JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Long-Term Effect of Phytoestrogens from *Curcuma comosa* Roxb. on Vascular Relaxation in Ovariectomized Rats

Suttira Intapad,[†] Vitoon Saengsirisuwan,[†] Mujalin Prasannarong,[†] Aporn Chuncharunee,[‡] Wisuda Suvitayawat,[#] Ratchanaporn Chokchaisiri,[§] Apichart Suksamrarn,[§] and Pawinee Piyachaturawat*[,†]

[†]Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand 10400

[‡]Department of Anatomy, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand 10700

[#]Department of Physiology, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand 10400

[§]Department of Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand 10240

ABSTRACT: Phytoestrogens have been implicated as promising therapeutic agents to treat the vascular impairment seen in menopausal women. The present study investigated the long-term effects of phytoestrogens from *Curcuma comosa* Roxb. on vascular relaxation of isolated thoracic aorta from ovariectomized (OVX) rats. Treatment of OVX rats for 12 weeks with *C. comosa* powder, hexane extract, and a novel phytoestrogen, diarylheptanoid-D3, [(3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol] prevented impairment of the endothelium-dependent relaxation response to acetylcholine in OVX, but not the endothelium-denude aortic ring relaxation in response to sodium nitroprusside. These data suggest that the vascular relaxation effect of *C. comosa* is mediated via endothelial cells. Treatment with D3 also increased endothelial nitric oxide synthase (eNOS) and estrogen receptor-α (ERα) protein expression in the aorta of OVX rats and suppressed elevated tumor necrosis factor-α (TNF-α) expression in OVX aortic rings. These results indicate that *C. comosa* treatment prevents impairment of vascular relaxation in estrogen-deficient animals via the ER-eNOS pathway as well as through its ability to promote an anti-inflammatory response.

KEYWORDS: *Curcuma comosa, diarylheptanoid, eNOS, phytoestrogens, vascular relaxation*

INTRODUCTION

Alterations in vascular function, especially a reduced response to relaxation stimuli, appear to be related to the onset of menopause.[1] Estrogen is thought to play a key role as a modulator of vasoprotection through its vasodilating, antioxidant, and anti-inflammation activities.[2] The protective effect of estrogen is mediated by binding to estrogen receptors (ERs) on blood vessel walls.[3] Both ERα and ERβ have been identified in various components of the vascular wall of experimental animals and humans; however, estrogen modulates vascular function mainly through ERα in both nongenomic and genomic pathways. Estrogen directly regulates the expression of endothelial nitric oxide synthase (eNOS) in cerebral microvessels[4] and the aorta.[5] In addition, estrogen can indirectly regulate eNOS expression through inhibition of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α), which down-regulates expression of eNOS by destabilizing its mRNA.[6] The role of estrogen in regulating eNOS production is also supported by the observation of elevated TNF-α serum levels in postmenopausal women and ovariectomized (OVX) rats.[7] The anti-inflammatory and vasoprotective effects of estrogen are ER-dependent and are not observed in the presence of the ER antagonist ICI 182,780 or in ER knockout mice.[8−11] Although estrogen has been proven to effectively prevent vascular impairment in postmenopausal women, its potential carcinogenic and feminizing effects on reproductive organs limit its therapeutic use. Thus, intensive investigations of alternatives to estrogen have been undertaken.

Currently, there is increasing interest in the use of phytoestrogens as an alternative approach to alleviate menopausal symptoms.[12] Phytoestrogens are naturally occurring, nonsteroidal compounds derived from plants that have biological activities similar to those of estrogen. Recently, a novel phytoestrogen, diarylheptanoid-D3 or (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (hereafter referred to as D3), was isolated and identified from *Curcuma comosa*, a member of the Zingiberaceae family.[13,14] *C. comosa* is extensively used by menopausal women in many forms, including dried rhizome powder and crude extracts,[16,17] and it is possible that D3 or other phytoestrogens may be the active compound in these preparations. D3 has the capacity to increase the endothelium-dependent relaxation of rat aortic rings, and its nongenomic mechanism is associated with enhanced phosphorylation of eNOS and Akt proteins.[15] The effect of D3 on eNOS and Akt proteins appears to be mediated by the same pathway as estrogen as the response to D3 is inhibited by the presence of an ER antagonist.[15] Due to the beneficial effects of D3 on vascular function, we hypothesized that D3 will restore endothelial function in OVX rats. The present study investigated the long-term effects of *C. comosa* treatment on the relaxation of isolated rat aortic rings and the underlying mechanisms that lead to relaxation. Our results provide mechanistic insight into the beneficial properties of *C. comosa* and its estrogenic effect, supporting a role for its further clinical use in vascular disorders.

Received: August 7, 2011
Revised: December 4, 2011
Accepted: December 21, 2011
Published: December 21, 2011

