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Raloxifene enhances nitric oxide release in rat aorta via increasing endothelial nitric oxide mRNA expression

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Abstract

We report the modulatory effects of chronic oral LY139481 (raloxifene) on basal release of nitric oxide (NO) and mRNA levels of endothelial NO synthase (eNOS) in rat thoracic aorta. Constrictor dose–response curves to phenylephrine were generated before and after pretreatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase. Aortic segments were obtained from four groups of rats gavaged orally for 21 days: (i) ovariectomized, (ii) sham, (iii) ovariectomized estradiol-treated, and (iv) ovariectomized raloxifene-treated. Intact aortic rings from sham rats and ovariectomized rats receiving raloxifene and estrogen showed a greater potentiation of the phenylephrine responses after L-NAME. Semi-quantitative reverse transcription-polymerase chain reaction indicated a gender-based difference in eNOS mRNA expression in thoracic aorta. Moreover, we demonstrated that eNOS mRNA expression in the upper thoracic aorta was significantly higher in treatment groups. These results show that chronically administered raloxifene is exerting a potentially important vasculo-protective effect by stimulating eNOS expression. © 2002 Published by Elsevier Science B.V.

Keywords: Estrogen; Raloxifene; Nitric oxide (NO); Nitric oxide (NO) synthase, endothelial

1. Introduction

Reduction of ovarian function at menopause is associated with a multitude of clinical symptoms that result from estrogen deficiency in various target organs such as bone, breast, heart and the urogenital system. The risk of myocardial infarction and stroke increases dramatically in women after menopause (Barrett-Connor and Bush, 1991). Many reports show that estrogen replacement therapy in postmenopausal women reduces mortality due to cardiovascular disease (Barrett-Connor and Bush, 1991; Stampfer and Colditz, 1991). Despite a wealth of supportive observational data (Grady et al., 1992), two recent clinical trials, the Heart and Estrogen/progestin Replacement Study trial (Hulley et al., 1998) and the Estrogen Replacement and Atherosclerosis trial (Herrington et al., 2000), have called into question whether current hormone replacement therapy regimens have cardioprotective effects. However, these studies only suggest that women with known coronary artery disease who are many years past menopause should not be started on hormone replacement therapy for secondary prevention of heart disease. Neither the Heart and Estrogen/progestin Replacement Study nor the Estrogen Replacement Therapy addresses the possibility that hormone replacement therapy may provide primary prevention of cardiovascular disease. Thus, the potential role of estrogens in protecting against cardiovascular disease remains controversial (Nabel, 2000). The protective effects of estrogen against cardiovascular disease are supported by many basic science studies demonstrating that estrogen not only indirectly impacts cardiovascular disease through effects on a variety of systemic factors but also directly benefits the heart and vasculature (Mendelsohn and Karas, 1999).

Although long-term estrogen replacement therapy alleviates many of the symptoms of menopause, it is associated

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with an increased risk of breast and endometrial cancer (Colditz et al., 1995; Grady et al., 1995). Chemical synthetic efforts have yielded a variety of non-estrogenic compounds with varying degrees of tissue selectivity known as selective estrogen receptor modulators (Kauffman et al., 1997). 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 benzothiophenes, LY117018 and LY139481 (raloxifene) are examples of highly promising selective estrogen receptor modulators. Raloxifene was shown to have antiestrogenic properties in breast tissue and to show minimal estrogenic effects in the uterus (Black et al., 1994). In addition, it has potentially beneficial estrogen-like effects in non-reproductive tissue such as bone (Yang et al., 1996).

Raloxifene significantly decreased circulating low density lipoprotein (LDL) by 12%, which is similar to observations made in most studies with estrogen replacement therapy (Walsh et al., 1998). Although circulating lipid levels and lipoprotein profiles improve with estrogen replacement therapy, these lipid effects still appear to account for only 25–50% of the observed cardiovascular risk reduction (Bush et al., 1987). Many of estrogen's beneficial cardiovascular effects may relate to its direct action on vascular reactivity, especially by modifying the functional state of the endothelium.

