Extracts from *Schizandra chinensis* Fruit Activate Estrogen Receptors: A Possible Clue to Its Effects on Nitric Oxide-Mediated Vasorelaxation

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**Schizandra chinensis** fruit has long been used for the treatment of cardiovascular symptoms associated especially with menopausal symptoms in Korea. To provide a scientific rationale for such uses, we have investigated the vasorelaxant effects of *Schizandra chinensis* fruit on the vasomotor tone of the rat thoracic aorta in an organ bath. The crude extracts of *Schizandra chinensis* fruit (SC-Ex) elicited a transient relaxing response in the endothelium-intact rat aorta contracted with norepinephrine. This relaxant effect was abolished by removal of the endothelium, and also by pretreatment with nitric oxide synthase inhibitor. We then examined whether this vasodilatory effect occurs through estrogen receptor by reporter assays. SC-Ex activated the estrogen-responsive luciferase gene in COS cells transiently transfected with estrogen receptor and reporter plasmids. The activation was maintained in the butanol-soluble fraction and further increased in the successively fractionated C_18 cartridge-adsorbed fraction (SC-ADF). Reporter gene activation by SC-ADF was inhibited by the specific estrogen receptor antagonist ICI 182,780, indicating that the effect is estrogen receptor dependent. However, SC-ADF failed to activate the androgen receptor in COS cells transfected with the corresponding receptor and reporter plasmids. These data show that extracts of *Schizandra chinensis* fruit act as a weak phytoestrogen.

**Key words** estrogen receptor; nitric oxide; phytoestrogen; *Schizandra chinensis*

The fruit of *Schizandra chinensis*, a member of the Magnoliaceae family, has an extensive history of medical use in the Orient. It has been used to increase the capacity to fight various aspects of stress, as an astringent, and is indicated in the treatment of such symptoms as dyspnea, irritability, palpitation, and insomnia. In China, it is one of the components of ‘Shengmai San’ that is commonly used for the treatment of coronary heart disease. It is used for postmenopausal women targeting cardiovascular disease in Korea. Research on the herb has shown that its components contain effects of antioxidation and lipid peroxidation inhibition. However, still only limited literature is available on the mechanism of action of *Schizandra*.

Numerous studies have shown that estrogen has favorable effects on cardiovascular disease by affecting endothelial cell function, blood vessel development, and vascular remodeling, etc. In addition to endogenous natural estrogen, phytoestrogens with nonsteroidal structures, such as isoflavones, are believed to be important for the prevention of cardiovascular disease, presumably due to their vasoprotective effect. Genistein, a phytoestrogenic compound in soybeans, improved endothelial function through increased nitric oxide synthase activity. Resveratrol, from grapes, acting through estrogen receptors, has been extensively studied for its cardioprotective benefit. It is very likely that *Schizandra chinensis* may function as phytoestrogen based on its traditional medical uses in Korea. However, there has been no conclusive scientific data yet to show that *Schizandra chinensis* contains phytoestrogens. This study was intended to determine the vasodilatory effect and possible mechanism of *Schizandra chinensis* fruit involved in alleviating the symptoms of menopause, possibly as phytoestrogens and/or by the activation of an estrogen receptor by measuring the transcription activation of an estrogen-responsive reporter plasmid.

**MATERIALS AND METHODS**

**Reagents** Carbachol, norepinephrine (NE), N^6^-nitro-L-arginine (L-NNa), 17β-estradiol (E2), and testosterone (Tes) were purchased from Sigma. ICI 182,780 (ICI) was obtained from ZENECA Pharmaceuticals. E2, ICI, and Tes were dissolved in 100% ethanol at a concentration of 1 mm.

**Plasmids** The ERE2-tk81-luc reporter plasmid, which was from Dr. Larry Jameson, contains two copies of the estrogen responsive element (ERE) of the vitellogenin gene, the herpes simplex thymidine kinase promoter, and the firefly luciferase gene. Plasmids for the expression of androgen receptor (AR) and (ARE)_2-Luc were from Dr. Chawnschang Chang.

