ESTROGEN POTENTIATES CONSTRICCTOR PROSTANOID FUNCTION IN FEMALE RAT AORTA BY UPREGULATION OF CYCLOOXYGENASE-2 AND THROMBOXANE PATHWAY EXPRESSION

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Running Head: Estrogen Upregulates COX-2--Thromboxane Pathway in Female Rat Aorta

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ABSTRACT

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 (AA) 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 TxA$_2$ and prostacyclin (PGI$_2$) were measured in male (M), intact female (InT-F), ovariectomized female (OvX-F) and OvX+17-β estradiol-replaced female (OvX+ER-F) rats. Expression of mRNA for 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. Expression of COX-1, COX-2, and TxS proteins by Endo and VSM was also estimated by immunohistochemistry (IHC). Basal release of TxA$_2$ and PGI$_2$ was similar in M (18.8±1.9, 1,723±153 pg/mg ring wt./45 min., respectively) and InT-F (20.2±4.2, 1,488±123 pg, respectively) rat aortas. VP stimulated dose-dependent release of TxA$_2$ and PGI$_2$ from both M and F rat aorta. OvX markedly attenuated and ER therapy restored VP-stimulated release of TxA$_2$ and PGI$_2$ in F rats. No differences in COX-1 mRNA levels were detected in either Endo or VSM of the four experimental groups ($P > 0.1$). 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 to M or OvX-F. Expression of TP mRNA was significantly higher in VSM of InT-F and OvX+ER-F, compared to M or OvX-F. IHC revealed 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. These data reveal that estrogen enhances constrictor
prostanoid function in F rat aorta by upregulating the expression of COX-2 and T$xS$ in both Endo and VSM and by upregulating the expression of TP in VSM.

**Keywords:** arginine vasopressin, cyclooxygenase, endothelium, thromboxane synthase, thromboxane receptor, vascular smooth muscle, vasoconstriction
INTRODUCTION

A SUBSTANTIAL SEXUAL DIMORPHISM exists in the vascular reactivity of the rat aorta and mesenteric vasculature to the systemic vasoconstrictor hormone vasopressin (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. 2,36,56,59,64) and endothelium (Endo; refs. 2,16,18) suggest 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 (NS) and the TXA₂/PGH₂ (TP) receptor antagonist SQ-29,548 (SQ) both attenuate contractile responses of the female rat aorta to VP to a similar extent (51). Further, 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 (232,51). Reactivity of the 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 (PGH$_2$ and TxA$_2$). This hypothesis is supported by the findings from recent companion studies that OvX abolished and ERT restored the inhibitory effect of NS and SQ 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 arachidonic acid (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+17-β estradiol-replaced female (OvX+ER-F) rat aortas. Thus, basal and VP-stimulated release of TxA$_2$ and prostacyclin (PGI$_2$) were first measured by radioimmunoassay (RIA). Second, 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, expression of COX-1, COX-2, and TxS proteins in aortic Endo and VSM was estimated by immunohistochemistry. The results reveal that estrogen enhances constrictor prostanoid function in the female rat aorta to VP 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 Labs, Inc, 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 (approx. 50%) and lighting cycle (12:12 L/D). 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 Tek-Lad, Houston, TX) 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 on vascular function (23,51). All experiments were reviewed and approved by the Texas A&M University Laboratory Animal Care Committee.

Animal Preparations. Animals were randomly separated into 4 experimental groups: males (M), intact-females (InT-F), ovariectomized females (OvX-F), and OvX+estrogen-replaced females (OvX+ER-F). Since previous studies established that neither contractile responses of the F rat aorta to VP or phenylephrine (PE; ref. 73), nor stimulation of prostanoid production by catechol estrogens (46) differ with phase of the estrous cycle, F rats were studied without regard to estrous cycle status. For each experimental group, sample sizes of 3-7 rats were used to account for known levels of statistical variability in prostanoid release and molecular experiments in vitro. Bilateral OvX of F rats was performed at 4-5 weeks age using standard methods. Half of the OvX-F rats received estrogen replacement therapy (ERT) beginning at 11-12 weeks
age for 21-28 days using 17β-estradiol (2 x 0.05mg-60 day release pellets, Innovative Research, Inc.; 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 sacrificed 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 radioimmunoassay (RIA) of plasma 17β-estradiol (see below). The thoracic aorta was rapidly but gently removed to avoid stretching or damaging the endothelium (Endo) and was placed in chilled (4°C), gassed (95% O₂/5% CO₂), Krebs-Henseleit-bicarbonate solution (KHB). The KHB was composed of (in mM): 118.0 NaCl, 25.0 NaHCO₃, 10.0 glucose, 4.74 KCl, 2.5 CaCl₂, 1.18 MgSO₄, and 1.18 KH₂PO₄ (pH 7.40; osmolality, 292 ± 1 mosmol/kgH₂O). The aorta was cleaned of all adipose and connective tissue, and the mid-thoracic region was cut into rings (3mm long) for prostanoid release experiments or kept intact for molecular experiments.