A number of reports indicate that nitric oxide (NO) production may play an important role in mediating the effects of estrogen on the vasculature. NO, a potent vasodilator (Furchgott and Zawadzki, 1980), is produced in vascular endothelial cells by the enzyme endothelial nitric oxide synthase (eNOS) (Palmer et al., 1988). We recently reported that estrogen and LY117018 enhance release of NO in rat aorta (Rahimian et al., 1997). High concentrations of raloxifene acutely induce NO-mediated relaxation in rabbit and human coronary arteries in vitro by endothelium- and estrogen receptor-dependent mechanisms (Figtree et al., 1999). In a recent study, Haynes et al. (2000) provided evidence that the stimulation of eNOS in endothelial cells via activation of estrogen receptor α involves the phosphatidylinositol 3-kinase Akt pathway. The Haynes study sheds light on the signal transduction pathway that mediates the rapid, nongenomic activation of eNOS by estrogen and raloxifene. However, to date the effects of chronic raloxifene therapy on vascular gene transcription and vascular function have not been addressed. Only a recent study showed that chronic treatment with raloxifene prevented decreased the Ca^{2+}-dependent constitutive NOS activity in ovariectomized rats (Pavo et al., 2000). Reports of increased eNOS expression in rat aorta during pregnancy provide evidence for physiological modulation of eNOS abundance (Goetz et al., 1994). By contrast, Barbacanne et al. (1999) found no effect of estrogen on NOS expression in rat aorta.

The aims of the present work were (1) to compare the effects of estrogen and raloxifene on rat arterial function as modulated by NO, (2) to investigate any gender-based differences in eNOS mRNA expression, and (3) to compare the possible effects of estrogen and raloxifene on eNOS mRNA expression in rat thoracic aorta. We report the effects of raloxifene, the first selective estrogen receptor modulator product to be marketed for the treatment of osteoporosis, on arterial function, secondary to its effects on endothelial NO synthesis/release.

2. Materials and methods

2.1. Animals and procedures

In the study comparing eNOS mRNA between male and female rats, four to five intact Sprague–Dawley rats per each group, weighing 275–300 g were used. In the drug administration study, 18 ovariectomized and 6 sham-operated female Sprague–Dawley rats weighing 275–300 g were assigned to four treatment groups. In both studies, the animals were housed in a temperature-controlled room (23 °C) on a 10-h dark/14-h light cycle and maintained on standard rodent chow. All animal care and treatment complied with Principles of Laboratory Animal Care the guidelines of the Canadian Council on Animal Care and was subject to prior local institutional review and approval. In the drug study, all groups (six animals per each group) received daily administration of drug or vehicle (placebo) via gavage for three weeks. Group 1: ovariectomized, dosed with vehicle (hydroxypropyl-β-cyclodextrin); group 2: ovariectomized, dosed with 17 α-ethinyl estradiol (0.1 mg/kg/day); group 3: ovariectomized, dosed with raloxifene (1 mg/kg/day) and group 4: sham-operated, dosed with vehicle.

2.2. Tissue collection and measurement of arterial tension

In the drug administration study, the rats were killed on day 21 with pentobarbital (65 mg kg^{-1}, i.p.) after an intravenous injection of heparin. The thoracic aorta was rapidly removed and divided into three consecutive segments: two segments of upper thoracic aorta and one segment of lower thoracic aorta. All segments were cleaned of fat and adhering connective tissue in ice-cold phosphate-buffered saline (PBS). For mRNA assays, one segment of upper thoracic aorta and one segment of lower thoracic aorta were frozen in liquid nitrogen. The tissues were then stored at -70 °C until processed. All dissection tools and instruments were treated with RNAseZap and diethylpyrocarbonate (DEPC)-treated distilled water, prior to use.

For arterial isometric tension assays, the remaining segment of upper thoracic aorta was cut into rings 3 to 4 mm in length. In some rings, the endothelium was removed by gently rubbing the intimal surface. For each experiment, four

or five rings from the same aorta (two or three with endothelium, two or three without endothelium) were used. Rings of aorta were suspended between two stainless steel hooks for measurement of isometric tension in individual organ baths containing 5 ml modified Krebs solution containing (in mM/l): NaCl, 119; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4$, 1.17; $NaHCO_3$, 24.9; EDTA, 0.023; $CaCl_2$, 1.6; Glucose, 11.1.at 37 °C, bubbled with 95% O_2 and 5% CO_2. Rings were equilibrated for 45 min under a resting tension of 1 g to allow development of stable basal tone and reproducible contractile responses to a high concentration (80 mM) of KCl. The responsiveness of rings to phenylephrine (2 μM) and acetylcholine (10 μM) was determined. Acetylcholine-induced relaxation of the phenylephrine pre-contracted vessels was taken as evidence for the preservation of an intact endothelium, whereas lack of relaxation confirmed the absence of functional endothelium.

2.3. Responses to acetylcholine

Rings of aorta were contracted with phenylephrine (2 μM), which represented a concentration that produced 80% of the maximal effect (EC_{80}). Relaxation–response curves were obtained by the addition of increasing concentrations of acetylcholine (10^{-8} to 10^{-5} M). Tissues were then washed with Krebs solution for 30 min to allow relaxation to basal tone.

