Estrogen and Selective Estrogen Receptor Modulator LY117018 Enhance Release of Nitric Oxide in Rat Aorta

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ABSTRACT

We report on the modulatory effects of chronic subcutaneous or oral estrogen and LY117018, a selective estrogen receptor modulator, on the release of nitric oxide in rings of rat aorta studied under isometric conditions. Dilator responses to acetylcholine (ACH; 10^{-8} to 10^{-6} M) were obtained in phenylephrine (PE; 2 μM)-contracted aorta, and constrictor dose-response curves to PE (10^{-8} to 10^{-5} M) were generated before and after pretreatment with N^\text{-}nitro-L-arginine methyl ester (L-NAME; 200 μM), an inhibitor of nitric oxide synthase. Tissue segments were obtained from five groups of rats implanted with a subcutaneous pellet delivery system for 21 days: (1) male, (2) sham-operated placebo-treated female, (3) ovariectomized, progesterone (15 mg/pellet) and 17β-estradiol (0.5 mg/pellet)-treated. Aortic rings from sham rats and ovariectomized rats receiving estrogen relaxed more to ACh (10^{-6} to 10^{-5} M) than did the rings from ovariectomized, progesterone plus estrogen-treated and male rats (P < .05). They were also characterized by a greater potentiation of the PE responses after L-NAME compared with male, progesterone plus estrogen-treated and ovariectomized rats (P < .05) and a similar sensitivity to PE. In addition, ACh-induced relaxation and L-NAME-induced potentiation of PE contractions in aortic rings from rats dosed orally with LY117018 were similar to responses of aortic rings from rats dosed orally with estrogen. These results demonstrate that chronically administered estrogen and LY117018 enhance the release of nitric oxide from endothelium in rat aortic rings.

Although it is clear that estrogen-mediated changes in total serum cholesterol are important factors in delineating the cardioprotective effects of estrogen, there is evidence suggesting that estrogen has effects that are independent of its lipoprotein effects.

Estrogen appears to have a direct beneficial effect on vessel wall physiology (Lobo, 1990). A number of reports indicate that NO production may play an important role in mediating the effects of estrogen on the vasculature. NO, a potent vasodilator (Furchgott and Zawadzki, 1980; Ignarro et al., 1987; Palmer et al., 1987) is produced in vascular endothelial cells by the enzyme NOS (Palmer et al., 1988). A positive correlation has been found between plasma 17β-estradiol concentrations and levels of stable metabolites of NO (nitrite/nitrate) during follicular development in women (Rosselli et al., 1994). Consistent with a role for NO, endothelium-dependent coronary artery vasodilation is enhanced by estrogen treatment in ovariectomized monkeys (Williams et al., 1994) and postmenopausal women (Gilligan et al., 1994). In vitro studies examining isometric tension development have also indicated enhanced endothelium-dependent relaxation in rabbit femoral arteries (Gisclard et al., 1988) and rat thoracic aorta (Williams et al., 1988) obtained from animals with similar age (Barret-Connor, 1994; Castelli, 1988). However, women experience a dramatic increase in the incidence of coronary heart disease with the onset of menopause. Epidemiological studies support the view that decreased levels of circulating estrogen may be an important factor for this increase in cardiovascular disease. Some reports show that estrogen replacement therapy in postmenopausal women reduces mortality due to cardiovascular disease (Barret-Connor and Bush, 1991; Stampfer and Colditz, 1991). The cardioprotective effect is in part related to the action of estrogen on blood lipid profiles and resultant inhibition of atherosclerotic coronary stenosis (Barrett-Connor and Bush, 1991). One of the best described actions of estrogen is to decrease serum low-density lipoprotein and increase high-density lipoprotein cholesterol concentrations (Walsh et al., 1991), such that at menopause, low-density lipoprotein levels become higher in women than in men (Jensen et al., 1990).

During their reproductive years, women have a lower incidence of coronary heart disease compared with men of a similar age (Barret-Connor, 1994; Castelli, 1988). However, women experience a dramatic increase in the incidence of coronary heart disease with the onset of menopause. Although it is clear that estrogen-mediated changes in total serum cholesterol are important factors in delineating the cardioprotective effects of estrogen, there is evidence suggesting that estrogen has effects that are independent of its lipoprotein effects.

