Estrogen potentiates constrictor prostanoid function in female rat aorta by upregulation of cyclooxygenase-2 and thromboxane pathway expression

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1Department of Veterinary Physiology and Pharmacology and 2Michael E. DeBakey Institute for Comparative Cardiovascular Science, College of Veterinary Medicine, Texas A&M University, College Station, Texas; and 3Department of Systems Biology and Translational Medicine, Texas A&M Health Sciences Center, Temple, Texas

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Li M, Kuo L, Stallone JN. Estrogen potentiates constrictor prostanoid function in female rat aorta by upregulation of cyclooxygenase-2 and thromboxane pathway expression. Am J Physiol Heart Circ Physiol 294: H2444–H2455, 2008. First published February 29, 2008; doi:10.1152/ajpheart.01121.2007.—Estrogen potentiates vascular reactivity to vasopressin (VP) by enhancing constrictor prostanoid function. To determine the cellular and molecular mechanisms, the effects of estrogen on arachidonic acid metabolism and on the expression of constrictor prostanoid pathway enzymes and endoperoxide/thromboxane receptor (TP) were determined in the female rat aorta. The release of thromboxane A2 (TxA2) and prostacyclin (PGI2) was measured in male (M), intact-female (Int-F), ovariectomized-female (OvX-F), and OvX + 17β-estradiol-replaced female (OvX + ER-F) rats. The expression of mRNA for cyclooxygenase (COX)-1, COX-2, thromboxane synthase (TxS), and TP by aortic endothelium (Endo) and vascular smooth muscle (VSM) of these four experimental groups was measured by RT-PCR. The expression of COX-1, COX-2, and TxS proteins by Endo and VSM was also estimated by immunohistochemistry (IHC). Basal release of TxA2 and PGI2 was similar in M (18.8 ± 1.9 and 1,723 ± 153 pg/mg ring wt/45 min, respectively) and Int-F (20.2 ± 4.2 and 1,488 ± 123 pg, respectively) rat aortas. VP stimulated the dose-dependent release of TxA2 and PGI2 from both male and female rat aorta. OvX markedly attenuated and ER therapy restored VP-stimulated release of TxA2 and PGI2 in female rats. No differences in COX-1 mRNA levels were detected in either Endo or VSM of the four experimental groups (P > 0.1). The expression of both COX-2 and TxS mRNA were significantly higher (P < 0.05) in both Endo and VSM of Int-F and OvX + ER-F compared with M or OvX-F. Expression of TP mRNA was significantly higher in VSM of Int-F and OvX + ER-F compared with M or OvX-F. IHC revealed the uniform staining of COX-1 in VSM of the four experimental groups, whereas staining of COX-2 and TxS was greater in Endo and VSM of Int-F and OvX + ER-F than in OvX-F or M rats. These data reveal that estrogen enhances constrictor prostanoid function in female rat aorta by upregulating the expression of COX-2 and TxS in both Endo and VSM and by upregulating the expression of TP in VSM.

Estrogen potentiates vascular reactivity to vasopressin (VP) by upregulating constrictor prostanoid function. To determine the cellular and molecular mechanisms, the effects of estrogen on arachidonic acid metabolism and on the expression of constrictor prostanoid pathway enzymes and endoperoxide/thromboxane receptor (TP) were determined in the female rat aorta. The release of thromboxane A2 (TxA2) and prostacyclin (PGI2) was measured in male (M), intact-female (Int-F), ovariectomized-female (OvX-F), and OvX + 17β-estradiol-replaced female (OvX + ER-F) rats. The expression of mRNA for cyclooxygenase (COX)-1, COX-2, thromboxane synthase (TxS), and TP by aortic endothelium (Endo) and vascular smooth muscle (VSM) of these four experimental groups was measured by RT-PCR. The expression of COX-1, COX-2, and TxS proteins by Endo and VSM was also estimated by immunohistochemistry (IHC). Basal release of TxA2 and PGI2 was similar in M (18.8 ± 1.9 and 1,723 ± 153 pg/mg ring wt/45 min, respectively) and Int-F (20.2 ± 4.2 and 1,488 ± 123 pg, respectively) rat aortas. VP stimulated the dose-dependent release of TxA2 and PGI2 from both male and female rat aorta. OvX markedly attenuated and ER therapy restored VP-stimulated release of TxA2 and PGI2 in female rats. No differences in COX-1 mRNA levels were detected in either Endo or VSM of the four experimental groups (P > 0.1). The expression of both COX-2 and TxS mRNA were significantly higher (P < 0.05) in both Endo and VSM of Int-F and OvX + ER-F compared with M or OvX-F. Expression of TP mRNA was significantly higher in VSM of Int-F and OvX + ER-F compared with M or OvX-F. IHC revealed the uniform staining of COX-1 in VSM of the four experimental groups, whereas staining of COX-2 and TxS was greater in Endo and VSM of Int-F and OvX + ER-F than in OvX-F or M rats. These data reveal that estrogen enhances constrictor prostanoid function in female rat aorta by upregulating the expression of COX-2 and TxS in both Endo and VSM and by upregulating the expression of TP in VSM.

