMC migration and MAP kinase activity suggests that the inhibition of the MAP kinase pathway by phytoestrogens contributes to the growth inhibitory effects of phytoestrogens. However, the participation of other mechanisms cannot be ruled out. In this regard, phytoestrogens have been shown to inhibit tumor cell growth by interfering with tyrosine kinase activity and to inhibit epidermal growth factor receptor autophosphorylation. Moreover, being phenoic in nature, the phytoestrogens express antioxidant effects and have been shown to favorably influence the profile of cholesterol/lipids and inhibit platelet aggregation. Since these effects may protect vascular endothelial cells against free radicals and LDL/oxidized LDL–induced injury and dysfunction as well as inhibit platelet adhesion, this provides another mechanism by which phytoestrogens may induce cardioprotection.

Could our in vitro finding that phytoestrogens inhibit mitogen-induced growth of male and female SMCs be of therapeutic relevance in vivo situations? The lowest concentration of phytoestrogens that significantly inhibit SMC growth is 1 μmol/L, which is several magnitudes higher than those required by 17β-estradiol (1 nmol/L). The effects of these series of experiments may be underestimated because, in addition to the phytoestrogens used in this study, several more phytoestrogens are present in the dietary products, and collectively the concentrations may be much higher. Indeed, in contrast to 17β-estradiol, the levels of phytoestrogens in humans is much higher. In this regard, collective levels of 3 individual phytoestrogens (equol, daidzein, and genistein) reach 1.8 μmol/L in human plasma. Moreover, if other phytoestrogens, such as enterolactone and enterodiol, are included, the plasma levels of phytoestrogens would reach
4 μmol/L. Earlier studies have shown that phytoestrogens improve cardiovascular risk factors, prevent neointima formation without affecting the reproductive system, and reduce the risk of cancer. Since HRT with estrogens is associated with potential side effects such as hot flushes, cancer, and bleeding, the use of dietary phytoestrogens in women may be an alternative. Moreover, unlike 17β-estradiol, phytoestrogens do not have feminizing effects. Thus phytoestrogens may provide a safe estrogen substitute to protect against cardiovascular disease in both women and men.

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