**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 minutes. The rings were then transferred into 12x75 mm plastic culture tubes with 2 ml chilled KHB, gradually warmed up to 37°C, and gassed continuously with 95% O₂/5% CO₂. After pre-incubation for 30 minutes at 37°C, the KHB solution was carefully aspirated, then 1.0ml of either KHB alone (basal) or KHB with agonists or inhibitors was added to the tissues, gassed continuously, and incubated for 45 minutes at 37°C. After incubation, the KHB was collected and stored in -70°C until radioimmunoassay of TxB₂ and 6-keto-PGF₁α.
For TxA\textsubscript{2} and PGI\textsubscript{2} release experiments, basal release reflected the steady-state release of the prostanoids into the incubation medium (KHB) during the 45 minute incubation period, and was normalized by dry weight of aortic rings and expressed as pg/mg 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 \times 10^{-8} M) or high (1 \times 10^{-6} M) concentration of VP, as used in previous contractile function experiments (51).

**RIA for TxB\textsubscript{2} and 6-keto-PGF\textsubscript{1\alpha}**. Concentrations of TxA\textsubscript{2} and PGI\textsubscript{2} in the KHB incubation media were each measured using specific RIAs for their stable metabolites TxB\text subscript{2} and 6-keto-prostaglandin F\textsubscript{1\alpha} (6-Keto-PGF\textsubscript{1\alpha}), respectively, as described in detail previously (75). Briefly, prostanoid standards (0.975-1,000 pg for TxB\textsubscript{2} and 1.95-1,000 pg for 6-Keto-PGF\textsubscript{1\alpha}) or unknown samples were incubated with [\textsuperscript{3}H]TxB\textsubscript{2} or [\textsuperscript{3}H]-6-keto-PGF\textsubscript{1\alpha} and with the appropriate prostanoid antiserum overnight at 4°C. The charcoal-dextran method was used to separate bound and free fractions of [\textsuperscript{3}H]TxB\textsubscript{2} or [\textsuperscript{3}H]-6-keto-PGF\textsubscript{1\alpha}. Bound radioactivity was counted by liquid scintillation spectroscopy. The limit of detection of the RIAs is 1.95 pg/tube for TxB\textsubscript{2} and 3.90 pg/tube for 6-Keto-PGF\textsubscript{1\alpha}; 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 PGH\textsubscript{2}/TxA\textsubscript{2} receptors**. Thoracic aortas from each of the four experimental groups, prepared as described above, 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 200µl chloroform, 5 µl glycogen (25 µg/ml; Ambion), 50 µl Na acetate (5 M), 500µl isopropyl 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 reverse transcription (RT) by reverse transcription polymerase chain reaction (RT-PCR), using 1.0 µg of specific antisense primers (Sigma-Aldrich, Genosys) and the Thermoscript RT-PCR system, (GibCoBrl, Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Two µl 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 10x PCR buffer without MgCl₂, 2 µl 10 mM dNTP, and 3 µl MgCl₂. After an initial 4 min denaturation step at 94°C, the thromboxane synthase (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; 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 primer for TxS were annealed for 60 s because of the longer length), and extension at 72°C for 30 s, followed by 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
laboratories, Hercules, CA, USA), and quantified by densitometry (Multi-analyst +
/Macintosh software, Bio-Rad laboratories). The PCR fragments were identified by size
using standard DNA (ϕX174RF DNA/Hae III Fragments, Life Technologies). The results
were expressed as the ratio of light intensity of a given mRNA controlled for the
expression of the housekeeping gene GAPDH amplified from the same tissues. The
negative control was performed under the same conditions without Taq polymerase.
The specific primers used were shown in Table 1 (8,32). The purity of Endo and VSM
preparations was assessed in preliminary experiments by probing for expression of
alpha smooth muscle actin and endothelial nitric oxide synthase (eNOS). Thus, in Endo
preparations, eNOS but not actin was detected, while in VSM preparations, actin but not
eNOS was detected, thereby confirming purity of Endo and VSM preparations.

**Immunohistochemistry (IHC) of COX-1, COX-2 and Txs.** Thoracic aortas, prepared
as described above, were perfusion-fixed with 10% neutral buffered formalin for 24
hours at a transmural pressure of ~80 mmHg, and then dehydrated with graded ethanol,
embedded in paraffin, and cut into 4-μm thick sections. After cleaning with xylene and
rehydration with graded alcohol, antigen was retrieved using the method of microwave
heating (71). Primary Txs, COX-1 or COX-2 antibody (Cayman Chemical, Ann Arbor,
Michigan) was then applied, and binding was detected using a biotinylated secondary
antibody (Cayman Chemical, Ann Arbor, Michigan) in combination with the avidin-biotin-
horseradish peroxidase method with 3,3'-diaminobenzidine (DAB, Vector Laboratories,
Inc., Burlingame, CA) as a chromagen. An adjacent section was used as a negative
control, and was treated with only secondary antibody without the first antibody.
Hematoxylin was used for the counterstain (11,30). The amount of positive IHC staining
of each specific protein was visualized under light microscopy (100X) and was scored on a scale of 1 (weakest) to 4 (strongest) by a naïve individual blinded to the sex or treatment of the experimental animal.