2.4. Contractile effect of phenylephrine

A cumulative concentration–contraction response curve to phenylephrine was generated (10^{-8} to 10^{-5} M). The rings were then washed with Krebs solution for 30 min and N^{ω}-nitro-L-arginine methyl ester (L-NAME; 200 μM), a NOS inhibitor, was added to the bath for 30 min. Selection of this concentration of L-NAME was based on our previous studies (Rahimian et al., 1997). The concentration–contraction response curves to phenylephrine (10^{-8} to 10^{-5} M) were then repeated. Contractions after L-NAME treatment were quantitated as the percent increase from maximum phenylephrine-induced tension before L-NAME treatment.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

2.5.1. RNA extraction

Total cellular RNA from intact thoracic aorta was extracted using a RNeasy mini kit™ according to manufacturer's instructions. RNA was quantified by measuring absorbance spectrophotometrically at 260 nm and its integrity was assessed after electrophoresis in nondenaturing 1% agarose gels stained with ethidium bromide.

2.5.2. Semi-quantitative RT-PCR

Reverse transcription of 2 μg total RNA was performed in 60 μl reaction volumes containing 200 units of Superscript II™ reverse transcriptase, 60 units RNase inhibitor, 3 mM $MgCl_2$, 1X Buffer II from Sigma and 0.3 μg random primers and 1 mM dNTP for 50 min at 42 °C. Contaminating genomic DNA present in the RNA preparations was removed by digesting the reaction with 5 units of DNase I for 45 min at 37 °C prior to the addition of reverse transcriptase. Five microliters of the RT product was used in each 100 μl PCR reaction. The PCR mixture contained 250 μM dNTP, 2 mM $MgCl_2$, 1X volume of buffer and 2.5 unit Hotstar™ Taq polymerase, and 1 μl of forward and 1 μl of reverse primers (100 ng). The temperature program for the amplification was 32 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The final extension was completed at 72 °C for 7 min.

Ten microliters of 6X loading buffer (containing 0.25% bromothymolblue, 0.25% xylene cyanol FF, and 15% Ficoll type 400, Pharmacia, in DEPC-treated distilled water) was added to the PCR products. Twenty microliters of PCR products was then analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide and gels were photographed under UV light. eNOS mRNA expression levels were normalized to 18S ribosomal RNA expression. Primers used for different amplifications were designed from published reports (Barbacanne et al., 1999) or sequences available in Genbank (Accession #AJ249546). Endothelial NOS, 340 bp (forward primer: 5′ -TTCCGGCTGCC ACCTGATCCTAA-3′ ; reverse primer: 5′ -AACATGTGT

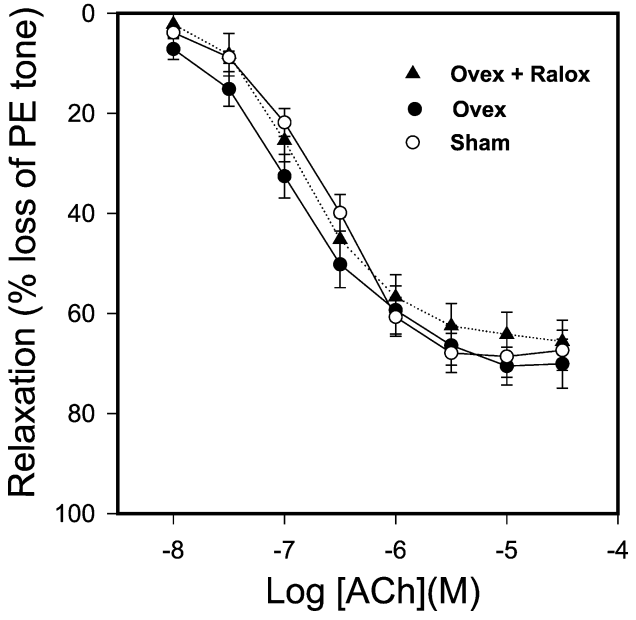

Fig. 1. Effect of chronic raloxifene treatment of ovariectomized rats on the relaxation–response to cumulative concentrations of acetylcholine (ACh) in intact aortic rings precontracted with phenylephrine (PE; 2 μM). Relaxation to acetylcholine is expressed as a percentage of phenylephrine (2 μM) maximum contraction. Each curve represents the mean ± S.E.M. of six animals per group. Relaxations of sham and raloxifene-treated ovariectomized rats (Ovex + Ralox) are not different from that of the ovariectomized (Ovex) ($P > 0.05$).