dx.doi.org/10.1021/jf203173b | *J. Agric.Food Chem.* 2012, 60, 758−764

MATERIALS AND METHODS

Chemicals and Plant Extract. Acetylcholine (ACh), L-phenylephrine (Phe), sodium nitroprusside (SNP), and 17β-estradiol (E2) were purchased from Sigma Chemical Co. (St. Louis, MO), and all other chemicals were of analytical grade. Preparations of plant extracts and isolation of diarylheptanoid from *C. comosa* rhizomes were conducted as previously described.[13] Rhizomes of *C. comosa* were purchased from Kampaengsaen district, Nakhon Pathom province, Thailand, and subjected to taxonomic identification[18] with voucher herbarium specimen (SCMU No. 300) deposited at the Department of Plant Science, Faculty of Science, Mahidol University, Bangkok. The rhizomes of *C. comosa* were cut into small pieces, dried, and ground to powder. The rhizome powder was extracted with *n*-hexane in a Soxhlet extractor, and after removal of the solvent in vacuo, a pale brownish viscous oil was obtained. The hexane extract was dissolved in corn oil (Mazola corn oil, ACH Food Companies Inc., Cordova, TN) for animal treatment. Characterization and standardization of the extract used in this study were performed by reversed-phase HPLC (column, Prodigy ODS-3, 250 mm × 4.6 mm, 5 μm, 100 Å; mobile phase, water/acetonitrile; detection at 260 nm), revealing one major diarylheptanoid (23.9%), (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (D3), as shown in Figure 1. Compound D3 was isolated from the hexane extract as a major component by repeated silica gel column chromatography eluting with hexane/dichloromethane; each step utilized an increasing quantity of the more polar solvent. The structure of D3 was confirmed from the spectroscopic data (NMR and mass spectra), and the absolute stereochemistry at the 3-position was determined to be *R*. The isolated compound D3 was assessed by TLC and NMR spectroscopy and was found to be approximately 99% pure. The dried rhizome powder for animal treatment was sieved through sieve no. 60.

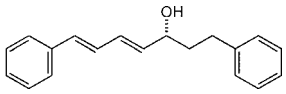

Figure 1. Structure of diarylheptanoids; (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (D3) isolated from the rhizome of *C. comosa*.

Animals and Aortic Ring Preparation. Female Sprague–Dawley rats at 8 weeks of age were supplied by the National Animal Center of Thailand, Mahidol University, Salaya Campus, Nakornpathom. All experimental animal protocols received approval from the Ethics Committee for Animal Care and Use, Faculty of Science, Mahidol University. Rats were subjected to ovariectomy and sham-operated animals served as controls, as previously described.[14] Animals were assigned into 10 groups with 6 rats each as follows: (1) sham-operated control; (2) ovariectomized (OVX); (3) OVX treated with estradiol, 2.5 μg/kg BW via subcutaneous injection; and (4) OVX treated with *C. comosa* dried powder, 1000 mg/kg BW via oral gavage. Groups 5, 6, and 7 were OVX treated with *C. comosa* hexane extract at doses of 125, 250, and 500 mg/kg BW, respectively, via intraperitoneal injection. Groups 8, 9, and 10 were OVX treated with D3 at doses of 25, 50, and 100 mg/kg BW, respectively, via intraperitoneal injection. Animals were treated 6 days/week for 12 weeks. The dose of powder (1000 mg/kg/day) used in the present study was based on our preliminary observation that it can protect against other adverse effects observed in OVX animals including bone loss. In addition, in a subacute toxicity study of dried powder in adult rats for 3 months, the low observed adverse effect level (LOAEL) was approximately 4000 mg/kg BW (unpublished data). After treatment on the day of the experiment, the animals were euthanized by intraperitoneal injection of pentobarbital sodium (50 mg/kg BW). The thoracic aorta was carefully excised and immediately placed in iced-cold Krebs–Henseleit solution with all connective tissue removed. The aorta was cut transversely into 3 mm wide strips for the relaxation response study and ~1 cm wide strips for Western blot analysis.

Vasodilator Responsiveness of Preconstricted Aortic Rings. Each aortic ring was placed between two parallel stainless steel hooks in an organ bath (20 mL). One hook was fixed, and the other was connected to a force transducer (model MLT1030, ADI Instrument, Australia) for the measurement of isometric tension. The transducers were connected to a PowerLab (ADI Instrument, Australia) to provide a continuous record of contractile tension. The organ bath contained a Krebs–Henseleit solution (pH 7.4) of the following composition (mM): NaCl (119.0), $NaHCO_3$ (25.0), glucose (11.1), $CaCl_2{\cdot}2H_2O$ (1.6), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4{\cdot}7H_2O$ (1.2), aerated with 95% O_2–5% CO_2 and maintained at 37 °C. Aortic ring functions were investigated either with the endothelium intact (Endo+) or denuded (Endo−), which was accomplished by mechanical de-endothelialization using a fine wire rubbed inside the lumen of the aortic ring. After the thoracic aortic ring was connected to an isometric tension transducer system, passive tension was gradually increased to 1.5 g. The vascular ring was allowed to equilibrate for at least 30 min, and the preparation was refreshed with Krebs–Henseleit solution every 15 min. Passive tension was adjusted to maintain 1.5 g throughout the equilibration and experimental periods. At the basal tension of 1.5 g, the rings were found to develop maximal active tension in response to stimulation with a contraction-inducing agent. After equilibration for 30 min, the maximal contractile response to a high concentration of potassium (80 mM) in Krebs solution (potassium exchanged with sodium on a molar basis) was determined. Then, rings were washed three times with fresh Krebs solution and allowed to equilibrate for 30 min before further evaluation of the relaxation response to either ACh 10^{-9} to 10^{-5} M (Endo+) or SNP 10^{-9} to 10^{-5} M (Endo−) in vessels precontracted with L-phenylephrine (Phe 10^{-7} M). The vasodilator effect of increasing concentrations of ACh and SNP was expressed as percent decrease of the peak Phe concentration.