**Preparation of Plant Extracts** The dried *Schizandra chinensis* fruit was obtained from a local market and ground with a commercial food mixer. This powder was consecutively extracted under reflux with water for 1 h. The resulting water extract was evaporated under reduced pressure at low temperature (37—40 °C) and lyophilized (SC-Ex). The solid was resuspended with water and then extracted with butanol. The butanol-soluble fraction was concentrated and lyophilized (SC-BSF). The solids were dissolved in water and applied to a C_18 cartridge (Sep-Pak cartridge, Waters Co., Milford, MA, U.S.A.) and then eluted with methanol. The eluted fraction was concentrated and lyophilized (SC-ADF). Each extract was dissolved in 100% ethanol at a concentration of 20—100 mg/ml for bioassay. Voucher specimens No. SC-001 (SC-Ex), SC-002 (SC-BSF), and SC-003 (SC-ADF) have been deposited in the Korea Food Research Institute, Gyeonggi-Do, Korea.

**Tissue Preparation and Tension Recording** Male Sprague-Dawley rats (200—250 g) were obtained from Han-Lym Lab. Animal Co. (Gyeonggi-Do, Korea). Animals were

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fed a standard laboratory rat chow (PICO-LAB Rodent Diet 20-5053, PMI Feeds, Richmond, IN, U.S.A.) for a 1—4 d period of acclimation, and were also given tap water. Food and water were allowed *ad libitum*. The air-conditioned animal room was maintained at 22±2 °C, with a relative humidity of 59±1% and a 12 h light/dark cycle (light period, 07—19 h). After acclimation, rats were killed by stunning and bleeding. The thoracic aorta was carefully isolated to protect the endothelial lining, then cut into rings (2—3 mm wide). These rings were mounted under 1 g of resting tension on stainless-steel hooks in 4 ml horizontal-type muscle chambers, and were bathed in physiological salt solution (PSS) at 37 °C containing (mM): NaCl, 136.9; KCl, 5.4; CaCl₂, 1.5; MgCl₂, 1.0; NaHCO₃, 23.8; glucose, 5.5, and ethylenediaminetetraacetic acid (EDTA) 0.01; then gassed with 95% O₂ and 5% CO₂. Tension was measured isometrically using a force-displacement transducer (FT03, Grass, Rhode Island, U.S.A.) connected to a polygraph system (RPS212, Grass, Rhode Island, U.S.A.) and a computer analyzer (Power Lab 400, MacLab System, Castle Hill, Australia). Tissues were allowed to equilibrate for 60 min before the experiments began. Endothelium was removed from some aortic rings by gently rubbing the intimal surface with a moistened cotton swab.

All tissues were repeatedly exposed to 70 mM KCl solution until responses were stable. Next, a control contraction was produced by exposing the tissues to 300 nm NE, which produced a maximal contraction. Then, the extracts or equivalent concentrations of the vehicle ethanol were added to the bath, and the relaxation response was studied. The high K⁺ solution was prepared by replacing NaCl with equimolar KCl. The functional activity of the endothelium was confirmed by the ability of carbachol (1 μM) to induce relaxation in aorta stimulated with 300 nm NE.14 In some experiments, L-NNA, a nitric oxide synthase inhibitor was used to pretreat the aorta strips for 30 min before tension stimulated by NE. Relaxations are expressed as the percentage of relaxation of NE-induced tone. Results are expressed as the mean±S.E.M.

