

ARTICLES

The Phytoestrogen α -Zearalenol Reverses Endothelial Dysfunction Induced by Oophorectomy in Rats

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SUMMARY: It has been shown recently that α -zearalenol, a resorcyclic acid lactone, prevents bone loss in a rat model of postmenopausal bone loss. We have therefore investigated the effects of this phytoestrogen on endothelial dysfunction induced by estrogen deficiency in rats. Female mature Sprague-Dawley rats underwent a bilateral oophorectomy (OVX rats). Sham-operated animals (sham OVX rats) were used as controls. Three weeks after surgery, animals were randomized to the following treatments: α -zearalenol (1 mg/kg/day, im, for 4 weeks), 17β -estradiol (20 μ g/kg/day, im, for 4 weeks), or their vehicle (100 μ l, im, of cottonseed oil). Two other groups of rats were treated with α -zearalenol or 17β -estradiol plus the pure estrogen receptor antagonist ICI 182780 (2.5 mg/kg/day, im, for 4 weeks). Mean arterial blood pressure (MAP), heart rate (HR), total plasma cholesterol, plasma estradiol, and plasma α -zearalenol were studied. We also investigated endothelial-dependent (acetylcholine, 10 nM to 10 μ M) and endothelial-independent (sodium nitroprusside, 15 nM to 30 nM) relaxation of aortic rings, as well as N(G)-methyl-L-arginine (L-NMA: 10 to 100 μ M)-induced vasoconstriction and calcium-dependent nitric oxide synthase (cNOS) activity in homogenates of lungs taken from both sham OVX rats and OVX rats. Untreated OVX rats had, compared with sham OVX animals, unchanged body weight, MAP, HR, and plasma cholesterol. In contrast oophorectomy reduced plasma estradiol levels (OVX, 2 ± 0.5 pg/ml; sham OVX, 35 ± 6 pg/ml), impaired endothelial-dependent relaxation and blunted L-NMA-induced contraction (L-NMA 100 μ M: sham OVX, 2.7 ± 0.3 g/mg tissue; OVX, 1.3 ± 0.1 g/mg tissue). Moreover OVX rats showed a reduced calcium-dependent NO synthase (cNOS) activity. Treatment with α -zearalenol or with 17β -estradiol reverted the endothelial dysfunction and increased cNOS activity in lung homogenates. These effects were abolished by the pure estrogen receptor antagonist ICI 182780. Our data suggest that α -zearalenol improves endothelial-dependent relaxation in OVX rats through an estrogen receptor-mediated effect. (*Lab Invest* 2001, 81:125-132).

A large body of evidence suggests that estrogens play a protective role in the cardiovascular system (Barrett-Connor and Bush, 1991). Observational studies have reported that postmenopausal estrogen therapy protects against cardiovascular disorders (Grady et al, 1992), although a randomized, blinded, placebo-controlled clinical trial for secondary prevention of coronary heart disease in postmenopausal women failed to confirm this evidence (Hulley et al, 1998). The mechanisms by which this protection is mediated remain largely unresolved because the beneficial effects of estrogen on the blood lipid profile only account for 20% to 30% of the overall protection (Lip et al, 1997). Growing evidence suggests that estrogens

have direct effects on the blood vessel wall (Oparil and Arthur, 1999; Seeger et al, 1999; Walsh et al, 1999) and, interestingly, it has been reported that vascular endothelium may play a key role in mediating these effects by producing several vasoactive factors and, more specifically, nitric oxide (NO) (Kleinert et al, 1998). Basal release of NO is higher in females than in males, and oophorectomy diminishes both circulating-estradiol concentration and basal release of NO to levels seen in males (Hayashi et al, 1992), thus offering an explanation for the protective effect of estradiol against the development of atherosclerosis. In agreement with experimental evidence, estrogen replacement therapy in postmenopausal women improves the impaired endothelial-dependent relaxation (Gerhard et al, 1998), but this evidence has been recently questioned (Van Baal et al, 1999). However, estrogens also have adverse effects on the reproductive system of females (Grady et al, 1995) and males (Robinson et al, 1963) that limits their therapeutic use.

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Therefore it is reasonable to explore the potential of other therapeutic strategies.

Phytoestrogens are nonsteroid compounds present in a variety of dietary products (Wang and Murphy, 1994; Whitten and Naftolin, 1992). The importance of phytoestrogens was first recognized by their effects on sheep fertility, but more recently epidemiologic studies have demonstrated that ingestion of food rich in phytoestrogens may provide protection against certain estrogen-dependent cancers, such as breast and prostate cancers (Barnes et al, 1995; Knight and Eden, 1995, 1996). Phytoestrogens also have antioxidant, radical scavenging, hypolipidemic, and serum cholesterol-lowering properties (Franke et al, 1994). Several categories of phytoestrogens have been identified, including lignans, isoflavonoids, coumestans, and resorcylic acid lactones. All of these compounds bind to estrogen receptors.