Western Immunoblotting Analysis. Western blot analyses of ERα, ERβ, eNOS, TNFα, and β-actin were conducted as previously described[19] with modifications. Frozen aortic tissue was homogenized in a lysis buffer of (in mM) 150 NaCl, 50 Tris·HCl, 5 EDTA·2Na, and 1 $MgCl_2$ containing 1% Triton X-100 and 0.5% SDS plus protease inhibitor cocktail and 1 mM sodium orthovanadate. Protein concentrations were determined according to the method of bicinchoninic acid protein assay (BCA). Proteins in the homogenates were separated on 7% polyacrylamide gels and transferred electrophoretically onto PVDF membrane (Bio-Rad, Hercules, CA). After blocking with 5% nonfat dry milk in TBS plus 0.1% Tween 20 for 2 h, protein blots were incubated with the appropriate dilution of antibodies against eNOS (Cell Signaling Technology, Beverly, MA), ERα (Santa Cruz Biotechnology, Santa Cruz, CA), ERβ (BD Transduction Laboratories, San Jose, CA), or TNFα (Santa Cruz Biotechnology) at 4 °C overnight. Subsequently, all blots were incubated with secondary anti-rabbit antibody at room temperature for 2 h. The immunocomplexes were detected by chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL) followed by densitometric analyses with ImageJ software. After detection, the membranes were stripped, blocked, and reprobed with mouse polyclonal anti-β-Actin (Santa Cruz). Results for ERα, ERβ, TNFα, and eNOS proteins were expressed as percent of the β-actin protein levels in each sample.

Statistics. The concentrations indicated in the text or in the figures represent the final organ bath concentrations of respective agonists. Data were expressed as the mean ± the standard error of the mean (SEM) and were analyzed using the statistical software package GraphPad Prism 4. Two-way ANOVA with Bonferroni multiple-comparisons post-test was used to compare concentration–response curves obtained in aortic rings. Western blotting results among different groups were analyzed by using one-way analysis of variance (one-way ANOVA), and the differences in pairs of means were determined by Newman–Keuls test. Statistical significance of the difference was accepted at P values of <0.05 or exceeding the 95% critical value ($P < 0.05$).

RESULTS

Effect on Vascular Endothelium-Dependent Relaxation. Figure 2 shows the relaxation of aortic rings with intact

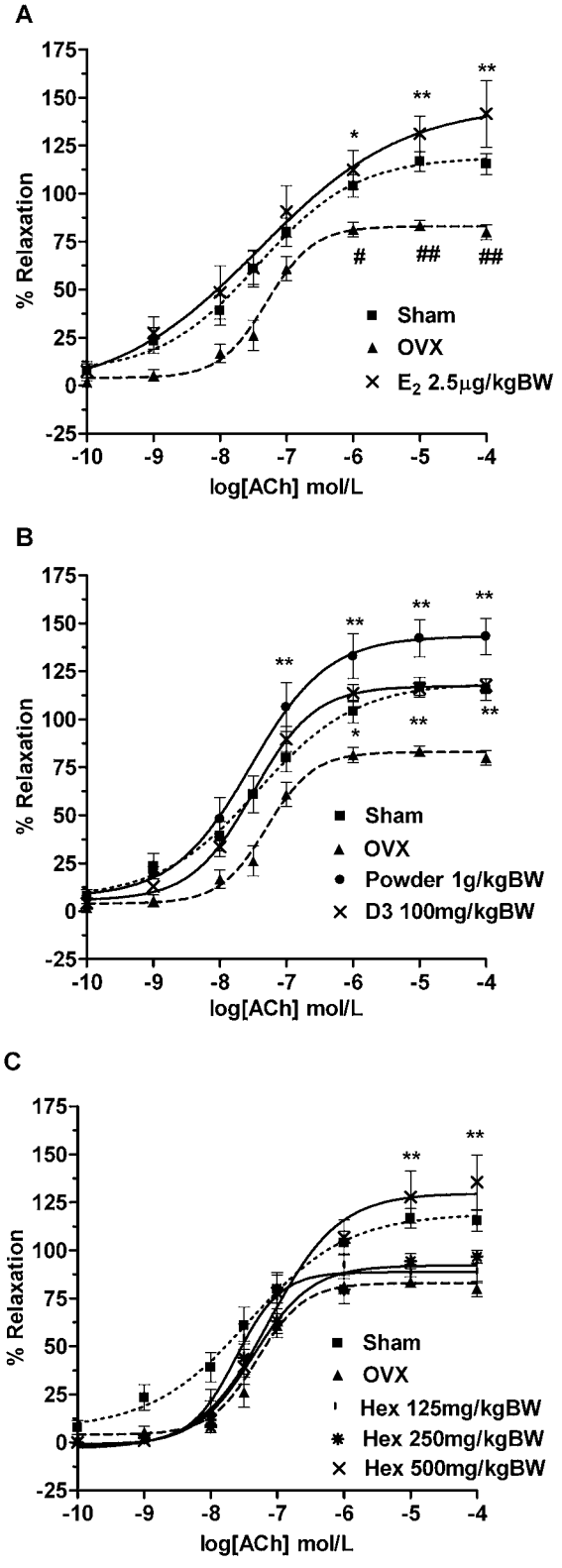

Figure 2. Effect of long-term treatment with (A) estradiol 2.5 μg/kg BW/day, (B) *C. comosa* powder 1 g/kg BW/day and compound D3 100 mg/kg BW/day, and (C) *C. comosa* hexane extract 125, 250, 500 mg/kg BW/day for 12 weeks on the endothelium-dependent relaxation in OVX rats. The OVX control received a similar treatment with olive oil. Data are the mean ± SEM (n = 6). (#) $P < 0.01$ and (##) $P < 0.001$, significant difference from sham control. (∗) $P < 0.01$ and (∗∗) $P < 0.001$, significant difference from OVX.