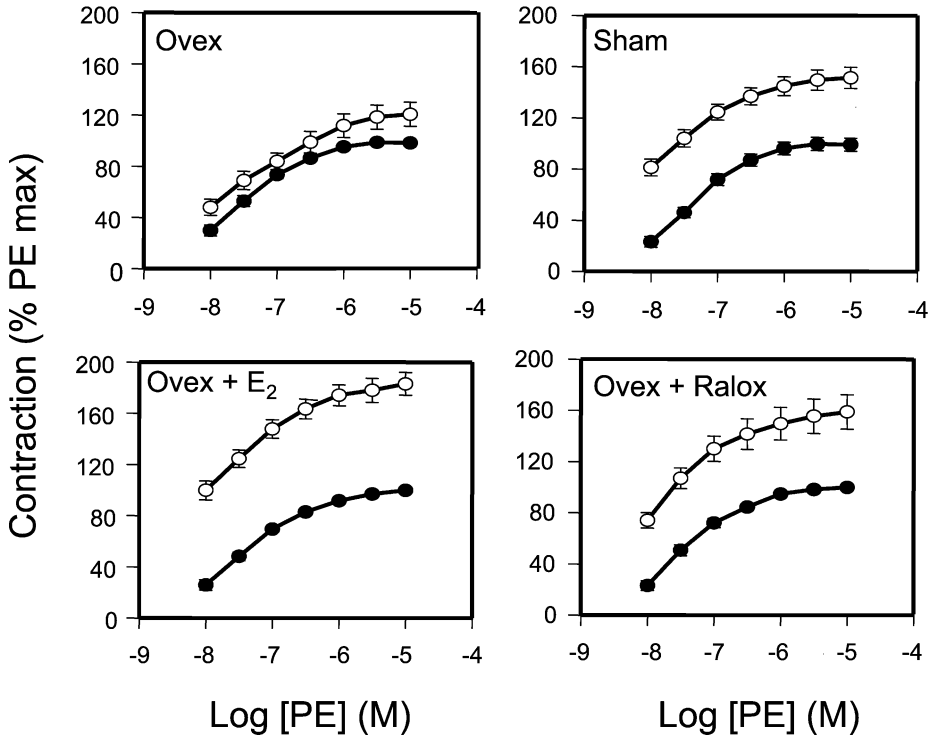

Fig. 2. Phenylephrine (PE) concentration–contraction response curves in intact thoracic aorta from vehicle-treated sham rats and raloxifene-, estrogen- and vehicle-treated ovariectomized rats, in the absence (●) or presence (○) of L-NAME (200 μM). Results are expressed as a percent of the maximal response to phenylephrine (10 μM) in the absence of L-NAME. The upward shift in the curves induced by L-NAME is significant ($P < 0.05$, ANOVA) in all groups and is significantly smaller in the Ovex group than in the other three. Each curve represents the mean ± S.E.M. of six animals per group.