Zearalenone, a resorcylic acid lactone, has been used previously in the food industry to promote growth in beef cattle. Zearalenone is metabolized in the presence of NADPH and UDPglucuronosyltransferase into α -zearalenol and β -zearalenol (Jimenez and Mateo, 1997). Because α -zearalenol showed a greater ability to bind to estrogen receptors than both zearalenone and β -zearalenol (Fitzpatrick et al, 1989), the α -isomer has been tested successfully in a model of postmenopausal bone loss (Draper et al, 1997). In this model the phytoestrogen was able to reduce bone loss and resorption, likely by acting as an estrogen agonist. However no data are available about the effects of α -zearalenol on the cardiovascular apparatus, especially in the presence of estrogen deficiency.

Using an experimental model of endothelial dysfunction induced by oophorectomy in female rats that resembles the impairment in endothelium-dependent vasodilatation observed in postmenopausal women, we investigated whether α -zearalenol and 17 β -estradiol exert the same degree of protection against the decline of vascular function in OVX rats.

Results

Mean Arterial Blood Pressure, Heart Rate, Body Weight, and Uterine Assay

Bilateral oophorectomy did not modify mean arterial blood pressure (MAP), heart rate (HR), and body weight. Supplementation with both 17 β -estradiol and α -zearalenol did not cause any significant change of these parameters in sham oophorectomized (sham OVX) and oophorectomized (OVX) rats throughout the study. MP and HR ranged from 101 \pm 12 mmHg to 108 \pm 13 mmHg and from 339 \pm 15 beats/minute to 366 \pm 13 beats/minute, respectively. Changes in body weight were similar among groups and ranged from 245 \pm 13 g to 290 \pm 14 g.

Uterine weights were decreased by more than 30% to 35% in the OVX control (vehicle) group compared with sham OVX rats, and α -zearalenol-supplemented OVX rats showed a similar decline (Fig. 1). The OVX rats treated with 17 β -estradiol had a significantly

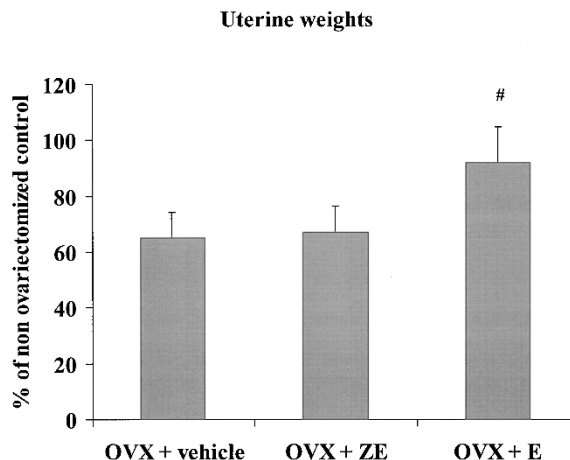


Figure 1.

Uterine weights in oophorectomized (OVX) rats treated daily with vehicle (100 μ l, im, of cottonseed oil), α -zearalenol (ZE; 1 mg/kg, im, in 100 μ l of cottonseed oil), or 17 β -estradiol (E; 20 μ g/kg, im, in 100 μ l cottonseed oil). Mean weights were determined for each treatment group and compared with untreated sham OVX rats. Each point represents the mean \pm SEM of seven experiments. # p < 0.01 versus vehicle.

higher mean uterine weight than either vehicle- or α -zearalenol-treated rats (Fig. 1).

Plasma 17 β -Estradiol, α -Zearalenol, and Total Cholesterol Levels

Plasma 17 β -estradiol levels were significantly reduced in OVX rats compared with sham OVX rats (Table 1). Estrogen replacement therapy markedly increased plasma 17 β -estradiol to levels resembling those observed in sham OVX rats (Table 1). In contrast, α -zearalenol supplementation did not cause any change of 17 β -estradiol levels (Table 1) in either OVX rats or sham OVX rats.

Plasma α -zearalenol levels were less than 1 ng/ml in OVX and sham OVX rats treated with both vehicle and 17 β -estradiol. Supplementation with α -zearalenol markedly increased the plasma levels of this phytoestrogen in both OVX and sham OVX rats.

Plasma total cholesterol levels were not significantly changed in OVX rats compared with sham OVX rats. Furthermore neither estrogen replacement therapy nor α -zearalenol supplementation produced any change of this lipid parameter in both OVX rats and sham OVX rats (Table 1).