endothelial cells in response to ACh in OVX rats receiving various treatments. The maximum vasodilation in the OVX rats after 12 weeks was significantly lower than that of sham control ($P < 0.05$). Treatment with 17β-estradiol at a dose of 2.5 μg/kg BW prevented impairment of relaxation in the OVX rats (Figure 2A). Treatment with *C. comosa* dried powder at a dose of 1 g/kg/day and isolated compound D3 at a dose of 100 mg/kg/day (Figure 2B) as well as the *C. comosa* hexane extract at a dose of 500 mg/kg effectively prevented impairment of endothelium-dependent relaxation in the OVX rats ($P < 0.05$). Their levels were similar to that observed in the estrogen-treated group. In Figure 2C, hexane extract at dose of 500 mg/kg BW/day prevented the impairment of relaxation induced by OVX. However, treatment with hexane extract at lower doses of 125 and 250 mg/kg BW/day was not sufficient to prevent the reduction of endothelium-dependent relaxation induced by OVX. EC_{50} values of ACh in inducing relaxation among different experimental groups were not significantly different from each other. These results indicate that the sensitivity of relaxation responses to ACh was not altered after 12 weeks of estrogen deficiency.

Effect on Vascular Endothelium-Independent Relaxation. Treatment of OVX rats with 17β-estradiol, *C. comosa* powder, compound D3, and *C. comosa* hexane extract for 12 weeks did not significantly alter the relaxation of aortic ring without endothelium (denuded) in response to SNP (Figure 3). EC_{50} values of the SNP-induced vascular relaxation in all experimental groups were comparable, suggesting that both the maximum relaxation and the sensitivity of the relaxation response to SNP in denuded vascular were not affected. These findings indicate that the site of action of estrogen, as well as *C. comosa*, in preventing impairment of vascular relaxation is at the endothelial lining cells and not smooth muscle cells.

Effect on the Expression Levels of ERs, eNOS, and TNF-α. To further investigate the involvement of the estrogenic activity of *C. comosa* on vascular dysfunction induced by OVX, we determined the expression level of ERs in blood vessels. As seen in Figure 4A, the expression of ERα in OVX groups was significantly reduced compared to the sham control. Treatment with *C. comosa* powder and compound D3 at 100 mg/kg BW/day significantly increased ERα expression ($P < 0.05$). In contrast, the ERβ expression level was not significantly altered by these treatments (Figure 4B).

Our analysis demonstrated that both E_2 and *C. comosa* treatments prevented impairment of the vascular relaxation response to ACh in OVX rats. To investigate the underlying mechanism, we examined the expression level of eNOS protein in aortic rings. Before determination of the protein levels by Western blotting, the semiquantitative RT-PCR analysis of gene expression for eNOS was examined. The results for both RT-PCR and Western blotting were similar, and we reported the data from the analysis of protein levels. Protein expression of eNOS was slightly lower in the OVX group compared to the sham group (Figure 5), but the difference did not reach statistical significance. Treatment with E_2 induced a significant increase in eNOS protein expression; however, *C. comosa* powder did not significantly alter eNOS expression. Treatment with a high dose of compound D3 was required to significantly increase eNOS expression compared to untreated OVX samples ($P < 0.05$).

TNFα has recently been reported to play an important role in the pathogenesis of vascular diseases during aging and