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ABBREVIATIONS: NO, nitric oxide; NOS, nitric oxide synthase; ACh, acetylcholine; PE, phenylephrine; L-NAME, N^\text{-}nitro-L-arginine methyl ester; SNP, sodium nitroprusside; SERM, selective estrogen receptor modulator; ANOVA, analysis of variance.
elevated estrogen levels. The acute exposure of porcine left circumflex coronary arteries to estrogen also potentiated endothelium-dependent relaxations (Bell et al., 1995).

There are some studies reporting that chronic estrogenic treatment has no effect on receptor-mediated release of NO. Hayashi et al. (1992) found no significant difference in the relaxant responses to acetylcholine in aortic rings from male, female or ovariectomized rabbits. On the other hand, basal NO production may be related to plasma estrogen status. Inhibition of NOS produced a greater increase in tension in partially contracted aortic segments from female rabbits compared with male or ovariectomized animals (Hayashi et al., 1992).

A direct action of estrogen on vascular smooth muscles has also been reported by several in vitro studies. High concentrations (≥1 μM) of exogenous 17β-estradiol produce an endothelium-independent relaxation in coronary arteries, with antagonism of calcium (Ca"^2+") entry as the proposed mechanism (Harder and Coulson, 1979; Jiang et al., 1991; Ravi et al., 1994). The mechanism of estrogen-mediated relaxation is thus controversial.

Although estrogen replacement therapy is both cardioprotective and bone preserving in postmenopausal women, it is accompanied by liabilities related to reproductive organs, including an elevated risk of breast and uterine cancers (Kauffman and Bryant, 1995). Chemical synthetic efforts have yielded a variety of nonestrogenic compounds with varying degrees of tissue selectivity known as selective estrogen receptor modulators or SERM (Kauffman and Bryant, 1995). The most selective of these compounds preserve the beneficial properties of estrogen in the cardiovascular and skeletal systems and minimize or eliminate estrogenicity in mammary and uterine tissue. Such compounds have considerable therapeutic potential in women's health. The benzo-thiophene LY177018 is an example of a highly promising SERM. Like estrogen, LY177018 has been demonstrated to lower serum total cholesterol and triglyceride concentrations and preserve bone against resorption in ovariectomized animals (Bryant et al., 1995; Kauffman et al., 1997). Unlike estrogen, LY177018 is nearly devoid of estrogenic activity in rat uterus (Jones et al., 1984). Furthermore, LY177018 antagonizes estrogen binding to the estrogen receptor (Black et al., 1983) and inhibits estrogen-induced proliferation of cultured MCF-7 cells from human mammary tumor (Sato et al., 1995; Wakeling et al., 1984).

In the present study, we report the effects of estrogen status on modulation of arterial function due to its effects on endothelial NO synthesis and release. We also compared LY177018 with estrogen in the oral administration phase of our work.

Materials and Methods

Animals and Procedures

Two types of treatment protocol were used. We implanted rats with a subcutaneous pellet delivery system for 21 days. However, because orally administered estrogen is of greater clinical interest, we also chose to administer estrogen via this route. An additional group was dosed orally with LY177018 (SERM).

Implantation group. Sixteen (12 ovariectomized and 4 sham-operated) female and five male Sprague-Dawley rats weighing 275 to 300 g were purchased from Charles River (Quebec, Canada). With a 10-gauge trochar, a pellet was implanted subcutaneously at the back of the neck of rats, where it remained until death 21 days later. Rats were assigned to five treatment groups (at least two or three aortic segments were taken from each animal). Group 1 rats were sham-operated, placebo-treated (sham) animals; group 2 rats were ovariectomized, placebo-treated animals; group 3 rats were ovariectomized, 17β-estradiol (0.5 mg/pellet)-treated (E2) animals; group 4 rats were ovariectomized, progesterone (15 mg/pellet)-plus-17β-estradiol (0.5 mg/pellet)-treated (PG/E2) animals and group 5 rats were male animals.