Vessel Reactivity

Endothelium-dependent relaxation (acetylcholine [ACh], 10 nM to 10 μ M) and endothelium-independent relaxation (sodium nitroprusside [SN], 15 nM to 30 nM) of thoracic aortas precontracted with phenylephrine (PE; 100 nM) were studied at the end of treatment. Oophorectomy markedly reduced the relaxant effect of ACh (Fig. 2), but did not produce any significant change in the relaxant effect caused by SN (Fig. 3). Estrogen replacement therapy or α -zearalenol supplementation did not modify the relaxant effects of ACh and SN in sham OVX rats (Figs. 2 and 3). In contrast

Table 1. Plasma 17β Estradiol, α-Zearalenol, and Cholesterol Levels in Oophorectomized (OVX) or Sham Oophorectomized (Sham OVX) Rats Treated with α-Zearalenol (ZE), 17β Estradiol (E), ZE Plus ICI 182780, E Plus ICI 182780, and Their Vehicle

Group	17β estradiol (pg/ml)	α-zearalenol (ng/ml)	Cholesterol (mmol/l)
Controls	34 ± 6	< 1	2.2 ± 0.2
Sham OVX + vehicle	35 ± 6	< 1	3.3 ± 0.7
Sham OVX + ZE	30 ± 7	4.3 ± 2.1	3.4 ± 0.5
Sham OVX + E	55 ± 5	< 1	2.5 ± 0.8
OVX + vehicle	2 ± 0.5*	< 1	3.5 ± 0.4
OVX + ZE	3 ± 2.1*	5.1 ± 1.8**	2.7 ± 0.5
OVX + E	27 ± 6**	< 1	2.5 ± 0.6
OVX + ZE + ICI 182780	4 ± 2.9	4.7 ± 1.9**	2.8 ± 1.2
OVX + E + ICI 182780	29 ± 67**	< 1	3.3 ± 0.9

Each point represents the mean ± SEM of six experiments.
* *p* < 0.001 vs sham OVX; ** *p* < 0.001 vs OVX + vehicle.