Arginine vasopressin; endothelium; thromboxane synthase; thromboxane receptor; vascular smooth muscle; vasoconstriction

A SUBSTANTIAL SEXUAL DIMORPHISM exists in the vascular reactivity of the female rat aorta to VP (23, 73–75). Previous studies have established that contractile responses of the female rat aorta to VP are three- to fourfold greater than those of the male aorta and that constrictor prostanoids potentiate responses of the female aorta to VP (23, 51). The presence of estrogen receptors in both vascular smooth muscle (VSM; Refs. 2, 36, 56, 59, and 64) and endothelium (Endo; Refs. 2, 16, and 18) suggests that female gonadal steroid hormones may modulate VSM and/or Endo function and thus contribute to male/female differences in the vascular reactivity to VP and other vasoactive hormones. Indeed, recent companion studies of vascular function (51) reveal that ovariectomy (OvX) attenuates and estrogen replacement therapy (ERT) restores contractile responses to VP and to the stable thromboxane A2 (TxA2) analog U-46619 in the female rat aorta. The greater response to VP appears to involve enhanced cyclooxygenase-2 (COX-2) and constrictor prostanoid activity, since the selective COX-2 inhibitor NS-398 and the TxA2/PGH2 (TP) receptor antagonist SQ-29548 both attenuate contractile responses of the female rat aorta to VP to a similar extent (51). Furthermore, the constrictor prostanoid TxA2 appears to be an end product of arachidonic acid (AA) metabolism in the vascular wall, as well as the platelet, and to play a role in the regulation of vascular tone in the normal female vasculature (23, 51). Reactivity of the female rat aorta to the stable TxA2 analog U-46619 is also significantly greater in female than in male rat aorta and estrogen enhances contractile responses of the female aorta to U-46619 (51). Thus estrogen may enhance contractile responses of the female rat aorta to VP by upregulating the expression of COX-2 and thereby enhancing the production of and/or the vascular reactivity to constrictor prostanoids (PGH2 and TxA2). This hypothesis is supported by the findings from recent companion studies that Ovx abolished and ERT restored the inhibitory effect of NS-398 and SQ-29548 on the contractile responses to VP (51). Therefore, in the present companion investigation, the cellular and molecular mechanisms underlying these vascular effects of estrogen were determined. Specifically, the effects of estrogen on AA metabolism and on the expression of constrictor prostanoid pathway enzymes and endoperoxide/thromboxane receptor (TP) were assessed in three sets of experiments utilizing male (M), intact-female (Int-F), ovariectomized-female (Ovx-F), and OvX + 17β-estradiol-replaced female (Ovx + ER-F) rat aortas. Thus basal and VP-stimulated release of TxA2 and prostacyclin (PGI2) were first measured by radioimmunoassay (RIA). Second, the expression of mRNA for the key enzymes involved in AA metabolism and for the TP receptor was quantified by reverse transcription-polymerase chain reaction (RT-PCR) for COX-1, COX-2, and thromboxane synthase (TxS) in both aortic Endo and VSM. Third, the

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expression of COX-1, COX-2, and TxS proteins in aortic Endo and VSM was estimated by immunohistochemistry (IHC). The results reveal that estrogen enhances constrictor prostanoid function in the female rat aorta by upregulating the expression of COX-2 and TxS message and protein in both Endo and VSM and by upregulating TP message in VSM.

**MATERIALS AND METHODS**

**Animals**

Age-matched (14–16 wk old) female and male Sprague-Dawley rats (Harlan, Houston, TX) were used in all studies. The rats were housed in vivarium facilities at the College of Veterinary Medicine (Laboratory Animal Resources and Research facility) with controlled temperature (22–24°C), relative humidity (∼50%), and 12-h:12-h light-dark cycle. The animals were segregated by sex and housed in pairs in standard plastic laboratory rat cages. Tap water and an alfalfa-and soy-free diet (Global Diet, formulation 2016, Harlan) were provided ad libitum. This latter special diet is free of phytoestrogens that are often contained in commonly used standard laboratory rat chow (68, 69) and that have been reported to confound the effects of OvX and molecular experiments in vitro. Bilateral OvX of female rats was performed at 4 to 5 wk age using standard methods. Half of the OvX-F rats received ERT beginning at 11 to 12 wk of age for 21–28 days using 17β-estradiol (2 × 0.05 mg/60 day release pellets, Innovative Research; Sarasota, FL). Previous studies have shown that this dose produces physiological plasma levels of 17β-estradiol in the OvX-F rat (51, 82).

**Preparation of Isolated Thoracic Aorta**

Animals were euthanized by rapid decapitation, in accordance with American Veterinary Medical Association and Texas A&M University Laboratory Animal Care Committee guidelines. Trunk blood was collected at the time of decapitation for RIA of plasma 17β-estradiol (see RIA for TxB2 and 6-Keto-PGF1α). The thoracic aorta was rapidly removed, opened longitudinally, and the Endo cells were gently removed with a cotton swab and placed directly into 1.0 ml TRIzol reagent and kept on ice. Freshly isolated Endo and VSM were stored at −80°C for poster incubation and molecular experiments in vitro. Bilateral OvX of female rats was performed at 4 to 5 wk age using standard methods. Half of the OvX-F rats received ERT beginning at 11 to 12 wk of age for 21–28 days using 17β-estradiol (2 × 0.05 mg/60 day release pellets, Innovative Research; Sarasota, FL). Previous studies have shown that this dose produces physiological plasma levels of 17β-estradiol in the OvX-F rat (51, 82).