Oral group. Twenty (15 ovariectomized and 5 sham-operated) female Sprague-Dawley rats weighing 275 to 300 g were assigned to four treatment groups. All groups received oral administration of drug or vehicle via gavage for 35 days. Group 1 rats were ovariectomized animals that had received vehicle (hydroxypropyl-β-cyclodextrin); group 2 rats were ovariectomized animals that had received 17α-ethinyl estradiol (0.1 mg/kg/day); group 3 rats were ovariectomized animals that had received LY177018 (1 mg/kg/day) and group 4 rats were sham-operated animals that had received vehicle (hydroxypropyl-β-cyclodextrin).

Measurement of Arterial Tension

The rats were killed on day 21 (implantation category) or 35 (oral category) with pentobarbital (65 mg/kg i.p.) after an intravenous injection of heparin. On the day on which they were killed, blood samples were collected from the vena cava, and the plasma fraction was frozen (-70°C) for later analysis of 17β-estradiol levels. Rats were exsanguinated by cutting both carotid arteries. The thoracic aorta was removed and placed in ice-cold modified Krebs' solution containing 119 mmol/liter NaCl, 4.7 mmol/liter KCl, 1.18 mmol/liter KH2PO4, 1.17 mmol/liter MgSO4, 24.9 mmol/liter NaHCO3, 0.023 mmol/liter EDTA, 1.6 mmol/liter CaCl2 and 11.1 mmol/liter glucose. The aorta was cleaned of fatty tissue and adhering connective tissue before being cut into rings 2 to 4 mm in length. Rings of aorta were suspended horizontally between two stainless steel hooks for measurement of isometric tension in individual organ baths containing 5 ml of Krebs' solution at 37°C, bubbled with 95% O2/5% CO2. Rings were equilibrated for 45 min under a resting tension of 1 g to allow development of a stable basal tone and reproducible evoked contractile responses. Stimulation of rings with 80 mM K+ was repeated every 15 min two or three times until responses were stable.

Responses to ACh

Rings of aorta were contracted with PE (2 μM), which represented a concentration that produced 80% of maximal effect (EC50). Dilator-response curves were obtained by the addition of increasing concentrations of ACh (10^-9 to 10^-5 M). Tissues were washed with Krebs' solution for 30 min to allow relaxation to basal tone. Figure 1 shows a typical tracing of a concentration-response curve to ACh (10^-8 to 10^-5 M) in PE-precontracted aortic rings from the control group of rats. Relaxation is expressed as the percent decrease from maximum PE-induced tension.

Contractile Effect of PE

A concentration-response curve to PE was obtained by the addition of increasing concentrations of PE (10^-3 to 10^-5 M). The rings were then washed with Krebs' solution for 30 min, and L-NAME (200 μM) was added for 30 min. The use of this concentration of L-NAME was based on studies by others (Hayashi et al., 1992; Paredes-Carbajal et al., 1995; Zheng et al., 1994). The concentration-response curves to PE (10^-9 to 10^-5 M) were then repeated. Figure 1 shows a typical tracing of a concentration-response curve to PE (10^-8 to 10^-5 M) before and after pretreatment with L-NAME (200 μM) in aortic rings from the control group of rats. Contraction was measured as the percent increase from maximum PE-induced tension.
Relaxing Effect of SNP

Concentration-response curves to SNP, an endothelium-independent vasodilator agent (10^-10 to 10^-4 M), were made in aortic rings precontracted with PE (2 μM) before and after pretreatment of the same ring with L-NAME (200 μM) for 30 min.

Radioimmunoassay for 17β-Estradiol Measurement

Plasma concentrations of 17β-estradiol were measured using an 125I radioimmunoassay kit (ICN Biomedical, Carson, CA). Briefly, 1 ml of the 125I-estradiol was added to assay tubes containing 100 μl of plasma or standard solution. They were incubated at 37°C for 90 min, and the content of tubes was aspirated or decanted. The empty tubes were counted for 125I in a gamma counter. A standard curve was used to estimate the 17β-estradiol concentration of each sample.