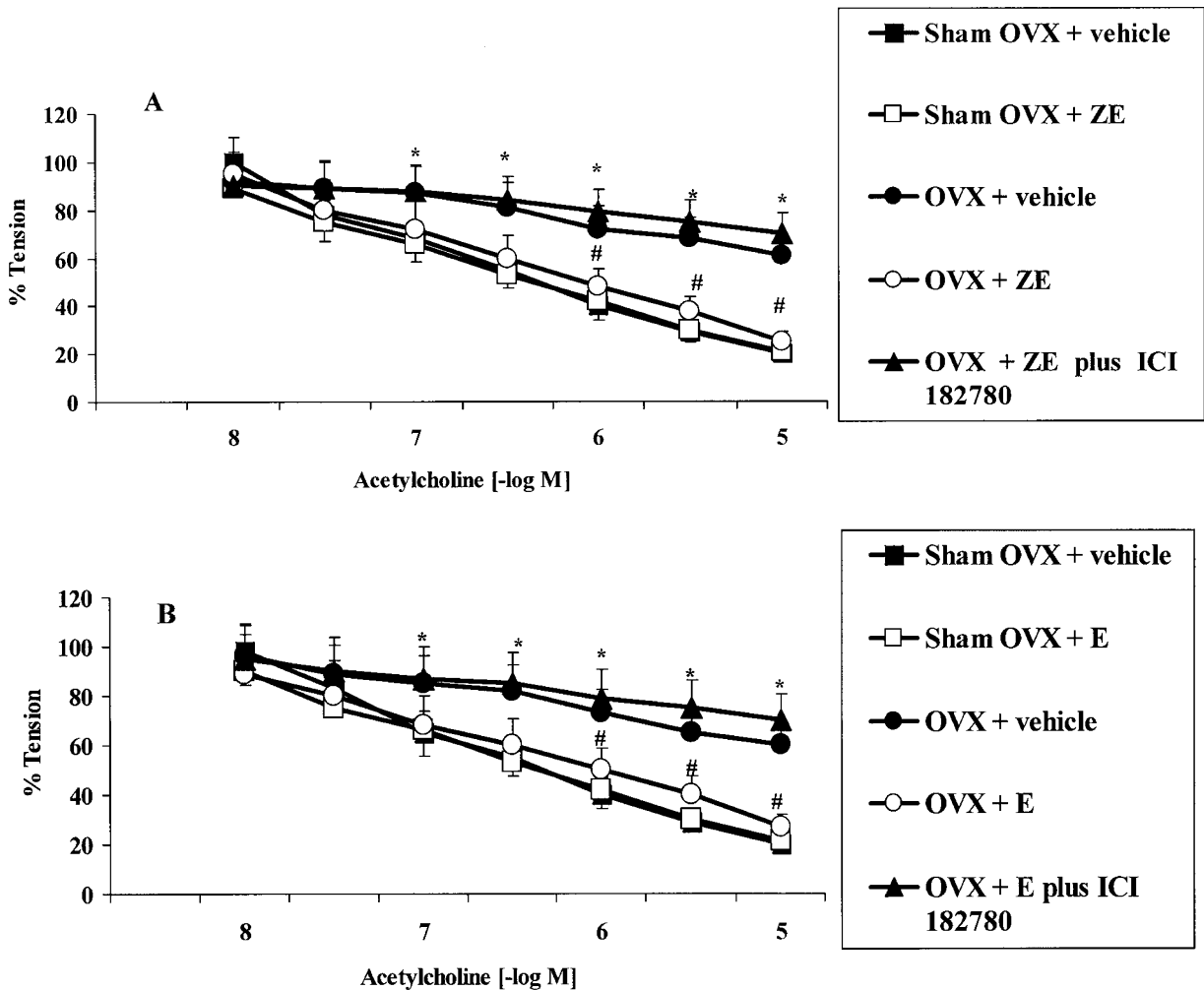


Figure 2.

A, Relaxant effect of acetylcholine (ACh) in aortic rings (contracted with phenylephrine [PE], 100 nM) from sham OVX and OVX rats treated daily with α-zearalenol (ZE; 1 mg/kg, im, in 100 μl of cottonseed oil), ZE plus ICI 182780 (2.5 mg/kg, im, in 100 μl of cottonseed oil), or vehicle (100 μl, im, of cottonseed oil). Each point represents the mean ± SEM of six experiments. * *p* < 0.001 versus sham OVX and OVX + ZE; # *p* < 0.005 versus OVX + vehicle. B, Relaxant effect of ACh in aortic rings (contracted with PE 100 nM) from sham OVX and OVX rats treated daily with 17β-estradiol (E; 20 μg/kg, im, in 100 μl cottonseed oil), E plus ICI 182780 (2.5 mg/kg, im, in 100 μl of cottonseed oil), or vehicle (100 μl, im, of cottonseed oil). Each point represents the mean ± SEM of six experiments. * *p* < 0.001 versus sham OVX and OVX + ZE; # *p* < 0.005 versus OVX + vehicle.

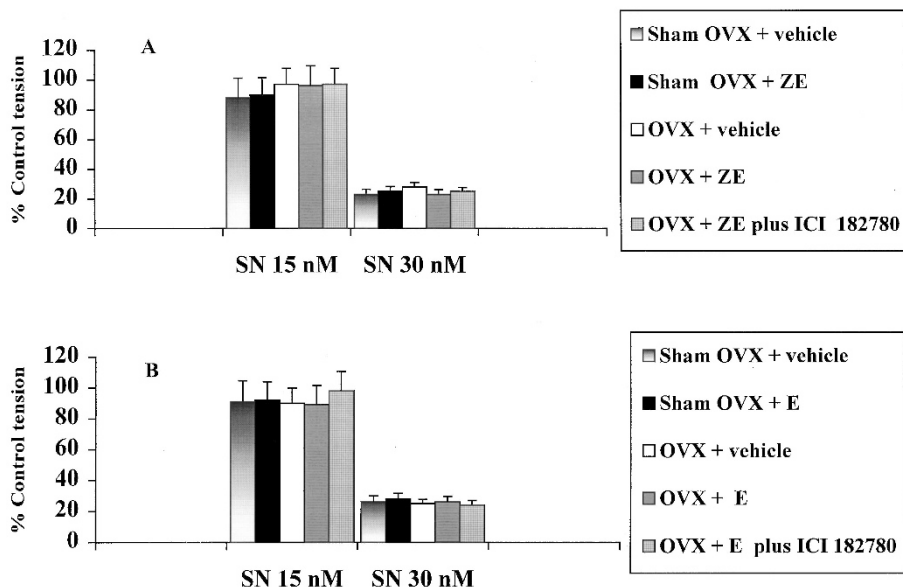


Figure 3.