**Vascular Prostanoid Release Studies**

Aortic rings (3 mm long) were prepared from Endo-intact thoracic aortas and were placed into chilled, gassed KHB solution and allowed to stabilize for at least 30 min. The rings were then transferred into 12 × 75-mm plastic culture tubes with 2 ml chilled KHB, gradually warmed up to 37°C, and gassed continuously with 95% O2-5% CO2. After preincubation for 30 min at 37°C, the KHB solution was carefully aspirated, and then 1.0 ml of either KHB alone (basal) or KHB with agonists or inhibitors was added to the tissues, gassed continuously, and incubated for 45 min at 37°C. After incubation, the KHB was collected and stored at −70°C until RIA of TxB2 and 6-keto-PGF1α.

For TxA2 and PGH2 release experiments, basal release reflected the steady-state release of the prostanoids into the incubation medium (KHB) during the 45-min incubation period and was normalized by dry weight of aortic rings and expressed as picogram/milligram dry tissue weight/45 min. Agonist-stimulated release reflects the total agonist-stimulated release of the prostanoids into the incubation medium in the presence of a low (1 × 10⁻⁶ M) or high (1 × 10⁻⁵ M) concentration of VP, as used in previous contractile function experiments (51).

**RIA for TxB2 and 6-Keto-PGF1α**

Concentrations of TxA2 and PGH2 in the KHB incubation media were each measured using specific RIAs for their stable metabolites TxB2 and 6-keto-PGF1α, respectively, as described in detail previously (75). Briefly, prostanoid standards (0.975–1.000 pg for TxB2 and 1.95–1.000 pg for 6-keto-PGF1α) or unknown samples were incubated with [3H]TxB2 or [3H]6-keto-PGF1α, and with the appropriate prostanoid antiserum overnight at 4°C. The charcoal-dextran method was used to separate bound and free fractions of [3H]TxB2 or [3H]6-keto-PGF1α. Bound radioactivity was counted by liquid scintillation spectroscopy. The limit of detection of the RIAs is 1.95 pg/tube for TxB2 and 3.90 pg/tube for 6-keto-PGF1α, the cross-reactivity of the antiserum to other prostanoids is <0.1% and the intra-assay and interassay coefficients of variation are 5.0% and 7.6%, respectively (75).

**RT-PCR for COX-1, COX-2, TxS, and PGH2/TxA2 Receptors**

Thoracic aortas from each of the four experimental groups, prepared as described in *Preparation of Isolated Thoracic Aorta*, were opened longitudinally, and the Endo cells were gently removed with a cotton swab and placed directly into 1.0 ml of TRIzol reagent and kept on ice. The remaining aorta (VSM) was gently scrubbed to remove the remaining Endo and was placed into 1.0 ml TRIzol reagent and kept on ice. Freshly isolated Endo and VSM were homogenized in the TRIzol reagent. The RNA was then extracted in 200 µl chloroform, 5 µl glycogen (25 µg/ml; Ambion), 50 µl Na acetate (5 M), 500 µl isopropl alcohol, and 1 ml 75% ethanol and then centrifuged at 4°C according to the manufacturer’s instructions. The extracted RNA was quantified by UV absorbance at 260 nm and stored at −80°C. Equal amounts of extracted RNA from each sample (0.2 µg) were subjected to RT by RT-PCR, using 1.0 µg of specific antisense primers (Sigma-Aldrich, Genosys) and the Thermocycler RT-PCR system, (GibCoBrl, Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Two microliters or 5 µl of cDNA, according to the anticipated level of gene expression, was then amplified in a total volume of 50 µl containing 1 µl 5’ specific primer, 1 µl 3’ specific primer, 1 µl Taq polymerase (Expand High Fidelity, Roche Molecular Biochemical, Indianapolis, IN), 5 µl 10× PCR buffer without MgCl2, 2 µl 10 mM dNTP, and 3 µl MgCl2. After an initial 4-min denaturation step at 94°C, the TxS cDNA was amplified for 35 cycles and the primers were annealed at 54°C; COX-1 and COX-2 cDNA were amplified for 30 cycles and the primers were annealed at 55°C; and TP cDNA was amplified for 35 cycles and the primers were annealed at 55°C. Each cycle consisted of 30 s denaturation at 94°C, 30 s annealing at specific temperature (except that primers for TxS were annealed for 60 s because of the longer length), and extension at 72°C for 30 s, followed by a 7-min final extension at 72°C. The RT-PCR product was electrically separated by gel electrophoresis on 1.8% agarose gel, visualized with ethidium bromide staining using Gel Doc 1000 system (Bio-Rad, Hercules, CA), and quantified by densitometry (Multi-analyst...
either alone or with the TxS inhibitor dazoxiben (Daz, 50 μM) for
45 min. Additional groups of aortic rings prepared in duplicate from
M or treatment of the experimental animal.
Thoracic aortas, prepared as described in Preparation of Isolated
Thoracic Aorta, were perfusion-fixed with 10% neutral-buffered for-
malin for 24 h at a transmural pressure of 100 mmHg and were
dehydrated with graded ethanol, embedded in paraffin, and cut into
4-μm-thick sections. After the sections were cleaned with xylene and
dehydrated with graded ethanol, they were embedded in paraffin and
cut into sections, which were then stained with hematoxylin and
eosin. The amount of positive IHC staining of each specific protein was
determined using a semiquantitative method by a naïve individual blinded to the sex
of the experimental animal. The following reagents and drugs were used: arginine VP
(Bachem; Torrance, CA); DAB (Vector); Daz (generously provided
by Pfizer Pharmaceuticals; Kent, UK); indomethacin (Sigma; St.
Louis, MO); 17β-estradiol (2× 0.05 mg pellets, 60-day release,
Innovative Research of America), and TRizol reagent (Life Technol-
gies). Daz and indomethacin were prepared fresh daily, whereas VP
was diluted daily from aliquots of 1 × 10^{-3} M stock solution stored at
−70°C.