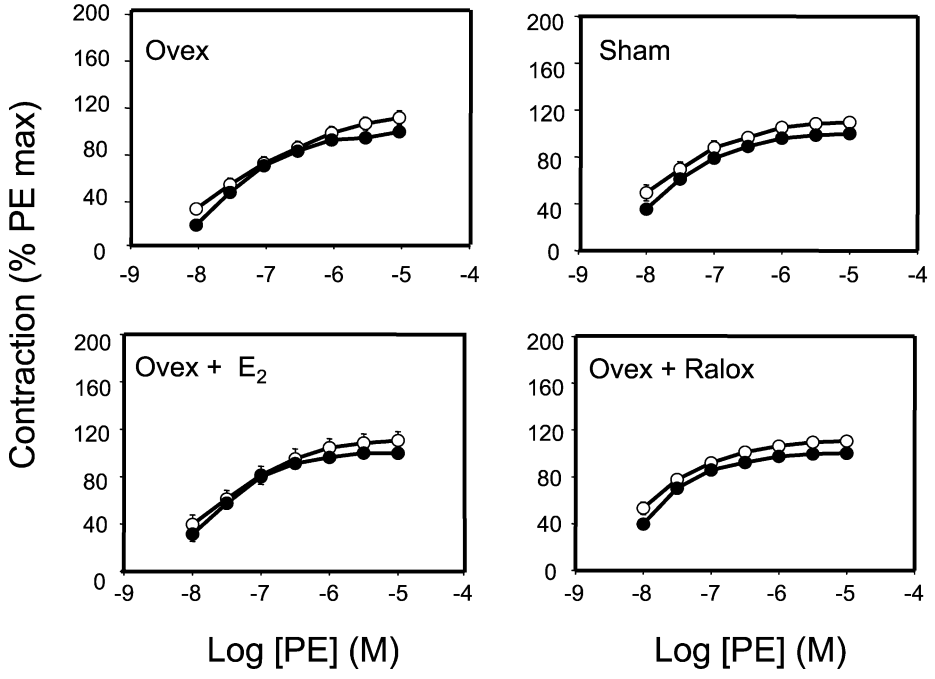

Fig. 3. Phenylephrine (PE) concentration–contraction response curves in thoracic aorta lacking endothelium from vehicle-treated sham rats and raloxifene-, estrogen- and vehicle-treated ovariectomized rats, in the absence (●) or presence (○) of L-NAME (200 μM). Results are expressed as a percent of the maximal response to phenylephrine (10 μM) in the absence of L-NAME. Each curve represents the mean ± S.E.M. of six animals per group.

CCTTGCTCGAGGCA-3′). Amplified eNOS PCR products from rat tissue were isolated from agarose gel, sequenced and found to be 100% identical to the authentic sequences of rat eNOS cDNA.

2.6. Chemical reagents and drugs

L-phenylephrine hydrochloride, acetylcholine, L-NAME, and 17 α-ethinyl estradiol were obtained from Sigma (St. Louis, MO, USA). Hydroxypropyl-β-cyclodextrin was purchased from Aldrich Chemical (Milwaukee, WI, USA). Raloxifene was a gift from Eli Lilly & Company (Indianapolis, IN, USA). SuperscriptII™ reverse transcriptase, RNase inhibitor and random primers were obtained from Gibco/BR, Canada). Buffer II (10 ×) was obtained from Sigma/Aldrich (Canada). $MgCl_2$, dNTP, 10 × volume PCR Buffer, Hotstar™ Taq polymerase and RNeasy mini kit™ were purchased from Qiagen (Canada). Primers for ribosomal RNA (18S) and RNAseZap were purchased from Ambion (TX, USA).

2.7. Data analysis

Values are expressed as means ± standard error of the mean (S.E.M.). Comparisons of means were made by one-way analysis of variance (ANOVA) and the all pairs Tukey–Kramer test and the Student–Newman–Keuls test. A probability value of less than 5% ($P<0.05$) was considered significant.

3. Results

3.1. Relaxation responses to acetylcholine

Relaxation in response to acetylcholine was used to examine the effect of raloxifene treatment on receptor-mediated endothelium-dependent release of NO. No significant differences in responses to the entire concentration range of the acetylcholine (10^{-8} to 10^{-5} M) occurred between aortic rings from ovariectomized, sham-operated, and ovariectomized rats receiving raloxifene (Fig. 1).

Table 1
Maximum tension (in grams) generated in response to phenylephrine (PE, 2 μM) before and after removal of endothelium in the aorta taken from various group of rats

Group	With endothelium	Without endothelium
Ovex	2.41 ± 0.15	2.55 ± 0.21
Sham	2.39 ± 0.17	3.01 ± 0.32
Ovex + E_2	2.05 ± 0.08	2.23 ± 0.18
Ovex + Ralox	2.65 ± 0.18	3.3 ± 0.22

Data are represented as the mean ± S.E.M. of six animals.
Ovex: ovariectomized rats, Ovex + E_2: estrogen-treated ovariectomized rats, Ovex + Ralox: raloxifene-treated ovariectomized rats.

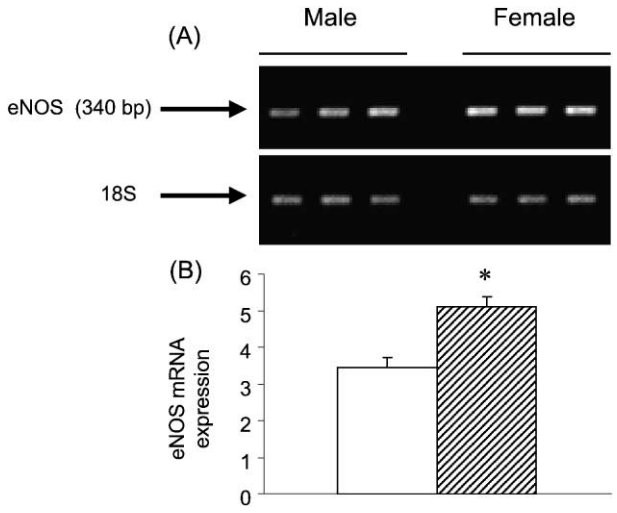

Fig. 4. Endothelial NOS (eNOS) mRNA expression in female and male rat upper thoracic aorta. (A) Typical gel from RT-PCR analysis of RNA extracted from aorta obtained from three males and three females. The expression level of 18S ribosomal RNA served as an internal control. (B) eNOS mRNA levels in aorta from male normalized to 18S rRNA (open bar) and female (slash bar) rats. Each bar represents the mean ± S.E.M. of four to five animals. * $P<0.01$ vs. males.