A, Relaxant effect of sodium nitroprusside (SN; 15 and 30 nM) in aortic rings (contracted with PE, 100 nM) from sham OVX and OVX rats treated daily with α -zearalenol (ZE; 1 mg/kg, im, in 100 μ l of cottonseed oil), ZE plus ICI 182780 (2.5 mg/kg, im, in 100 μ l of cottonseed oil), or vehicle (100 μ l, im, of cottonseed oil). Each point represents the mean \pm SEM of six experiments. B, Relaxant effect of SN (15 and 30 nM) in aortic rings (contracted with PE, 100 nM) from sham OVX and OVX rats treated daily with 17 β -estradiol (E; 20 μ g/kg, im, in 100 μ l cottonseed oil), E plus ICI 182780 (2.5 mg/kg, im, in 100 μ l of cottonseed oil), and vehicle (100 μ l, im, of cottonseed oil). Each point represents the mean \pm SEM of six experiments.

both treatments succeeded in improving the impairment in endothelium-dependent relaxation of OVX rats and no significant difference was observed between these two active treatments (Fig. 2). Furthermore, ICI

182780, a pure estrogen receptor antagonist, abolished the beneficial effect of both 17 β -estradiol and α -zearalenol on endothelial-dependent relaxation (Fig. 2).

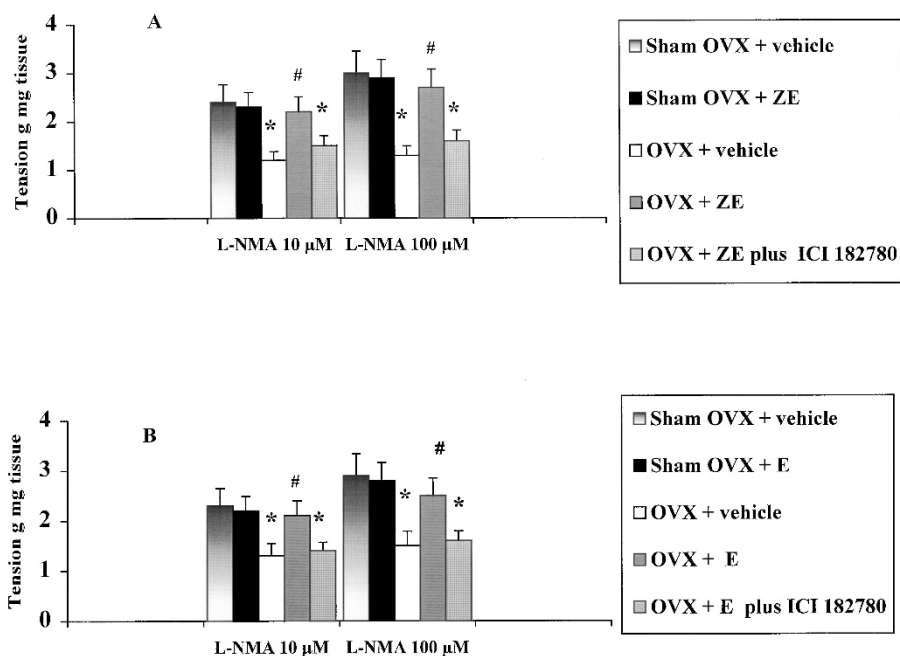


Figure 4.

A, Effects of N(G)-methyl-L-arginine (L-NMA; 10 and 100 μ M) on maximal contractile response to PE in aortic rings taken from sham OVX and OVX rats treated daily with α -zearalenol (ZE; 1 mg/kg, im, in 100 μ l of cottonseed oil), ZE plus ICI 182780 (2.5 mg/kg, im, in 100 μ l of cottonseed oil), or vehicle (100 μ l, im, of cottonseed oil). Each point represents the mean \pm SEM of six experiments. # $p < 0.001$ versus OVX + vehicle; * $p < 0.005$ versus sham OVX and OVX + ZE. B, Effects of L-NMA (10 and 100 μ M) on maximal contractile response to PE in aortic rings taken from sham OVX and OVX rats treated daily with 17 β -estradiol (E; 20 μ g/kg, im, in 100 μ l cottonseed oil), E plus ICI 182780 (2.5 mg/kg, im, in 100 μ l of cottonseed oil), and vehicle (100 μ l, im, of cottonseed oil). Each point represents the mean \pm SEM of six experiments. # $p < 0.001$ versus OVX + vehicle; * $p < 0.005$ versus sham OVX and OVX + E.

Addition of N(G)-methyl-L-arginine (L-NMA; 10 to 100 μM) to the organ bath caused a significant contraction of the aortic rings precontracted with PE (Fig. 4). L-NMA-induced vasoconstriction was significantly blunted in OVX rats compared with sham OVX rats. Estrogen replacement therapy or α -zearalenol supplementation markedly increased the constrictor response elicited by L-NMA and no significant difference was observed between these two regimens (Fig. 4). The pure estrogen receptor antagonist ICI 182780 abated these effects (Fig. 4). Finally, neither of the active treatments changed L-NMA-induced responses in sham OVX rats (Fig. 4).