Data Analysis

All data are expressed as means ± SE; n indicates the number of animals studied. Statistical analysis of the data was performed using one- or two-way ANOVA to detect differences among the means of the experimental groups. If significant differences were detected by ANOVA, pairwise comparisons between means of the experimental groups were made using a Student’s t-test. A P value < 0.05 was considered significantly different. Prostanoid release data were analyzed by sex (M vs. Int-F vs. OvX-F vs. OvX + ER-F) and tissue (basal vs. low-dose VP vs. high-dose VP) using two-way ANOVA and Student’s t-tests. COX-1, COX-2, and TxS mRNA data were analyzed by sex (M vs. Int-F vs. OvX-F vs. OvX + ER-F) and tissue (Endo vs. VSM) using two-way ANOVA and Student’s t-tests. TP mRNA data were analyzed by sex (M vs. Int-F vs. OvX-F vs. OvX + ER-F) using one-way ANOVA and Student’s t-tests.

RESULTS

Effects of OvX and ERT on Plasma Estradiol Concentrations

Plasma 17β-estradiol concentration of Int-F rats averaged
43.9 ± 13.0 pg/mL. OvX reduced plasma estradiol by 97% in
OvX-F rats (1.3 ± 0.4 pg/mL, P < 0.05), whereas ERT restored

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COX, cyclooxygenase; TxS, thromboxane synthase; TP, TxA2 receptor.
plasma 17β-estradiol in OvX + ER-F rats (27.9 ± 5.6 pg/ml) to concentrations that did not differ from those of Int-F rats (P = 0.05). The plasma estradiol concentration of M rats was similar to that of OvX-F rats (P > 0.05).

**Male-Female Difference in Basal and VP-Stimulated Prostanoid Release**

Basal release of TxB2 did not differ significantly between M (18.8 ± 1.9 pg/mg ring wt/45 min) and Int-F (20.2 ± 4.2 pg) rat aortas (P = 0.388; Fig. 1). VP stimulated TxB2 release in a concentration-dependent manner, and both low (1 × 10⁻⁸ M) and high (1 × 10⁻⁶ M) concentrations of VP significantly stimulated more release of TxB2 by F than by M rat aortas. In M aorta, the low concentration of VP increased TxB2 release by only 37% (25.7 ± 4.2 pg, P = 0.08), and the high concentration of VP increased TxB2 release by only 134% (43.9 ± 8.3 pg, P = 0.014). In contrast, in the Int-F aorta, low and high concentrations of VP increased TxB2 release by 121% (44.6 ± 7.0 pg, P = 0.006) and 334% (87.7 ± 12.7 pg, P < 0.001), respectively, compared with basal release (20.2 ± 4.2 pg) (Fig. 1).

Aortas of all four experimental groups released substantially more 6-keto-PGF1α than TxB2 under basal as well as VP-stimulated conditions. Basal release of 6-keto-PGF1α did not differ significantly between M (1,723 ± 153 pg/mg ring wt/45 min) and Int-F (1,488 ± 123 pg) rat aortas (P = 0.388; Fig. 2). VP also stimulated the release of 6-keto-PGF1α in a concentration-dependent manner, and similar to the release of TxB2, responses of Int-F were greater than M aortas. In M, low and high concentrations of VP increased the release of 6-keto-PGF1α by 63% (2,811 ± 184 pg, P = 0.002) and 199% (5,155 ± 1,046 pg, P = 0.012), respectively, compared with basal. In contrast, in Int-F, low and high VP increased the release of 6-keto-PGF1α by 159% (3,860 ± 800 pg, P = 0.013) and 750% (12,646 ± 2,683 pg, P < 0.001), respectively (Fig. 2).

**Effects of OvX and ERT on Prostanoid Release**

OvX and ERT had no effects on basal release of either TxB2 or 6-keto-PGF1α (Figs. 1 and 2). In contrast, OvX significantly decreased both low- and high-concentration VP-stimulated release of TxB2 (P ≤ 0.011) to levels similar to those of M aortas, whereas ERT of OvX-F rats restored both low- and high-concentration VP-stimulated release of TxB2 by OvX + ER-F aortas to levels not different from those of Int-F aortas (Fig. 1; P > 0.05). Similarly, OvX significantly decreased both low and high VP-stimulated release of 6-keto-PGF1α (P ≤ 0.045) to levels similar to those of M aortas, whereas ERT of OvX-F rats restored both low and high VP-stimulated release of 6-keto-PGF1α by OvX + ER-F aortas to levels not different from those of Int-F aortas (Fig. 2; P > 0.05).