3.2. Effect of L-NAME on phenylephrine-induced contractions

3.2.1. Aortic rings with functionally intact endothelium

In Fig. 2, we show that incubation of intact aortic ring segments with L-NAME (200 μM) resulted in potentiation of the contractile responses to phenylephrine in all four groups of aortae through the entire concentration–contrac-

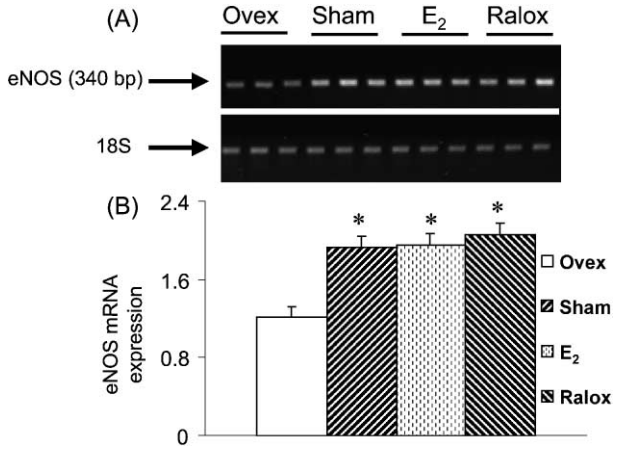

Fig. 5. Endothelial NOS mRNA (eNOS) expression in rat upper thoracic aorta from vehicle-treated sham female rats (Sham) and raloxifene- (Ralox), estrogen- (E2) and vehicle-treated (Ovex) ovariectomized rats. (A) Typical gel from RT-PCR analysis of RNA extracted from aortic tissue. The expression level of 18S ribosomal RNA served as an internal control. (B) Ratio of eNOS mRNA levels in aorta normalized to 18S rRNA from Ovex (open bars), Sham (slash bars), E2 (dash bars), and Ralox (back slash bars) rats. Each bar represents the mean ± S.E.M. of three to four animals. * $P<0.05$ vs. the Ovex group.

tion response range of phenylephrine (10^{-8} to 10^{-5} M). Aortic rings from sham, estrogen and raloxifene-treated rats had greater maximal potentiation of the phenylephrine responses after inhibition of NOS than did rings from vehicle-treated, ovariectomized rats ($P<0.05$).

3.2.2. Aortic rings lacking functional endothelium

The same phenylephrine concentration–response experiment as above was performed simultaneously on aortic rings lacking a functional endothelium. However, in this condition, there was little or no L-NAME-dependent potentiation of contractile responses to phenylephrine in all treatment groups (Fig. 3). The maximum tension generated by phenylephrine in the rings without endothelium exceeded those of the rings with endothelium in all groups (Table 1).

3.3. Effect of estrogen and raloxifene on eNOS mRNA expression

In order to determine whether there is a gender-dependent difference in eNOS expression in rat thoracic aorta, mRNA eNOS expression was measured using semi-quantitative RT-PCR. A single band of the predicted size (340 bp) was detected in aorta (Fig. 4A). The expression level of 18S ribosomal RNA was used as an internal control to correct for inter-sample variations in total RNA abundance. RT-PCR reactions run in the absence of reverse transcriptase or cDNA were used as negative controls (data not shown). As shown in Fig. 4B, eNOS mRNA expression was significantly higher in thoracic aorta ($P<0.01$) from female rats compared to that seen in male rats. In the drug administration study, the thoracic aorta was divided into two segments: the upper and lower thoracic aorta. We studied the effect of estrogen and raloxifene on the eNOS mRNA abundance in both segments. A single band of the predicted size for eNOS (340 bp) was detected in aorta taken from all treatment groups (Fig. 5A). RT-PCR analysis revealed that eNOS mRNA levels were significantly elevated in upper thoracic aorta taken from sham-operated rats dosed with vehicle (by 158%) and ovariectomized rats dosed with estrogen or raloxifene (by 161% and 169%, respectively) ($P<0.05$) compared to eNOS mRNA levels seen in the corresponding regions of ovariectomized animals dosed with vehicle (Fig. 5B). However, in the lower thoracic aorta, eNOS expression was similar between groups (Fig. 6).