**Transient Transfection and Luciferase Assays** COS cells were maintained in phenol red-free Dulbecco’s modified Eagles medium (DMEM) containing antibiotic/antimycotic mix, 5 mM N-(2-hydroxyethyl)-piperazine-N-2-ethanesulfonic acid, and 0.37% sodium bicarbonate, supplemented with 10% fetal bovine serum (FBS). Cells were grown at 37 °C in a humidified atmosphere of 95% air/5% CO₂ and fed every 2—3 d. Before hormone induction, the cells were washed with phosphate-buffered saline and cultured in DMEM/10% charcoal-dextran stripped FBS (CD-FBS) for 2 d to eliminate any estrogenic source before treatment. All E2 treatments were done with DMEM/10% CD-FBS. Ten nanomolar E2 was used to maximize the response, unless otherwise noted. Controls were treated with the vehicle. All the compounds were treated such that the total ethanol concentration was never higher than 0.1—0.4%. Cells were seeded in 24-well plates at a density of 7×10⁴ cells/well. After 24 h, plasmids were transiently transfected by the calcium phosphate precipitation method. The next day, transfected cells were washed with PBS and treated with the compounds for 24 h. Cell extracts were prepared and analyzed with a luminometer (Berthold Lumat LB 9501) using the luciferase assay system (Promega). Each sample was normalized for total protein concentration, as measured by the Bradford method using bovine serum albumin as a standard.

**RESULTS**

**Endothelium and Nitric Oxide Dependent Relaxation Induced by SC-Ex** In the quiescent preparation, SC-Ex (0.03—10 mg/ml) did not evoke any changes in tension in intact or endothelium-denuded arteries. Figure 1A shows a typical trace of the effect of SC-Ex (0.3 mg/ml) on muscle tension stimulated with 300 nm NE in endothelium-intact rat aorta. SC-Ex (0.3 mg/ml) transiently relaxed (41±7%, n=6) the contractions induced by NE on endothelium-intact rat aorta. Tension could be regained by NE and completely relaxed by 1 μM carbachol after washing of the aorta. In contrast, SC-Ex (0.3 mg/ml) did not affect the contractions induced by NE on endothelium-denuded arteries (Fig. 1B). We evaluated the effect of L-NNA, a nitric oxide synthase inhibitor, on the endothelium dependent relaxation induced by SC-Ex on endothelium-intact arteries. Pretreatment with L-NNA (10 μM) led to a significant suppression of SC-Ex induced relaxation on the aortic rings contracted with NE (Fig. 1C). Fractionated active fractions, SC-BSF and SC-ADF, also showed an endothelium and nitric oxide dependent relaxation in the same manner as SC-Ex (data not shown). The ICI 182,780 (10 μM), a specific estrogen receptor antagonist, significantly reduced the relaxation to SC-Ex (0.1 mg/ml) (manuscript in submission).

**Activation of an Estrogen-Responsive Luciferase Reporter Construct** Studies have shown that estrogen plays an important role in vasodilation through the stimulation of nitric oxide release produced by endothelial nitric oxide synthase.15 We therefore investigated whether components in *Schizandrae* may have estrogen-like activity. Current evidence suggests that nitric oxide release by estrogen is through the plasma membrane ER by nongenomic action.16 The plasma membrane ER has not been isolated or sequenced.17 In a classical genomic action model, estrogenic ligand binding to the ER initiates transcriptional activation through ERE in certain target genes.13 As stated, due to the lack of plasma membrane ER and other appropriate experimental systems, we have studied the mechanism of vasorelaxation by *Schizandra* to examine the possibility that *Schizandra* extracts may act through the nuclear ER to activate the transcription of an ERE containing reporter plasmid by transiently transfecting ER and reporter plasmids in ER-negative

**Fig. 1. Endothelium and Nitric Oxide Dependent Relaxation of SC-Ex on NE Induced Contraction**

Endothelium-intact (A) or -denuded aorta (B) were repeatedly exposed to 70 mm KCl solution until responses became stable. The rings were contracted with NE 300 nm, then SC-Ex (0.3 mg/ml) was added to the muscle. The endothelium-intact strip was pre-treated with L-NNA for 30 min in the quiescent preparation, then SC-Ex (0.3 mg/ml) was added to NE-contracted aorta (C). The presence and absence of endothelium on aortic rings is indicated by (+) and (−). Representative tracings.
However, the response is not as high as that with 10 nM E2. The highest activation was observed in the fraction of SC-ADF. We were not able to observe saturation effects of the estrogen receptor because the maximal treated concentration under our experimental conditions was insufficient in such therapies, in contrast to the extensive studies no conclusive scientific data has shown that herb for the alleviation of menopausal symptoms, however is ef