Nitric Oxide Synthase Activity

Ca^{2+} -dependent NO synthase (cNOS) activity was investigated in lung homogenates at the end of treatment (Table 2). OVX rats showed a marked decrease in NO synthase (NOS) activity compared with sham OVX animals (Table 2). Estrogen replacement therapy or α -zearalenol supplementation markedly enhanced the activity of NOS in lung homogenates. This effect was completely reverted by the concomitant treatment with ICI 182780. Neither estrogen therapy nor α -zearalenol treatment modified cNOS activity in sham OVX rats. Furthermore the addition of L-NMA to the incubation buffer effectively inhibited NOS activity in the lung, providing further evidence that NOS was being measured.

Discussion

Several reports have suggested that some effects of phytoestrogens are mediated through their ability to bind to estrogen receptors (Makela et al, 1995; Miksicsek, 1994). This is supported by the observation that certain phytoestrogens, such as genistein, coumestrol, or zearalenol, are able to bind to the estrogen receptors and modulate a variety of estrogen-dependent processes (Stahl et al, 1998). Furthermore it has been suggested that α -zearalenol shows interesting bioactivity in preventing bone loss in a well-

recognized model of postmenopausal osteoporosis in the rat (Draper et al, 1997).

With this background, we attempted to determine if the protective effects of α -zearalenol supplementation on the cardiovascular apparatus may be mediated, in part, by an increased production of endothelial NO from the vascular endothelium. To test this hypothesis we performed a comparison study on the effects of α -zearalenol and 17β -estradiol in endothelial dysfunction induced by bilateral oophorectomy in female rats.

Our results suggest that OVX rats developed a typical endothelial dysfunction resembling that observed in postmenopausal women. More specifically they had a markedly reduced relaxant response to ACh, an endothelium and NO-dependent relaxant agent, and unchanged response to SN, an endothelium and NO-independent relaxing compound. In addition aortic rings prepared from OVX rats showed a reduced contraction in response to L-NMA, an inhibitor of NO synthase. These data suggest that OVX rats show a marked impairment in the L-arginine/NO pathway characterized by a blunted production of endothelial NO. This blunted endothelial NO release seems to be the consequence of a decreased activity of the enzyme devoted to its regulation, the constitutive NO synthase. Indeed lung homogenates prepared from OVX rats showed a reduced constitutive NO synthase activity compared with sham OVX rats. This disturbance in the L-arginine/NO pathway has already been proposed to explain the endothelial dysfunction observed in postmenopausal women (Gerhard et al, 1998). In contrast the plasma levels of cholesterol were unchanged in OVX rats, which suggests that this present model (4 weeks of estrogen deficiency) causes a selective impairment in the L-arginine/NO pathway (endothelial dysfunction) without other important confounding alterations. The major finding of this study is that α -zearalenol supplementation succeeded in reverting the impaired endothelial dysfunction observed in OVX rats. This compound was able to restore the endothelium-dependent relaxation, likely through an increased production of basal endothelial NO release; in fact α -zearalenol treatment increased

Table 2. Calcium-Dependent (cNOS) and Calcium-Independent (iNOS) Nitric Oxide Synthase Activity in Lung of OVX or Sham OVX Rats Treated with α -Zearalenol (ZE), 17β Estradiol (E), ZE Plus ICI 182780, E Plus ICI 182780 and Their Vehicle

Group	cNOS	iNOS	Total NOS + L-NMA
Controls	18.5 \pm 3.5	2.5 \pm 0.7	2.3 \pm 1.1
Sham OVX + vehicle	19.3 \pm 4.1	2.8 \pm 1.2	2.1 \pm 0.7
Sham OVX + ZE	21.2 \pm 3.4	2.8 \pm 1.7	2.3 \pm 1.1
Sham OVX + E	23.8 \pm 6.7	2.8 \pm 1.5	1.9 \pm 1.2
OVX + vehicle	10.3 \pm 2.8*	2.7 \pm 1.8	1.2 \pm 0.7
OVX + ZE	18.1 \pm 3.6**	2.3 \pm 1.5	2.1 \pm 0.5
OVX + E	19.6 \pm 1.9**	2.2 \pm 1.8	1.7 \pm 0.6
OVX + ZE + ICI 182780	8.9 \pm 2.1*	2.5 \pm 2.2	2.3 \pm 1.8
OVX + E + ICI 182780	9.3 \pm 2.5*	2.4 \pm 1.9	1.8 \pm 0.5

NOS activity was expressed as pmol citrulline 20 min per mg protein and shown as mean \pm SEM of six experiments.