dx.doi.org/10.1021/jf203173b | J. Agric.Food Chem. 2012, 60, 758–764

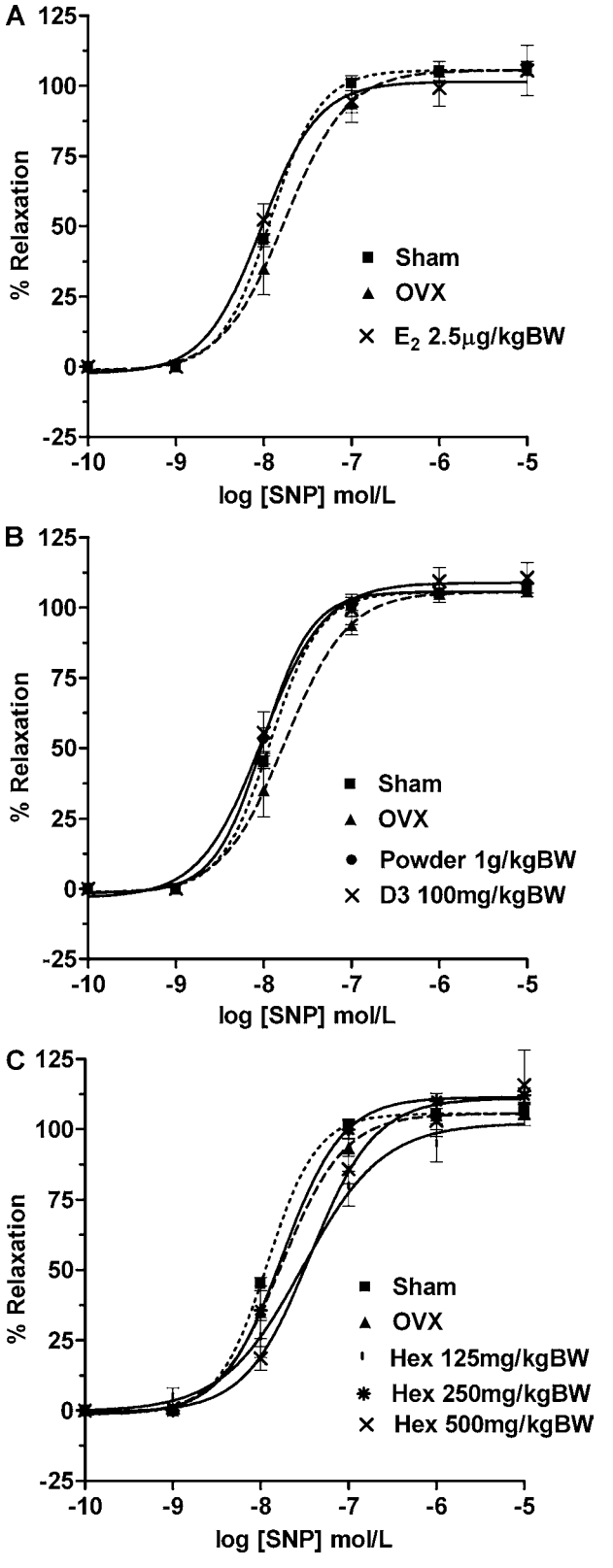

Figure 3. Effect of long-term treatment with (A) estradiol 2.5 μg/kg BW/day, (B) *C. comosa* powder 1 g/kg BW/day and compound D3 100 mg/kg BW/day, and (C) *C. comosa* hexane extract 125, 250, 500 mg/kg BW/day on the endothelium-independent relaxation in OVX rats for 12 weeks. The OVX control received a similar treatment with olive oil. Data are the mean ± SEM (n = 6).

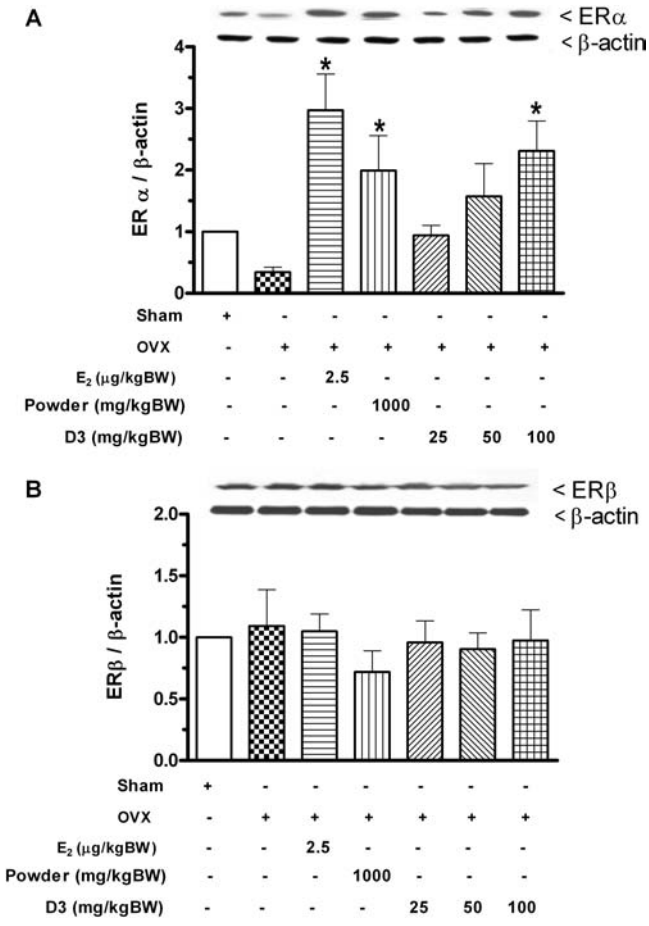

Figure 4. Effect of long-term treatment with estradiol 2.5 μg/kg BW/day and *C. comosa* powder 1 g/kgBW/day and compound D3 25, 50, 100 mg/kg BW/day on the expression levels of ERα (A) and ERβ (B). Data are the mean ± SEM (n = 4). (*) $P < 0.05$, significant difference from OVX.

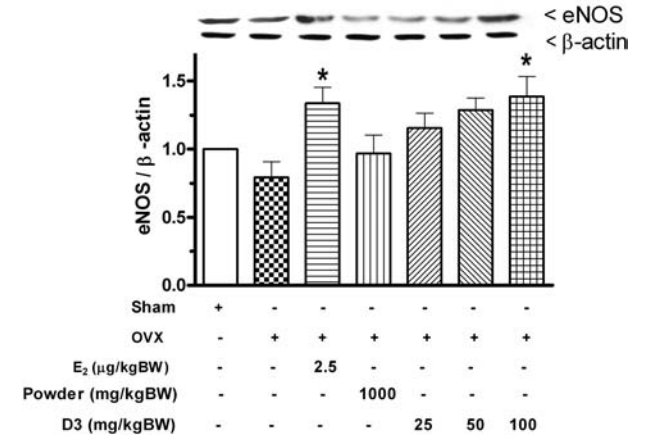

Figure 5. Effect of long-term treatment with estradiol 2.5 μg/kg BW/day and *C. comosa* powder 1 g/kg BW/day and compound D3 25, 50, 100 mg/kg BW/day on the expression levels of eNOS. Data are the mean ± SEM (n = 4). (*) $P < 0.05$, significant difference from OVX.