**Effects of TxA2 Inhibition on VP-Stimulated Prostanoid Release**

The TxA2 inhibitor Daz (50 μM) inhibited high-concentration VP-stimulated TxB2 release from M, Int-F, OvX-F, and OvX + ER-F rat aortas significantly (P ≤ 0.008) (Fig. 3). The inhibitory effect of Daz on TxB2 release in Int-F (79 ± 3%) and OvX + ER-F (77 ± 3%) did not differ significantly from M (74 ± 2%) or OvX-F (70 ± 9%) (P > 0.11). Interestingly, Daz did not decrease but, rather, significantly increased VP-stimulated release of 6-keto-PGF1α in all four experimental groups (P < 0.015) (Fig. 4). Daz increased 6-keto-PGF1α release to a similar extent (P = 0.12) in Int-F (66% ± 11%) and in the OvX + ER-F (42% ± 17%) groups, whereas it increased release substantially more (P ≤ 0.015) in the M (298% ± 52%) and OvX-F (178 ± 57%) groups.

**mRNA Expression of COX-1, COX-2, and TxA2 in Aorta and Effects of OvX and ERT on mRNA Expression**

RT-PCR measurements showed that COX-1 mRNA was expressed in both aortic Endo and VSM cells. There were no significant differences in COX-1 mRNA expression in M vs.
Int-F, either in Endo (0.203 ± 0.044 vs. 0.193 ± 0.043, P = 0.879) or in VSM (0.463 ± 0.113 vs. 0.440 ± 0.087, P = 0.879). OvX-F and ER therapy (OvX + ER-F) had no effect on COX-1 mRNA expression, either in aortic Endo or VSM (Figs. 5 and 6).

COX-2 mRNA was also expressed in both aortic Endo and VSM. In contrast to findings for COX-1, Int-F rat aortas expressed substantially more COX-2 mRNA in both Endo (0.360 ± 0.050 ± 0.040 and 0.052 ± 0.032, respectively). OvX markedly attenuated COX-2 mRNA expression by 79% in both Endo (0.077 ± 0.047, P = 0.031) and VSM (0.093 ± 0.018, P = 0.006) of OvX-F aortas to levels similar to those detected in M aortas. ERT of OvX-F rats restored COX-2 mRNA expression in both Endo (0.387 ± 0.018, P = 0.960) and VSM (0.363 ± 0.117, P = 0.69).

Fig. 3. High-dose (10⁻⁶ M) VP-stimulated release of TxB₂ by aortic rings from male, Int-F, OvX-F, and OvX + ER-F rats, in the presence of dazoxiben (Daz, 50 μM) or its vehicle-control (Veh). Bars represent means ± SE; n, no. of animals. **Among the 4 groups (male vs. Int-F vs. OvX-F vs. OvX + ER-F) and 2 experimental treatments (Veh vs. Daz), mean values for TxB₂ release without common script are significantly different (0.0001 ≤ P < 0.03). The effects of the 2 experimental treatments were measured in rings from each aorta.

Fig. 5. Representative RT-PCR gels for expression of cyclooxygenase (COX)-1, COX-2, thromboxane synthase (TxS), and thromboxane receptor (TP) mRNAs from aortic endothelium (Endo) and vascular smooth muscle (VSM) cells of male, Int-F, OvX-F, and OvX + ER-F rats. These data suggest that expression of mRNA for COX-2, TxS, and TP mRNA (but not COX-1) differed by sex and was altered by OvX and estrogen replacement, M, male; Fem, female; Est, OvX + ER (estrogen replacement); SM, smooth muscle.
Expression of TP mRNA in Aortic VSM Cells and Effects of OvX and ERT on TP Expression

The expression of TP mRNA in aortic VSM was measured by RT-PCR. TP mRNA levels were significantly higher in Int-F (0.51 ± 0.04) than in M (0.27 ± 0.03, P = 0.004) aortic VSM. OvX markedly attenuated TP mRNA expression in OvX-F aortic VSM (0.26 ± 0.04, P = 0.010), whereas ERT of OvX-F rats restored receptor mRNA expression in OvX + ER-F aortic VSM (0.37 ± 0.03, P = 0.102) to levels similar to those in Int-F aortic VSM (Figs. 5 and 9).

IHC of COX-1, COX-2, and Txs Enzymes and Effects of OvX and ERT on Enzyme Expression

IHC techniques provided a way to quantify COX-1, COX-2, and Txs protein expression in the aortic wall. Qualitatively, there was no stain observed on the Endo or VSM from the negative controls (=absence of primary antibodies). Very light expression (Fig. 10, and Table 2). In contrast, significantly more staining for COX-2 was observed in the VSM of the Int-F (2.9 ± 0.5) than in the M (1.6 ± 0.4, P = 0.041) group. OvX markedly attenuated COX-2 staining in VSM of the OvX-F (1.1 ± 0.1, P = 0.011) aortas, whereas ERT of OvX-F rats fully restored the staining for COX-2 in OvX + ER-F aortas (3.1 ± 0.5, P = 0.849) to levels similar to those in Int-F aortas (Fig. 10, and Table 2). Similar to the pattern observed for COX-2 protein expression, significantly more staining for Txs was observed in the VSM of the Int-F (2.8 ± 0.5) than in the M (1.4 ± 0.2, P = 0.031) group. OvX markedly attenuated Txs staining in the VSM of the OvX-F (1.2 ± 0.2, P = 0.017) group, whereas ERT restored the staining for Txs in the OvX + ER-F (2.05 ± 0.166, P = 0.247) group (Fig. 10, and Table 2).