Chemical Reagents and Drugs

ACh, PE, SNP, L-NAME and 17α-ethinyl estradiol were obtained from Sigma Chemical (St. Louis, MO). Hydroxypropyl-β-cyclodextrin was purchased from Aldrich Chemical (Milwaukee, WI). LY117018 was obtained from Eli Lilly Co. (Indianapolis, IN). 17β-Estradiol (0.5 mg/pellet), progesterone (15 mg/pellet) and placebo pellets were purchased from Innovative Research of America (Toledo, OH) and designed to release 17β-estradiol and progesterone over a 21-day period.

Data Analysis

Values are expressed as mean ± S.E.M. Comparisons of mean values were made by using the Student’s t test for unpaired values; when more than two groups were compared, one-way ANOVA and Newman-Keuls test for multiple comparison were used to identify differences among groups. A probability value of <.05 (P < .05) was considered significant. Sensitivity is expressed as negative log molar concentration required for 50% of maximal relaxation or contraction (EC50) determined.

Results

Plasma estradiol level. Estrogen treatment significantly increased the concentrations of plasma 17β-estradiol (table 1). 17β-Estradiol concentrations were significantly (P < .01, ANOVA) lower in male and ovariectomized rats compared with those in female and estrogen-treated ovariectomized rats. In agreement with Ferrer et al. (1996), the body weight of rats treated with estrogen and LY117018 was significantly (P < .01) lower than that of nontreated rats at the time of death (339 ± 3.46 and 330.83 ± 10.0 g, respectively, vs. 388.8 ± 6.60 g).

TABLE 1

<table>
<thead>
<tr>
<th>Animal</th>
<th>17β-Estradiol (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td>Female</td>
<td>67.01 ± 5.50</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>11.76 ± 1.05a</td>
</tr>
<tr>
<td>Ovariectomized + 17β-estradiol</td>
<td>54.96 ± 1.71</td>
</tr>
<tr>
<td>Male</td>
<td>35.86 ± 3.96a</td>
</tr>
</tbody>
</table>

Each value ± S.E.M. represents a mean of four or five animals.
a Value is significantly less (P < .01, ANOVA) compared with that in intact female and 17β-estradiol-treated ovariectomized rats.

Relaxation responses to ACh. Relaxation to ACh was used to examine the effect of estrogen treatment on receptor-mediated endothelium-dependent release of NO (fig. 2). In the parenteral treatment study, no significant differences in responses to low concentration of ACh (10^-8 to 10^-6 M) occurred between aortic rings from male, sham-operated and
ovariectomized rats receiving progesterone plus estrogen, estrogen and LY117018. However, aortic rings from sham and ovariectomized rats receiving estrogen relaxed more (P < .05) to ACh (10^{-6} to 10^{-5} M) than those from ovariectomized, progesterone-plus-estrogen-treated and male rats (fig. 2A). Aortic rings from LY117018-treated rats in the orally treated group also relaxed more (P < .05) to ACh (10^{-6} to 10^{-5} M) than ovariectomized rats (fig 2B). These results have been summarized in figure 3; this figure shows the differences in the maximum dilator responses to ACh (10^{-5} M) in the various groups of rats. Only the LY117018-treated ovariectomized rats are from the orally treated category in this figure.

Effect of L-NAME on contraction induced by PE. To examine whether estrogen affects endothelial NO production, concentration-response curves to PE were generated in rings of aorta before and after pretreatment with L-NAME, an NOS inhibitor. Significant changes as a result of L-NAME pretreatment would reveal effects of basal NO production on contraction. In figure 4, we show that incubation of the aortic ring segments with L-NAME (200 μM) resulted in a significant potentiation of the contractile responses to PE in all five groups of aortae through the entire concentration-response range of PE (10^{-9} to 10^{-5} M). Aortic rings from sham, estrogen-treated and LY117018-treated rats had a greater maximal (P < .05) potentiation of the PE responses after inhibition of NOS than those in male, ovariectomized and progesterone plus estrogen-treated ovariectomized rats (fig. 5). The sensitivity of alpha adrenoceptors is not significantly affected by estrogen status either before or after inhibition of NOS by L-NAME, as indicated by no significant differences at the level of 5% in PE EC_{50} values between treatment groups before and after L-NAME treatment (fig. 6). However, pretreatment with L-NAME increased significantly the sensitivity to PE of all groups of aortae.