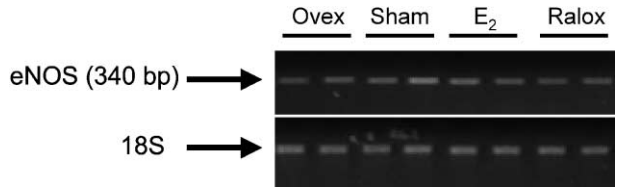

Fig. 6. Exemplary agarose gel electrophoresis from RT-PCR analysis of endothelial NOS (eNOS) in the in rat lower thoracic aorta from vehicle-treated sham female rats (Sham) and raloxifene- (Ralox), estrogen- (E2) and vehicle-treated (Ovex) ovariectomized rats. PCR products were generated through the use of specific primers for eNOS. eNOS mRNA expression was similar in lower thoracic aorta from different animals ($P>0.05$). The expression of 18S ribosomal RNA was used as an internal control.

4. Discussion

In the present study, we have demonstrated that estrogen and raloxifene exert in vivo stimulatory effects on eNOS mRNA expression in rat thoracic aorta resulting in the enhanced generation of basal NO. This was demonstrated by (1) an elevated expression of aortic eNOS mRNA and (2) enhancement of the upward shift of the concentration–contraction response curve to phenylephrine following L-NAME pretreatment of thoracic aortic rings taken from estrogen- and raloxifene-treated ovariectomized rats compared to non-treated rats.

We recently reported that chronically administered estrogen and LY117018, a close structural analog of raloxifene, enhances release of NO in the rat aorta without changing the sensitivity of smooth muscle cells to either NO donors or to an adrenoceptor agonist (Rahimian et al., 1997). The effects of acute, high concentrations of raloxifene on rabbit coronary artery vasoreactivity in vitro have also been investigated (Figtree et al., 1999). Since the vasoactive mechanism(s) of raloxifene have not yet been fully investigated, we undertook this study to evaluate one potential regulatory pathway involved in mediating raloxifene effects on arterial function in vivo. Raloxifene is currently prescribed to women for the treatment and prevention of osteoporosis (Ettinger et al., 1999). These patients may derive additional benefits from this drug in terms of cardiovascular protection secondary to improved endothelial function.

Basal NO release from rat aortic endothelial cells was indirectly monitored by observing the effects of L-NAME on phenylephrine concentration–response curves. L-arginine is converted to L-citrulline in endothelial cells by the enzyme NOS (Palmer et al., 1988). NO synthesis is competitively inhibited by certain analogs of L-arginine such as L-NAME (Rees et al., 1989). Since the endothelial cells appear to be devoid of α-adrenoceptors (Rahimian et al., 1998a), differences in basal release of NO are manifested as differences in the degree of phenylephrine-induced tension in the presence and absence of L-NAME.

In the current study, intact aortic rings from sham-operated rats dosed with vehicle and ovariectomized rats dosed with estrogen or raloxifene demonstrated greater L-NAME-potentiation of phenylephrine-induced contractions than did similarly treated rings from ovariectomized rats dosed with vehicle. Since these differences were not observed in aorta from which the endothelium had been removed, it is concluded that raloxifene restores basal endothelial NO release. However, raloxifene treatment did not affect stimulated release of NO, as judged by similar acetylcholine relaxation–response curves in various groups.

Enhancement of NO by estrogen and raloxifene may result from either stimulation of eNOS or greater expression of eNOS. It has recently been shown that raloxifene prevents a decrease in Ca^{2+}-dependent constitutive NO synthase in ovariectomized rat aorta as assessed by the citrulline assay (Pavo et al., 2000). Our recent data on isolated rat valvular endothelial cells also support the hypothesis that estrogen increases eNOS activity, at least in part, by raising the Ca^{2+} level in endothelial cells (Rahimian et al., 1998b).

eNOS is classified as a constitutively expressed gene, the activity of which is partly regulated by $[Ca^{2+}]_i$, in contrast to the inducible NOS isoform (iNOS), which is regulated solely at the transcriptional level. However, the increased expression of constitutive NOS in rat aorta during pregnancy has been reported by other investigators (Goetz et al., 1994; Xu et al., 1996). The promoter regions of the human and bovine eNOS gene have been sequenced and several half palindrome sequences of the estrogen response element have been recognized (Marsden et al., 1993; Venema et al., 1994), suggesting possible regulation at the level of gene expression. It has recently been appreciated that eNOS expression can be modulated to a certain extent by physical and hormonal stimuli. Estrogen can stimulate eNOS expression in cultured human endothelial cells (Kleinert et al., 1998) and in tissues from guinea pigs treated with estrogen (Weiner et al., 1994). It has also been reported that estrogen stimulates the expression of eNOS and iNOS in neonatal and adult cardiomyocytes in vitro and in vivo (Nuedling et al., 1999). However, there are conflicting data showing stimulation of in vivo endothelium derived relaxing factor (EDRF) activity by estrogen without accompanying upregulation of eNOS in rat aorta (Barbacanne et al., 1999). Therefore, the role of estrogen in regulation of eNOS activity and expression remained controversial.