Phytoestrogens are nonsteroidal compounds that occur naturally in plants and that possess weak estrogenic activity.22) Estrogen-like responses have been reported for chemicals such as flavonoids, coumestans, and stilbenes.23) The potential biological impact of phytoestrogens has generated considerable interest. Phytoestrogens interact with nuclear ER through which they modulate a variety of estrogen-dependent processes. Schizandra has been used as a medical herb for the alleviation of menopausal symptoms, however no conclusive scientific data has shown that Schizandra is efficient in such therapies, in contrast to the extensive studies on the estrogenic activity of isoflavones found in soybeans. It is clear that the molecular mechanisms and efficacy of Schizandra need to be elucidated for safer use of this traditional therapy. Further studies will allow us to isolate single

Fig. 2. Effects of Schizandra Extracts on an Estrogen Responsive Reporter Gene in COS Cells
Cells were transiently co-transfected with hER and ERE-tk81-luc and treated with 10 nM E2, 20 μg/ml SC-ADF, 100 μg/ml SC-BSF, or 20 μg/ml SC-Ex for 24 h, then assayed for luciferase activity as described in Materials and Methods. Transfections were performed in triplicate more than three times. One representative result is shown in this figure. Data are expressed as the mean±S.E.M. for relative luciferase units.

Fig. 3. SC-ADF Dose-Dependently Activates Estrogen Responsive Reporter System, Which is Blocked by Estrogen Receptor Specific Antagonist
Cells were transiently transfected with hER and ERE-tk81-luc and treated with ethanol vehicle control, 10 nM E2, or 20 μg/ml (A), or as indicated in the figure (B) of SC-ADF, with or without 1 μM ICI, as marked in phenol-red-free DMEM plus 10% CD-CS, then assayed for luciferase activity after 24 h treatments. One representative result is shown in this figure. Data are expressed as the mean±S.E.M. Data are representative of at least three independent experiments performed in triplicate.

Fig. 4. SC-ADF Does Not Activate Androgen Receptor
Cells were transiently transfected with hAR and AR-responsive luciferase plasmids under indentical conditions, as in Fig. 3, then treated with the compounds indicated in the Figure. Transfections were performed in triplicate more than three times, and one representative data is shown.

Discussion
In rat aorta SC-Ex, a hot water soluble fraction of Schizandra chinensis fruit elicited potent relaxation in the endothelium-intact rat thoracic aorta contracted with NE. The SC-Ex induced relaxation was abolished by the removal of the endothelium, and was completely suppressed by pretreatment with nitric oxide synthase inhibitor. We have considered the possible involvement of endothelial nitric oxide synthase in the endothelium-dependent relaxation induced by SC-Ex. Estrogens and plant-derived estrogens confer vasoprotection through nitric oxide synthase with subsequent nitric oxide release.19,20) In line with this reasoning, we examined further and report here that SC-Ex contains compounds that activate estrogen receptors.

Schizandra has long been used in the Orient as a tonic. Studies have shown that it contains antioxidative, anti-inflammatory, hepato-protective, and cardiovascular properties. It is consumed in various ways, such as in tea, liquor and juice.21) However, it is not as extensively studied as ginseng, despite its famed pharmacological effects. The purpose of this study was to understand the mechanism of the vasorelaxation effect of Schizandra. This is the first in vitro study to evaluate the estrogenic efficacy of Schizandra.

Effects on Androgen Receptor
To exclude the possibility that SC-ADF activation of an estrogen receptor is a spurious, nonspecific event, we transiently transfected COS cells with the AR and reporter plasmids containing the androgen responsive elements. 10 nM Tes was used as a positive control to examine the androgen activity of SC-ADF. SC-ADF failed to elicit any activity through the AR (Fig. 4).
compounds responsible for the effects, and increase our understanding of the rationale for traditional usages of *Schizandra*.

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**REFERENCES**