Effect of L-NAME on dilation induced by SNP. SNP is a NO donor, leading to a rise of cGMP-mediated endothelium-independent relaxation in smooth muscle cells (Ignarro et al., 1981). The addition of L-NAME (200 μM for 30 min) did not inhibit SNP (10^{-9} to 10^{-6} M)-induced relaxation (data not shown). The sensitivity of smooth muscle to SNP was not significantly different in aortic rings from sham-operated, LY117018-treated and untreated ovariectomized rats (EC_{50}}
The main findings of our study are that (1) treatment of ovariectomized rats with estrogen and LY117018 enhances cholinergic, endothelium-dependent vasodilation of the aorta and (2) inhibition of NOS causes a greater enhancement of adrenergic vasoconstriction in estrogen- and LY117018-treated animals than in male, ovariectomized, or ovariectomized progesterone-plus-estrogen-treated animals. These effects occur without changes in the sensitivity of smooth muscle cells to either NO donors or an adrenergic receptor agonist. We propose that estrogen and LY117018 exert their vasomotor effects primarily through enhancement of endothelium-dependent vasodilation by increasing basal and stimulated release of NO.

A number of reports indicate that NO production may play an important role in the cardiovascular protective effect of estrogen. Gisclard et al. (1988) reported that femoral arteries from estrogen-treated rabbits show an enhanced endothelium-dependent relaxation to ACh. However, there are also studies reporting that chronic estrogenic treatment did not affect receptor-mediated release of NO by ACh. For example, Miller and Vanhoutte (1990, 1991) observed no differences in receptor-mediated (ACh, ADP, bradykinin) relaxations of arteries from estrogen-treated and untreated ovariectomized rabbits. Similar findings were reported by Hayashi et al. (1992). In view of these conflicting reports, our objectives were to (1) compare NO-dependent responses in intact aortic rings from sham-operated, estrogen-treated and untreated ovariectomized and male rats both in the basal state and after stimulation by ACh, an endothelium-dependent vasodilator, and (2) compare the effects of estrogen and LY117018 on modulation of arterial function due to its effects on endothelial NO synthesis and release. LY117018 has been previously shown (Jones et al., 1984; Sato et al., 1995; Wakeling et al., 1984) to act as an inhibitor of estrogen-induced proliferation of cancer cells, making it a selective estrogen replacement compound that would be safe for women at risk of developing breast or uterine cancer. Some cardiovascular effects of LY117018 are described in this study.

ACh stimulates the production of NO from L-arginine within the endothelium, which then relaxes the underlying smooth muscle by stimulating production of cGMP within the vascular smooth muscle (Furchgott and Zawadzki, 1980). Nitrovasodilators (endothelium-independent vasodilators) such as nitroglycerin and SNP are metabolized to NO in smooth muscle, which then activates guanylyl cyclase (Ignarro et al., 1981). The effect of basal release of NO was monitored indirectly by observing the effects of L-NAME on the concentration-response curve to PE. L-Arginine is converted to L-citrulline in endothelial cells (Palmer et al., 1988) by the enzyme NOS (Forstermann et al., 1991; Furchgott and Zawadzki, 1980). NO synthesis is competitively inhibited by certain analogs of L-arginine, such as L-NAME (Rees et al., 1989). Differences in basal release of NO would be reflected as differences in the degree of PE-induced contraction in the presence and absence of L-NAME.