DISCUSSION

In the present studies, OvX and ERT were employed to determine the role of estrogen in the regulation of basal and VP-stimulated release of constrictor prostanoids. The underlying molecular mechanisms of estrogen action on constrictor prostanoid pathway function were also determined by measuring the expression of COX-1, COX-2, Txs, and TP mRNA by RT-PCR and by estimating COX-1, COX-2, and Txs protein production by IHC. The major findings of this study reveal that...
endogenous estrogen is an important regulator of constrictor prostanoid function by the female rat aorta, which involves the potentiation of COX-2 and TxS message and protein expression in both Endo and VSM and upregulation of TP message in VSM of this blood vessel.

Male-Female Differences in Vascular Prostanoid Release and Effects of OvX and ERT on Female Aorta

The present study reveals that VP stimulated the release of TxA₂ and PGI₂ to a greater extent from female than from male rat aorta and that estrogen potentiates VP-stimulated prostanoid release in the female aorta. Previous studies support these findings, although most experiments have focused on the effects of estrogen-mediated increases in PGI₂ in the systemic or TxA₂ in the pulmonary vasculatures. For example, estrogen treatment enhances PGI₂ production in intact rat aorta (43) and in cultured rat aortic VSM (13) and ovine fetal pulmonary arterial endothelial cells (70). In the isolated, perfused rat lung, AA-induced production of PGI₂ and TxA₂, which reflects COX activity, varies during the course of the estrous cycle and peaks during the estrogen surge at proestrus (3). Similarly, the pretreatment of isolated, blood-perfused lungs of juvenile female
The present study also revealed that TxS mRNA is expressed in both male and female rats. Ovx attenuated and ERT restored the expression of both mRNA and protein. In contrast, estrogen had no effect on expression of COX-1 mRNA or protein in the female rat aorta. Previous cellular and molecular studies in cultured endothelial cells suggested that estrogen may regulate vascular reactivity by upregulation of COX-2 and prostaglandin (1, 62); however, the present and recent companion studies (51), involving measurements of vascular reactivity, prostaglandin release, and gene expression, are the first to establish an important physiological role for COX-2 function in the female systemic vascular wall and consistently support the idea that estrogen enhances prostaglandin-potentiated contractile responses to VP in female rat aorta by upregulating COX-2 activity.

Similarly, it was also demonstrated that estrogen enhances the expression of TxS mRNA and protein in the female aorta. Although TxS inhibition with Daz in previous studies failed to attenuate constrictor prostaglandin-potentiated vasoconstriction (23), as discussed in Male-Female Differences in Vascular Prostanoid Release and Effects of Ovx and ERT on Female Aorta, the inhibition of TxS will result in the accumulation of the upstream intermediate, PGH, which is also a vasoconstrictor and interacts with the same TP (PGH/Tx2) receptor as TXA2. Thus the failure of the TxS inhibitor to attenuate vasoconstriction establishes the involvement of PGH but does not exclude the involvement of TXA2 in potentiating contractile responses of the female aorta to VP. Indeed, PGH2 may be more important than TXA2 in activating the TP receptor in this system; however, it is difficult to determine their relative importance given the instability of PGH2 and the inability to measure its release from vascular tissues.

The present study also revealed that TxS mRNA is expressed in both Endo and VSM and that Ovx attenuated and ERT restored TxS mRNA and protein expression in both Endo and VSM. Although previous studies have already revealed that COX-2 mRNA and protein are expressed in cultured sheep with estradiol enhances the production of TXA2 (76). Although estrogen-induced increases in TXA2 production have been reported previously in rat endothelial cells (87), the present study is the first to establish the importance of endogenous estrogen in the regulation of vascular TXA2 production by the systemic vasculature of the female rat. TxA2 causes platelet aggregation and is a vasoconstrictor; in contrast, PGI2 inhibits platelet aggregation and is a vasodilator. The balance between TXA2 and PGI2 is known to be important in the regulation of vascular TXA2 production by the systemic vasculature of the female rat.

The present study also reveals the existence of a unique dynamic interrelationship between TXA2 and PGI2 synthesis within the prostaglandin biosynthesis pathway. In the presence of the TxS inhibitor Daz, VP-stimulated release of TXA2 was markedly attenuated, whereas the production of PG2 was increased. This finding reveals that inhibition of the TXA2 pathway and accumulation of the common upstream intermediate PGI2 enhances the production of PG2. The Daz-mediated reduction in TXA2 production may itself enhance PG2 synthase activity and the production of PG2. Indeed, there is evidence to suggest that TXA2 (via activation of protein kinase C) causes tyrosine nitration of PGI2 synthase, which attenuates the activity of this enzyme (19); thus Daz-mediated reductions in TXA2 production would be expected to enhance PGI2 synthase activity and the production of PG2, as observed in the present study. Since PGI2 is an important local vasodilator and inhibitor of platelet aggregation, these data imply that TxS inhibitors may be an effective therapy for constrictr prostanooid-mediated systemic vascular diseases not only by inhibiting the production of vasoconstrictor TXA2 but also by potentiating the production of vasodilator PGI2.

A related finding of interest is that Daz tremendously increased 6-keto-PGF1α release in M (298%) and Ovx+F (178%) aortas but only increased the release of this prostaglandin by 66% in Int-F and by 42% in Ovx+ER-F aortas, suggesting that estrogen may exert a regulatory effect on the dynamics between TXA2 and PGI2 biosynthesis pathways. The prolonged use of combined oral contraceptives is associated with decreased production of PGI2 (88), and estrogen decreases PGI2 production in both intact and castrated male rats (17). Thus the increased release of 6-keto-PGF1α in the presence of Daz, especially the remarkable increase in M and Ovx+F aortas, implies that a combined inhibitory effect of TXA2 and estrogen on PGI2 production may exist, perhaps the involving regulation of PGI2 synthase activity, so that in the presence of Daz and loss of estrogen, the PGI2 synthesis is dramatically increased.