* $p < 0.001$ vs sham OVX; ** $p < 0.001$ vs OVX + vehicle.

the constrictor response elicited by L-NMA. This result strongly supports the idea that the phytoestrogen treatment was able to enhance the depressed L-arginine/NO pathway. From a mechanistic point of view it can be proposed that α -zearalenol increases the activity of endothelial NOS; indeed lung homogenates prepared from phytoestrogen-supplemented OVX rats showed an increased activity of this enzyme isoform. As far as we know, this paper represents the first report indicating that this naturally occurring phytoestrogen stimulates in vivo the activity of endothelial NOS. It is likely that α -zearalenol increases endothelial NOS activity in the rat aorta through a rapid receptor-mediated Ca^{2+} translocation of the enzyme without causing any change in endothelial NO synthase gene expression, as has been shown recently for 17β -estradiol (Barbacanne et al, 1999; Goetz et al, 1999).

The second important finding of this study is that α -zearalenol and 17β -estradiol showed overlapping effects in endothelial dysfunction. In fact, in our experimental model, the well-known ability of estrogen replacement therapy to revert endothelial dysfunction during estrogen deficiency was nicely reproduced by α -zearalenol supplementation. No statistical differences were, in fact, observed between these two active treatments. In contrast, although 17β -estradiol significantly modifies uterine weights, the beneficial effects of α -zearalenol on the cardiovascular apparatus was not accompanied by any change in uterus weight.

Estrogen receptors are present in the blood vessel wall (Losordo et al, 1994) and α -zearalenol acts as estrogen agonist on these structures (Collins and McLachlan, 1997). To confirm this we carried out experiments with the pure estrogen receptor antagonist ICI 182780 (al-Matubsi et al, 1998). Our results clearly indicate that blocking the estrogen receptor abolished the beneficial effects of α -zearalenol on endothelium dysfunction, thus strongly supporting the hypothesis that the α -zearalenol serves as an estrogen receptor agonist.

In conclusion, our findings suggest that α -zearalenol-induced augmentation of NO production may contribute to its beneficial cardiovascular effects.

Materials and Methods

Animals

All procedures performed on animals were approved by the Ethical Committee of the University of Messina and were consistent with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 8523, revised 1987). OVX female mature Sprague-Dawley rats (Charles River Labs, Calco, Italy) were maintained at constant humidity ($60 \pm 5\%$), temperature ($24 \pm 1^\circ\text{C}$), and light cycle (6 am to 6 pm), and were given free access to a semipurified chow with poor phytoestrogen content and to drinking water. Sham OVX rats were used as controls.

Estrogen and α -Zearalenol Supplementation

Three weeks after surgery, OVX rats and sham OVX animals were randomly assigned to one of the five treatment groups. The first group received daily injections of 17β -estradiol ($20 \mu\text{g}/\text{kg}$, im, in $100 \mu\text{l}$ cottonseed oil, daily), the second group was given α -zearalenol ($1 \text{ mg}/\text{kg}$, im, in $100 \mu\text{l}$ cottonseed oil, daily), the third group was treated with 17β -estradiol plus the pure estrogen antagonist ICI 182780 (al-Matubsi et al, 1998) ($2.5 \text{ mg}/\text{kg}$, im, in $100 \mu\text{l}$ cottonseed oil, daily), the fourth group was treated with α -zearalenol plus the pure estrogen antagonist ICI 182780, and the fifth group received the 17β -estradiol, α -zearalenol, and ICI 182780 vehicle ($100 \mu\text{l}$, im, in cottonseed oil daily). The treatment lasted 4 weeks. The doses of 17β -estradiol and α -zearalenol were selected according to previous studies of the effects of both compounds in OVX rats (Draper et al, 1997; Squadrito et al, 1996). It has been suggested that the estrogenic potency of α -zearalenol relative to 17β -estradiol is 50 times less (Everett et al, 1987).

Mean Arterial Blood Pressure, Heart Rate, Body Weight, and Uterine Assay

MAP and HR were measured using the tail cuff method at baseline conditions and after 4 weeks of treatment. Body weight (in milligrams) was also monitored at the same time points. At the end of the experiment, the uteri were removed and weighed.