In the current study, chronic treatment with estrogen increased the plasma estradiol levels in rats after both routes (implantation and oral) of administration. The ranges of plasma estradiol values reported in the literature for estrogen-treated and untreated ovariectomized rats are 26.6 ± 3.3 to 180 ± 17.5 pg/ml and 12.2 ± 4.7 to 21 ± 2.4 pg/ml, respectively (Cheng et al., 1994, Hayashi et al., 1992). Chronic treatment with estrogen also decreased body weight (Conrad et al., 1994; Ferrer et al., 1996). In our study, aortic rings from sham and ovariectomized rats receiving estrogen and LY117018 showed a significantly greater potentiation of the PE responses in the presence of L-NAME compared with L-NAME-mediated potentiation of PE contractions in ovariectomized rats receiving placebo or progesterone-plus-estrogen and male rats. These results are consistent with chronic estrogen- and LY117018-dependent maintenance of basal NO release from rat aortic endothelium after ovariectomy. In agreement with this result, Hayashi et al. (1992) reported that basal release of NO is greater from the endothelium of aortic rings from female rabbits than from either ovariectomized or male rabbits. It is important to note that estrogen...
and LY117018 treatment did not directly affect the sensitivity of rat aorta to PE contraction or SNP relaxation.

In addition to the gender difference in the basal release of NO, we observed that chronic treatment of ovariectomized rats with estrogen or LY117018 enhanced endothelium-dependent relaxation to high concentrations (10^{-6} to 10^{-5} M) of ACh in PE-precontracted aortic rings. Activation of endothelial muscarinic receptors induces synthesis and release of NO (Furchgott and Zawadzki, 1980). Our results show that estrogen treatment can increase receptor-mediated NO release. In agreement with these results, Weiner et al. (1989, 1991) observed that ACh-induced NO-mediated relaxation of guinea pig uterine and carotid arteries was increased during pregnancy.

The enhanced NO production may result from elevated basal Ca^{2+} concentrations in endothelial cells or greater expression of NOS. Weiner et al. (1994) showed that estrogen treatment and pregnancy in the guinea pig increase the activity of Ca^{2+}-dependent NOS in the uterine artery, heart, kidney, skeletal muscle and cerebellum as well as the levels of mRNA expression for both the endothelial and neuronal isoforms of the constitutive NOS (eNOS and nNOS) in skeletal muscle. In agreement with this finding, Hishikawa et al. (1995) demonstrated that treatment of cultured human aortic endothelial cells with estrogen enhances both Ca^{2+}-dependent NO production and NOS protein. These findings suggest that estrogen increases release of NO, at least in part, by enzyme induction.

Harder and Coulson (1979) demonstrated that a synthetic estrogen, diethylstilbestrol, directly hyperpolarizes coronary smooth muscle cells by activating an outward K^{+} current, a finding further supported by our recent report that acute administration of 17β-estradiol (1–30 μM) markedly enhanced the activity of the large Ca^{2+}-activated K^{+} channels in rabbit aortic endothelial cells and caused an increase in intracellular Ca^{2+} concentration (Rusko et al., 1995). Taken together, our results may be explained by assuming that estrogen hyperpolarizes the endothelial cell and increases the electrochemical gradient for Ca^{2+} entry through leak- or store-operated channels (the probability of these openings appears to be voltage sensitive) (Adams et al., 1989). The resultant increase in endothelial intracellular Ca^{2+} concentration would be expected to augment NO release. Although the above interpretation is consistent with the data presented, it is not possible to rule out estrogen modulation of endothelial NOS expression or changes in its Ca^{2+} sensitivity.

Although the focus of our study was to examine the effects of estrogen and LY117018 on vascular function, estrogen is usually administered in combination with progesterone when used therapeutically. We therefore included an progesterone-plus-estrogen-treated group of rats in our study. Interestingly, the vasomotor effects of chronic estrogen were reduced when progesterone was combined with estrogen. The mechanism of the interaction we report in the current study is unclear, but it is known that estrogen and progesterone can act in ways that are antagonistic to each other. Related to this, it has been demonstrated that progesterone attenuates estrogen-induced stimulation of the endothelium-dependent responses in isolated dog coronary artery rings (Miller and Vanhoutte, 1991).

In conclusion, our study indicates that chronic estrogen and LY117018 treatment enhances both basal and receptor-mediated release of NO in aortic rings of rats and the vaso-motor effects of chronic estrogen are reduced when progesterone is combined with estrogen. We conclude that the endothelium is an important therapeutic site for the cardioprotective effect of estrogen and LY117018.

References


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