**Male-Female Differences in Expression of COX and TxS mRNA and Protein and Effects of Ovx and ERT on Female Aorta**

In the present study, the expression of COX-2 mRNA and protein were higher in female than in male rat aorta. Ovx attenuated and ERT restored the expression of both mRNA and protein. In contrast, estrogen had no effect on expression of COX-1 mRNA or protein in the female rat aorta. Previous cellular and molecular studies in cultured endothelial cells suggested that estrogen may regulate vascular reactivity by upregulation of COX-2 and prostaglandin production (1, 62); however, the present and recent companion studies (51), involving measurements of vascular reactivity, prostaglandin release, and gene expression, are the first to establish an important physiological role for COX-2 function in the female systemic vascular wall and consistently support the idea that estrogen enhances prostaglandin-potentiated contractile responses to VP in female rat aorta by upregulating COX-2 activity.

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**Table 2. Immunohistochemical staining intensity for COX-1, COX-2, and TxS proteins in aortic VSM from male, Int-F, Ovx-F, and Ovx + ER-F rat aortas**

<table>
<thead>
<tr>
<th></th>
<th>InT-F</th>
<th>Male</th>
<th>Ovx-F</th>
<th>Ovx + ER-F</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td><strong>COX-1</strong></td>
<td>3.6 ± 0.1b</td>
<td>3.5 ± 0.2a</td>
<td>3.7 ± 0.2a</td>
<td>3.2 ± 0.3a</td>
</tr>
<tr>
<td><strong>COX-2</strong></td>
<td>2.9 ± 0.5b</td>
<td>1.6 ± 0.4a</td>
<td>1.1 ± 0.1a</td>
<td>3.1 ± 0.5a</td>
</tr>
<tr>
<td><strong>TxS</strong></td>
<td>2.8 ± 0.5b</td>
<td>1.4 ± 0.3a</td>
<td>1.2 ± 0.2a</td>
<td>2.1 ± 0.2b</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. Staining intensity is expressed on a relative scale of 1−4, as scored by a naive observer. a, b, c, d, e: Within rows for each specific protein (COX-1, COX-2, or TxS), mean values for each group (male vs. intact female (Int-F) vs. ovariectomized male (Ovx-F) vs. Ovx-F plus estrogen replaced female (Ovx+ER-F]) without common superscript are significantly different (P ≤ 0.041).
vascular Endo (60–62) and cultured VSM cells (60), the present study is the first to establish the expression of TxS mRNA and protein in both the Endo and VSM of Int-F systemic blood vessels. Previous studies reported the production of TxA2 from cultured vascular Endo but not VSM (26, 27); however, preliminary experiments in the present study revealed that TxA2 is released from both Endo + and Endo − (VSM) aortic rings (data not shown), which is consistent with the molecular findings that both COX-2 and TxS are expressed in both Endo and VSM cells of the female rat aorta.

Effects of OvX and ERT on Thromboxane Receptor Expression

Interestingly, the present study also established that TP mRNA is expressed in aortic VSM cells and that the expression is higher in female than in male aorta. Furthermore, OvX attenuates and ERT restores TP mRNA expression in VSM cells. These findings are consistent with the recent report that contractile responses to U-46619 are higher in female than in male aorta and are attenuated by OvX in the female aorta (51) and with the previous finding that the contractile responses to U-46619 in isolated rat lung are enhanced by estrogen (21). Thus the present findings reveal that estrogen potentiates contractile responses of the female aorta to VP not only by increasing constrictor prostanoid production (via upregulation of both message and protein for COX-2 and TxS) but also by enhancing the reactivity to TxA2 and PGH2 (via increased TP receptor expression).

Clinical Significance of the Present Findings

The findings of the present study are consistent with the extensive epidemiological data that reveal the existence of a prominent sexual dimorphism in the incidences of primary vascular diseases that involve excessive vasoconstriction. Thus primary pulmonary hypertension (PPH; 24, 84), migraine headache (5, 63), and Raynaud’s Disease (9, 29, 83) all occur in premenopausal women at rates as much as fourfold higher than in men. Furthermore, an association has been reported between Raynaud’s Disease and these other afflictions, suggesting that a common mechanism of vasospasm may be involved (6a). Elevated vascular production of TxA2 may be the common mediator of vasospasm in all of these diseases, since the production of this constrictor prostanoid is increased in patients with PPH (15), migraine headache (49), and Raynaud’s Disease (67). Animal studies have also revealed a similar relationship between TxA2 and excessive pulmonary vasoconstriction (11, 22). Furthermore, TxS inhibitors such as CGS-13080 and Daz have been used successfully in the treatment of PPH and Raynaud’s Disease (7, 65).