In the present study, we demonstrated a gender difference in aortic eNOS mRNA expression. In accordance, Morschl et al. (2000) have reported that the constitutive NOS activity is higher in the aorta of the female rat compared to its activity of male aortic tissue. Therefore, we tested the hypothesis that raloxifene may also stimulate mRNA overexpression of eNOS. Our data show for the first time that chronic raloxifene administration, like estrogen, stimulates eNOS mRNA expression in upper thoracic aorta, identifying a novel mechanism that may explain raloxifene's vasoprotective effects as well as its anti-atherogenic activity (Bjarnason et al., 2001). Enhanced eNOS expression and basal NO production may similarly explain raloxifene's capacity to inhibit the expression of cell adhesion molecules (Simoncini et al., 1999) since NO is known to inhibit endothelial leukocyte adhesion (De Caterina et al., 1995). Although, our observed raloxifene-induced eNOS mRNA upregulation was genomically mediated, Simoncini and Genazzani (2000) showed that clinically effective concentrations of raloxifene can trigger a rapid, concentration-dependent stimulation of eNOS activity in cultured human umbilical vein endothelial cells in the absence of changes in eNOS mRNA expression. It is important to note that these investigators made their observations in vitro using a fetal endothelial cell line that may differ significantly from the in vivo setting.

In the present study, although we did not measure eNOS protein levels in thoracic aorta, our detection of eNOS mRNA upregulation in aortae from estrogen-treated rats is in complete agreement with the findings of others (Goetz et al., 1994; Kleinert et al., 1998) who showed that the expression of constitutive NOS in rat aorta and human endothelial cells was increased during pregnancy and treatment with estrogen. These findings differ from those of Barbacanne et al. (1999) who found that estradiol increased rat aorta NO activity without changes in eNOS gene expression. The basis for discrepancy between the work of Barbacanne et al. (1999) and our results is unclear. It is possible that in the study by Barbacanne et al. (1999) the duration of estrogen administration was short to elicit detectable eNOS upregulation. In time course studies, we found that 21 days of estrogen administration is required to optimally restore EDRF activity in ovariectomized rats (unpublished data). Alternatively, the optimal estrogen level may not have been achieved or they may have studied a region of rat aorta that does not respond to estrogen with elevated eNOS expression. Our results indicate that eNOS mRNA responsiveness to estrogen is highly region-dependent. In accordance with this possible explanation, it has been shown that the eNOS activity in the aortic arch and upper, but not lower, thoracic aorta of rabbits, is significantly increased by long-term treatment with 17 β-estradiol (Andersen and Stender, 2000). Shear stress is directly linked to blood flow and blood vessel diameter; it is high when blood flow is high and can be reduced by increasing the vessel diameter (Zarins et al., 1987). Interestingly, it has been shown that rat aorta, characterized by chronically high hemodynamic sheer stress, and cultured endothelial cells exposed to arterial levels of shear stress, have elevated levels of eNOS mRNA and protein expression (Nadaud et al., 1996; Ranjan et al., 1995). Thus, the regional variation in aortic eNOS expression in the present study may be due in part, to regional-dependent differences in the endothelial activation state. In support of this notion, Tsao et al. (1995) proposed that the preferential distribution of atherosclerotic plaque in areas of low shear stress is partly explained by reduced NO production at these sites.

Our study indicates that the enhanced basal NO production observed in aorta from estrogen- and raloxifene-treated ovariectomized rats is likely due, at least in part, to increased aortic eNOS expression. In this context, it is peculiar that acetylcholine-induced relaxation was not affected. Further investigation is required to determine the relative importance of basal vs. stimulated NO release in vasoprotection. We conclude that the endothelium is an important therapeutic site for the cardioprotective effect of raloxifene. Understanding the mechanisms underlying the

cardioprotective action of estrogen is expected to contribute significantly to the development of new therapeutic strategies, whereby the beneficial activities of selective estrogen receptor modulators can be optimized and the undesirable activities of estrogen receptor activation can be minimized or eliminated.

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