estrogen deficiency. We observed increased TNFα expression in aortic ring homogenate of the OVX rats compared to that of the sham group (Figure 6). Interestingly, *C. comosa* and compound D3 treatment significantly decreased TNFα protein expression to levels similar to those seen with E_2 treatment,

dx.doi.org/10.1021/jf203173b | *J. Agric.Food Chem.* 2012, 60, 758–764

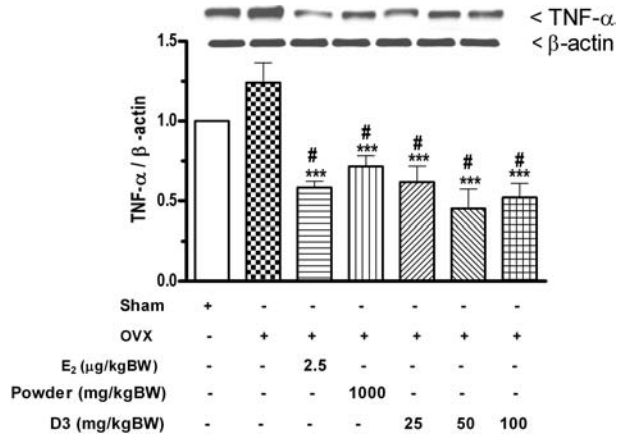

Figure 6. Effect of long-term treatment with estradiol 2.5 μg/kg BW/day and *C. comosa* powder 1 g/kg BW/day and compound D3 25, 50, 100 mg/kg BW/day on the expression levels of TNF-α. Data are the mean ± SEM (n = 4). (#) $P < 0.05$, significant difference from sham group. (***) $P < 0.001$, significant difference from OVX group, by one-way ANOVA.

suggesting that *C. comosa* is also acting through an anti-inflammatory mechanism. These results suggest that the potential mechanism of *C. comosa* in preventing impairment of vascular relaxation is mediated through alterations in ERα, eNOS, and TNFα protein expression during estrogen deficiency.

■ DISCUSSION

Our present study demonstrates that long-term treatment with *C. comosa* extracts is capable of preventing altered endothelium-dependent relaxation in the vasculature of OVX rats. This prevention was associated with the preservation of key signaling molecules for estrogenic action in vascular tissues including eNOS, ERα, and TNF-α during estrogen deficiency. The underlying mechanism for the effect of *C. comosa* appears to be mediated by the estrogenic activity of *C. comosa* compounds binding to ER as well as through anti-inflammatory activities.

OVX animals have been used to mimic the endothelial dysfunction developed in postmenopausal women.[20,21] Although endothelial cells produce and release many relaxing factors, endothelium-dependent vasodilatation relies almost entirely on the endothelial release of NO. OVX animals exhibited a reduced relaxant response to ACh, an endothelium- and NO-dependent vasodilator, but the response to SNP, an NO donor, was unchanged. Endothelial dysfunction induced by OVX is characterized by reduced NO bioactivity.[20,22,23] Likewise, long-term *C. comosa* supplementations were found in the present study to clearly improve the endothelium-dependent vasodilator response to ACh in OVX rats without affecting the response to SNP. These results suggest that *C. comosa* may improve endothelial function in OVX by increasing NO bioactivity.

Several mechanisms could account for modulation of NO bioactivity, including changes in expression of eNOS, posttranslational modification of the eNOS enzyme (e.g., phosphorylation), and changes in the sensitivity of the NO-cGMP pathway in vascular smooth muscle cells. As the response to nitroprusside was not modified by ovariectomy, impairment in OVX rats is likely to be independent of the NO-cGMP pathway in vascular smooth muscle cells. However, the expression of eNOS protein in aorta was increased by D3 treatment, suggesting increased NO bioactivity. Increased eNOS expression by *C. comosa* may be the major modulating factor that contributes to the protection of endothelium-dependent relaxation of OVX rat aorta. In addition, increased eNOS phosphorylation has been observed during acute exposure to *C. comosa*[15] and may also contribute to improve vascular function observed in the present study.

The estrogen receptor is the key molecule linking estrogen status to the function and health of the vascular endothelium. ERα is highly expressed in endothelial cells and is suggested to play a role in mediating the effects of estrogen in the vascular endothelium and the regulation of eNOS function. Decreased ERα expression is seen in several vascular beds of ovariectomized animals and postmenopausal women.[24,25] In the ERα knockout mouse model, the basal level of NO was not affected;[26] however, up-regulation of eNOS protein expression in estrogen-supplemented OVX animals is abolished in ERα knockout mice.[27] In the present study we also found decreased protein expression of ERα but not ERβ in thoracic aorta of OVX rats. Treatment with *C. comosa* prevented the decrease in ERα protein levels and resulted in ERα levels similar to that seen by treatment with estrogen. The protective effect of *C. comosa* treatment on vascular relaxation we observed appears to be strongly correlated to ER and eNOS expression, as these two proteins were increased in a dose-dependent manner by D3 treatment. The positive relationship between circulating estrogen and ERα expression in endothelial cells has been reported in peripheral veins of the premenopausal woman in different phases of their menstrual cycle and in postmenopausal women.[28] A number of studies in animals and in vitro cell culture also support the expression of ERα in vascular cells and its regulation by estrogenic-like activity.[24,25] The increase in ERα by *C. comosa* in this study is consistent with previous studies that indicate D3 induces transactivation of the ERα–ERE-driven pathway.[14,29] Although the circulating level of D3 was not determined in our analysis, a pharmacokinetic investigation recently reported that high D3 levels were detected in the plasma and tissues of rats given the *C. comosa* hexane extract.[30] Hence, the increased ERα expression in rat aorta following *C. comosa* treatment we observed may be due to circulating D3. Both nongenomic and genomic estrogenic activity may contribute to the protective effect of *C. comosa* and D3 found in the present study, and long-term treatment may affect the clinical use of *C. comosa* due to the possible genomic influence.