The higher incidences of peripheral vascular diseases in premenopausal women than in men suggest that estrogen and/or other ovarian steroids may be involved in the pathogenesis of primary vascular diseases in women. Indeed, the Heart and Estrogen-progestin Replacement Study (HERS; 33, 34, 38) and the more recent HERS II (28) reported no overall benefit of ERT and that untoward cardiovascular events, including venous thrombosis, actually increased significantly in the first year of the study. Similar deleterious effects of ERT on the incidences of coronary artery disease, stroke, and venous thrombosis were also reported in the more recent Women’s Health Initiative studies (54, 66). Furthermore, oral contraceptive use in young women is also associated with an increased risk of thrombosis (6, 20) and acute myocardial infarction (41). The increased production of and reactivity to TxA2 in the female systemic vasculature with ERT, as established in this and recent companion studies (51), may underlie these deleterious effects of estrogen on the cardiovascular system. Indeed, postmenopausal women undergoing ERT have elevated urinary levels of TxA2 (81). Interestingly, the present study demonstrated that estrogen upregulates the release of TxA2 by increasing the expression of message for COX-2 and TxS, as well as the COX-2 and TxS proteins. These findings raise the intriguing possibility that the deleterious effects of estrogen on vascular function and/or thrombosis, suggested by the earlier HERS (33, 34, 38) and more recent HERS II (28) and Women’s Health Initiative (54, 66) clinical studies, may involve the effect of estrogen to potentiate activity of TxA2 and constrictor prostanoid pathway function.

Although the rat aorta is a large conduit vessel not involved in the regulation of peripheral resistance, and its sensitivity to vasoconstrictor and vasodilator agonists, at least in vitro, is often much lower than smaller resistance-level vessels, it is well established that the rat aorta serves as a relevant model for the study of gonadal steroid effects on vascular function. Furthermore, many, if not all, of the male-female differences in vascular reactivity to vasoconstrictor agonists such as VP and phenylephrine identified in the rat aorta are qualitatively similar to those observed in peripheral microvascular preparations such as the rat mesenteric vasculature (75). The estrogen-dependent constrictor prostanoid mechanism identified in the present and recent companion studies (51) appears to be quite relevant to the regulation of systemic arterial blood pressure. Preliminary studies reveal that intravenous infusion of the TP receptor antagonist SQ-29548 into conscious normotensive rats reduces mean arterial blood pressure by 17% in female but has no effect in male; even more striking, the infusion of this antagonist into aortic coarctation-hypertensive rats reduces mean arterial blood pressure by 32% in female but only 17% in male (4) rats. Taken together, these data suggest that the estrogen-dependent constrictor prostanoid mechanism in the vascular wall may play an important role in the regulation of vascular tone in the female but not in the male rat, in both normal and pathophysiological states.

In conclusion, the present studies examined the mechanisms underlying the greater contractile response of the female rat aorta to VP and TxA2. The results reveal that VP stimulated a much greater release of TxA2 from female than from male aorta and that estrogen potentiated the release of TxA2 from the female aorta by upregulating mRNA and protein expression of COX-2 and TxS. Estrogen also upregulated TP mRNA expression in aortic VSM. These findings are fully consistent with recent companion studies of vascular function (51), which revealed that contractile responses to VP are attenuated by selective inhibition of COX-2 and by blockade of the TP receptor and that these effects are absent in vessels obtained from OvX-F rats but are restored in vessels from OvX-F rats following ERT. Taken together, the present and recent companion studies (51) demonstrate consistent effects of estrogen to enhance COX-2, TxS, and TP message and protein expression, the production of TxA2, and TP receptor-dependent...
potentiation of contractile responses to VP and TxA2/PGH$_2$ in the female rat aorta.

**Perspective**

In the normal physiological state, there are a number of male-female differences in cardiovascular function; for example, basal blood pressure (47, 86), production of NO (31, 44), and vascular reactivity to vasopressors such as phenylephrine, VP, and TxA2 (U-46619; Refs. 21, 23, and 42) all exhibit pronounced sexual dimorphism. Many but not all of these sex differences appear to result from the “protective” actions of estrogen on target tissues in the cardiovascular system, which result in lower vascular tone and blood pressure in the female than in the male. The results of this and recent companion studies (51) advance our knowledge of male-female differences in normal cardiovascular function and reveal a unique perspective on the physiological relevance of this presumably deleterious effect of estrogen on cardiovascular function, and how is it reconciled with the widely accepted dogma that estrogen upregulates the expression of eNOS and enhances the production of nitric oxide and other local prostanoid function through the upregulation of COX-2, TxS, and TP expression, thereby enhancing vascular tone and BP.

What is the physiological relevance of this presumably deleterious effect of estrogen on cardiovascular function, and how is it reconciled with the widely accepted dogma that estrogen plays a protective role in cardiovascular function? It is well known that estrogen upregulates the expression of eNOS and enhances the production of nitric oxide and other local vasodilators such as PGI$_2$ (13, 35, 48, 70, 85). Clearly, the balance between vasodilatation and vasoconstriction is crucial to the normal regulation of vascular tone and blood pressure in both sexes. Thus the presence of multiple, parallel, estrogen-sensitive vasodilator mechanisms in the female vasculature may necessitate a “backup” vasoconstrictor mechanism to counterregulate vascular tone and blood pressure and defend against hypotension. Indeed, the coexistence of eNOS and COX-2 in caveolin (52, 58, 72) provides intriguing suggestive evidence that estrogen plays a dual role in the regulation of local dilator and constrictor mechanisms important in the control of normal cardiovascular homeostasis in the female and that an imbalance among these mechanisms may underlie the pathogenesis of vascular diseases involving excessive vasoconstriction, which are more common in females than in males.

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