Measurement of Plasma-Estradiol, α -Zearalenol, and Total Cholesterol Levels

Plasma 17β -estradiol, α -zearalenol, and total cholesterol levels were studied when the animals were killed. Plasma 17β -estradiol levels were determined by radioimmunoassay with a commercially available kit. Assay sensitivity was $1.0 \text{ pg}/\text{ml}$, and intra-assay and interassay coefficients of variation for estradiol were 5.3% and 6.4%, respectively. Plasma α -zearalenol levels were assayed using high-performance liquid chromatography with fluorescence detection (Prelusky et al, 1989). Assay sensitivity was $1 \text{ ng}/\text{ml}$ and recovery was 85% to 93%. The cholesterol in the blood was determined by an enzymatic cholesterol esterase/cholesterol oxidase using an automatic analysis technique on a chemical analyzer.

Vessel Reactivity Studies

At the end of the treatment period, heparinized rats were killed with an overdose of sodium pentobarbital ($75 \text{ mg}/\text{kg}$, ip). Thoracic aortas were removed and placed in cold Krebs solution of the following composition: NaCl 118.4 mM , KCl 4.7 mM , MgSO_4 1.2 mM , CaCl_2 2.5 mM , KH_2PO_4 1.2 mM , NaHCO_3 25.0 mM , and glucose 11.7 mM . Aortas were then cleaned of adherent connective and fat tissue and cut into rings approximately 2 mm in length. Rings were then placed under 1 g of tension in an organ bath containing 10 ml Krebs solution at 37°C and bubbled with 95% O_2 and

5% CO₂ (pH 7.4). All experiments were carried out in the presence of indomethacin (10 μM) to exclude the involvement of eicosanoids and their metabolites. Developed tension was measured with an isometric force transducer and recorded on a polygraph (Ugo Basile, Varese, Italy). After an equilibration period of 60 minutes during which the rings were washed with fresh Krebs solution at 15- to 20-minute intervals, basal tension was readjusted to 1 g and the tissue was exposed to PE (100 nM). When the contraction was stable, the functional integrity of the endothelium was assessed by a relaxant response to ACh (100 nM). The tissue was then washed occasionally for 30 minutes.

Endothelium-dependent relaxation was evaluated with cumulative concentrations of ACh (10 nM–1 μM) in aortic rings precontracted with PE (100 nM). Endothelium-independent relaxation was investigated by analyzing the relaxant effects of SN (15–30 nM), as previously described (Squadrito et al, 1997). Relaxation of the rings was calculated as the percentage decrease of contractile force. Some rings were precontracted with PE and then incubated with L-NMA (10–100 μM) and the results were expressed as grams of tension per milligram of tissue.

NOS Activity

To measure NOS activity, the lungs were removed at the end of the experiment, immediately frozen in liquid nitrogen, and later assayed according to the method of Bredt and Snyder (1990) with slight modifications. Briefly, tissues were homogenized on ice in 10 volumes of homogenizing buffer: Tris HCl 50 mM, EDTA 0.1 mM, Pefabloc 1 mM, and dithiothreitol 1 mM. A 50-μl sample of the homogenate was incubated with 50 μl of incubation buffer for 20 minutes at 37° C. The incubation buffer contained the following cofactors: L-arginine 10 μM, [³H]L-arginine 5 pmol, NADPH 1 mM, calmodulin 30 nM, tetrahydrobiopterin 5 μM, calcium 2 mM, and L-valine 60 mM in a Tris buffer at pH 7.4. To determine calcium-independent NOS activity, a buffer replacing the calcium with EGTA (5 mM) was used. Nonspecific conversion of arginine to citrulline was determined in the absence of NADPH and a separate buffer containing L-NMA (1 mM) provided further evidence that NOS was being measured.

After the 20-minute incubation, the reaction was stopped by the addition of 1 ml of ice-cold stop buffer (containing HEPES 20 mM, EGTA 2 mM, and EDTA 2 mM at pH 5.5). The reaction mixture was applied to 1-ml Dowex columns (NA⁺ form, 50 W, mesh size 100 to 200) that had previously been equilibrated with 1 ml of stop buffer. The eluate was collected and the columns washed twice with 0.75 ml deionized water to retrieve any residual activity. A 400-μl sample of the elute was taken for scintillation counting. The protein concentration of the homogenates was determined by the Bradford assay (Bradford, 1976) using BSA (fraction V) as a standard.

Drugs

α-Zearalenol and 17β-estradiol were obtained from Sigma Chemical Company (St. Louis, Missouri). ICI 182780 was a kind gift of Dr. Salvatore Guarini, University of Modena, Italy.

Statistical Analysis

Data are expressed as means ± SEM. Results of the before versus after treatment measurements (MAP, HR, and body weight) were analyzed using the paired *t* test. For the other data, comparison between the means of the two groups was performed using ANOVA followed by the Bonferroni test, and was considered significant at *p* < 0.05.

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