Estrogen has multiple sites of action, and estrogen deficiency can influence both the short- and long-term regulation of vascular dilating factors. In addition, estrogen itself provides an anti-inflammatory action to vascular cells. Elevation of serum pro-inflammatory cytokines has been reported in postmenopausal women and in OVX rats.[7] TNF-α is a key pro-inflammatory cytokine involved in the pathogenesis of vascular disorder, and its formation is regulated by estrogen. Therefore, the change in the expression of TNF-α in OVX rat aorta in the present study is consistent with previous observations in which the decline of ovarian function in postmenopausal women is associated with an elevation of TNF-α.[7] TNF-α inhibition was also found to enhance agonist-mediated vasorelaxation and increase eNOS expression in the vasculature of female rats.[31] Moreover, the increase in TNF-α reduced NO availability in endothelial cells either by decreasing eNOS expression[6] or by increasing NO inactivation via enhanced superoxide anion production from NADPH oxidases.[32] Another possible reason

for the increase in vasorelaxation by *C. comosa* may be related to the decrease in TNF-α levels, leading to an increase in NO availability and eNOS expression.

In conclusion, the present study is the first to demonstrate the long-term protective effects of various components of *C. comosa* on the relaxation of OVX rat aortic rings. The potential mechanisms may be due to both the direct nongenomic and genomic estrogenic actions of *C. comosa* on the signaling proteins ERα, eNOS, and TNF-α. These data indicate that diarylheptanoid D3, a novel phytoestrogen, is, at least, an active compound responsible for the observed effect on vasorelaxation and may have therapeutic potential for the treatment of vascular diseases in postmenopausal women.

AUTHOR INFORMATION

Corresponding Author

*Postal address: Department of Physiology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok, Thailand 10400. Phone: (662) 2015611. Fax: (662) 3547154. E-mail: scppy@mahidol.ac.th.

Funding

This study was supported by the Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. Program (grant PHD/0094/2547 to S.I. and P.P.) and research grants from the TRF (DBG180020), the Strategic Basic Research Grant of TRF (to A.S.), and the Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative.

ACKNOWLEDGMENTS

We thank Prof. Chumpol Pholpramool and Dr. L. T. Jensen for a critical reading of the manuscript.

ABBREVIATIONS USED

ER, estrogen receptor; Ach, acetylcholine; Phe, phenylephrine; NO, nitric oxide; SNP, sodium nitroprusside; eNOS, endothelium nitric oxide synthase; TNF-α, tumor necrosis factor-α.

REFERENCES

(1) Xing, D.; Nozell, S.; Chen, Y. F.; Hage, F.; Oparil, S. Estrogen and mechanisms of vascular protection. *Arterioscler. Thromb. Vasc. Biol.* **2009**, *29*, 289−295.

(2) Orshal, J. M.; Khalil, R. A. Gender, sex hormones, and vascular tone. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2004**, *286*, R233−R249.

(3) Farhat, M.; Lavigne, M. C.; Ramwell, P. W. The vascular protective effects of estrogen. *FASEB J.* **1996**, *10*, 615−624.

(4) Stirone, C.; Chu, Y.; Sunday, L.; Duckles, S. P.; Krause, D. N. 17β-Estradiol increases endothelial nitric oxide synthase mRNA copy number in cerebral blood vessels: quantification by real-time polymerase chain reaction. *Eur. J. Pharmacol.* **2003**, *478*, 35−38.

(5) Goetz, R. M.; Morano, I.; Calovini, T.; Studer, R.; Holtz, J. Increased expression of endothelial constitutive nitric oxide synthase in rat aorta during pregnancy. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 905−910.

(6) Yoshizumi, M.; Perrella, M. A.; Burnett, J. C. Jr.; Lee, M. E. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ. Res.* **1993**, *73*, 205−209.

(7) Pfeilschifter, J.; Koditz, R.; Pfohl, M.; Schatz, H. Changes in proinflammatory cytokine activity after menopause. *Endocr. Rev.* **2002**, *23*, 90−119.

(8) Bakir, S.; Mori, T.; Durand, J.; Chen, Y. F.; Thompson, J. A.; Oparil, S. Estrogen-induced vasoprotection is estrogen receptor dependent: evidence from the balloon-injured rat carotid artery model. *Circulation* **2000**, *101*, 2342−2344.

(9) Mori, T.; Durand, J.; Chen, Y.; Thompson, J. A.; Bakir, S.; Oparil, S. Effects of short-term estrogen treatment on the neointimal response to balloon injury of rat carotid artery. *Am. J. Cardiol.* **2000**, *85*, 1276−1279.

(10) Karas, R. H.; Hodgin, J. B.; Kwoun, M.; Krege, J. H.; Aronovitz, M.; Mackey, W.; Gustafsson, J. A.; Korach, K. S.; Smithies, O.; Mendelsohn, M. E. Estrogen inhibits the vascular injury response in estrogen receptor β-deficient female mice. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 15133−15136.

(11) Brouchet, L.; Krust, A.; Dupont, S.; Chambon, P.; Bayard, F.; Arnal, J. F. Estradiol accelerates reendothelialization in mouse carotid artery through estrogen receptor-alpha but not estrogen receptor-β. *Circulation* **2001**, *103*, 423−428.

(12) Lissin, L. W.; Cooke, J. P. Phytoestrogens and cardiovascular health. *J. Am. Coll. Cardiol.* **2000**, *35*, 1403−1410.

(13) Suksamrarn, A.; Ponglikitmongkol, M.; Wongkrajang, K.; Chindaduang, A.; Kittidanairak, S.; Jankam, A.; Yingyongnarongkul, B. E.; Kittipanumat, N.; Chokchaisiri, R.; Khetkam, P.; Piyachaturawat, P. Diarylheptanoids, new phytoestrogens from the rhizomes of *Curcuma comosa*: isolation, chemical modification and estrogenic activity evaluation. *Bioorg. Med. Chem.* **2008**, *16*, 6891−6902.

(14) Winuthayanon, W.; Suksen, K.; Boonchird, C.; Chuncharunee, A.; Ponglikitmongkol, M.; Suksamrarn, A.; Piyachaturawat, P. Estrogenic activity of diarylheptanoids from *Curcuma comosa* Roxb. requires metabolic activation. *J. Agric. Food Chem.* **2009**, *57*, 840−845.

(15) Intapad, S.; Suksamrarn, A.; Piyachaturawat, P. Enhancement of vascular relaxation in rat aorta by phytoestrogens from *Curcuma comosa* Roxb. *Vasc. Pharmacol.* **2009**, *51*, 284−290.

(16) Piyachaturawat, P.; Ercharuporn, S.; Suksamrarn, A. Estrogenic activity of *Curcuma comosa* extract in rats. *Asia Pac. J. Pharmacol.* **1995**, *10*, 121−126.

(17) Piyachaturawat, P.; Ercharuporn, S.; Suksamrarn, A. Utero-trophic effect of *Curcuma comosa* in rats. *Int. J. Pharmacogn.* **1995**, *33*, 334−338.

(18) Soontornchainaksaeng, P.; Jenjittikul, T. Chromosome number variation of phytoestrogen-producing *Curcuma* (Zingiberaceae) from Thailand. *J. Nat. Med.* **2010**, *64*, 370−377.

(19) Segal, S. S.; Brett, S. E.; Sessa, W. C. Codistribution of NOS and caveolin throughout peripheral vasculature and skeletal muscle of hamsters. *Am. J. Physiol. Heart Circ. Physiol* **1999**, *277*, H1167−H1177.

(20) Catania, M. A.; Crupi, A.; Firenzuoli, F.; Parisi, A.; Sturiale, A.; Squadrito, F.; Caputi, A. P.; Calapai, G. Oral administration of a soy extract improves endothelial dysfunction in ovariectomized rats. *Planta Med.* **2002**, *68*, 1142−1144.

(21) Chanawirat, A.; Khemapech, S.; Patumraj, S.; Siriviriyakul, P. Genistein replacement therapy on endothelial dysfunction and bone loss in bilateral ovariectomized rats. *Clin. Hemorheol. Microcirc.* **2006**, *34*, 309−314.

(22) Khemapech, S.; Monsiri, K.; Patumraj, S.; Siriviriyakul, P. Genistein replacement therapy for vasodilation disorder in bilateral ovariectomized rats. *Clin. Hemorheol. Microcirc.* **2003**, *29*, 271−277.

(23) Pelligrino, D. A.; Ye, S.; Tan, F.; Santizo, R. A.; Feinstein, D. L.; Wang, Q. Nitric-oxide-dependent pial arteriolar dilation in the female rat: effects of chronic estrogen depletion and repletion. *Biochem. Biophys. Res. Commun.* **2000**, *269*, 165−171.

(24) Stirone, C.; Duckles, S. P.; Krause, D. N. Multiple forms of estrogen receptor-α in cerebral blood vessels: regulation by estrogen. *Am. J. Physiol. Endocrinol. Metab.* **2003**, *284*, E184−E192.

(25) Christian pinna, A. C.; Sanvito, P.; Pelosi, V.; Bolego, C. Prolong ovarian hormone deprivation impairs the protective vascular action of estrogen receptor α agonists. *Hypertension* **2008**, *51*, 1210−1217.

(26) Geary, G. G.; McNeill, A. M.; Ospina, J. A.; Krause, D. N.; Korach, K. S.; Duckles, S. P. Selected contribution: cerebrovascular

nos and cyclooxygenase are unaffected by estrogen in mice lacking estrogen receptor-α. *J. Appl. Physiol.* **2001**, *91*, 2389−2399.
(27) Glazier, M. G.; Bowman, M. A. A review of the evidence for the use of phytoestrogens as a replacement for traditional estrogen replacement therapy. *Arch. Intern. Med.* **2001**, *161*, 1161−1172.
(28) Gavin, K. M.; Seals, D. R.; Silver, A. E.; Moreau, K. L. Vascular endothelial estrogen receptor α is modulated by estrogen status and related to endothelial function and endothelial nitric oxide synthase in healthy women. *J. Clin. Endocrinol. Metab.* **2009**, *94*, 3513−3520.
(29) Winuthayanon, W.; Piyachaturawat, P.; Suksamrarn, A.; Ponglikitmongkol, M.; Arao, Y.; Hewitt, S. C.; Korach, K. S. Diarylheptanoid phytoestrogens isolated from the medicinal plant *Curcuma comosa*: biologic actions in vitro and in vivo indicate estrogen receptor-dependent mechanisms. *Environ. Health Perspect.* **2009**, *117*, 1155−1161.
(30) Su, J.; Sripanidkulchai, K.; Suksamrarn, A.; Hu, Y.; Piyachaturawat, P.; Sripanidkulchai, B. Pharmacokinetics and organ distribution of diarylheptanoids phytoestrogens from *Curcuma comosa* in rats. *J. Nat. Med.* **2011**, DOI: 10.1007/s11418-011-0607-x.
(31) Arenas, I. A.; Xu, Y.; Davidge, S. T. Age-associated impairment in vasorelaxation to fluid shear stress in the female vasculature is improved by TNF-α antagonism. *Am. J. Physiol. Heart Circ. Physiol.* **2006**, *290*, H1259−H1263.
(32) Murphy, H. S.; Shayman, J. A.; Till, G. O.; Mahrougui, M.; Owens, C. B.; Ryan, U. S.; Ward, P. A. Superoxide responses of endothelial cells to C5a and TNF-α: divergent signal transduction pathways. *Am. J. Physiol. Lung Cell Mol. Physiol.* **1992**, *263*, L51−L59.