**Experimental Protocols**

*Male-female differences in prostanoid release by rat aorta and the role of estrogen in the regulation of the prostanoid biosynthesis pathway.* Aortic rings, prepared in triplicate from M, InT-F, OvX-F, or OvX+ER-F rats, were incubated in 1.0 ml of KHB either alone (basal) or with low-dose (10^{-8} M) VP or high-dose (10^{-6} M) VP, for 45 min. Additional groups of aortic rings prepared in duplicate from M, InT-F, OvX-F and OvX+ER-F rats were pre-incubated in KHB either alone or with the TxS inhibitor Dazoxiben (DAZ, 50 µM) for 30 min. The rings were then incubated (45 min) with KHB containing 10^{-6} M VP either alone, or in the presence of DAZ, respectively.

*Molecular mechanism(s) by which estrogen regulates constrictor prostanoid release in rat aorta.* Isolated aortic Endo and VSM cells from M, InT-F, OvX-F, and OvX+ER-F rats were prepared for analysis of mRNA levels of COX-1, COX-2, TxS and TP using RT-PCR methods. Aortic rings prepared from male, InT-F, OvX-F and OvX+ER-F rats were used for IHC determination of COX-1, COX-2, and TxS expression in aortic Endo and VSM.

**Chemical Reagents and Drugs.** The following reagents and drugs were used: arginine VP (Bachem; Torrance, CA); DAB (Vector Laboratories Inc., Burlingame, CA); DAZ (generously provided by Pfizer Pharmaceuticals; Kent, UK); Indo (Sigma; St. Louis, MO);
17β-estradiol (2 x 0.05mg, pellets, 60-day-release, Innovative Research of America, Sarasota, FL), and Trizol reagent (Life Technologies, Rockville, MO, USA). DAZ and Indo were prepared fresh daily, while VP was diluted daily from aliquots of 1 x 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-way or two-way ANOVA to detect differences among the means of the experimental groups. If significant differences were detected by ANOVA, pair-wise 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 treatment (basal vs. low dose VP vs. high dose VP) using two-way ANOVA and Student's t-tests. COX-1, COX-2, and TX-synthase 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. COX-1, COX-2, and TX-synthase IHC 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 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). 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 x 10⁻⁸ M) and high (1 x 10⁻⁶ M) concentrations of VP stimulated significantly 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 to 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-PGF$_{1\alpha}$ in a concentration-dependent manner and similar to the release of TxB$_2$, responses of InT-F were greater than M aortas. In M, low and high concentrations of VP increased the release of 6-keto-PGF$_{1\alpha}$ by 63% (2,811 ± 184 pg, $P = 0.002$) and 199% (5,155 ± 1,046 pg, $P = 0.012$), respectively, compared to basal. In contrast, in InT-F, low and high VP increased the release of 6-keto-PGF$_{1\alpha}$ by 159% (3,860 ± 800 pg, $P = 0.013$) and 750% (12,646 ± 2683 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 TxB$_2$ or 6-keto-PGF$_{1\alpha}$, Figs. 1 and 2). In contrast, OvX significantly decreased both low and high concentration VP-stimulated release of TxB$_2$ ($P \leq 0.011$) to levels similar to those of M aortas, while ERT of OvX-F rats restored both low- and high-concentrations VP-stimulated release of TxB$_2$ 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-PGF$_{1\alpha}$ ($P \leq 0.045$) to levels similar to those of M aortas, while ERT of OvX-F rats restored both low and high VP-stimulated release of 6-keto-PGF$_{1\alpha}$ by OvX+ER-F aortas to levels not different from those of InT-F aortas (Fig. 2; $P > 0.05$).

**Effects of TxS inhibition on VP-stimulated prostanoid release.** The TxS inhibitor DAZ (50 µM) inhibited high concentration VP-stimulated TxB$_2$ release from M, InT-F, OvX-F and OvX+ER-F rat aortas significantly ($P \leq 0.008$) (Fig. 3). The inhibitory effect of DAZ on TxB$_2$ 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-PGF$_{1\alpha}$ in all four experimental groups ($P < 0.015$) (Fig. 4). DAZ increased 6-keto-PGF$_{1\alpha}$ release to a similar extent ($P = 0.12$) in InT-F (66% ± 11%) and in OvX+ER-F (42% ± 17%), while it increased release substantially more ($P \leq 0.015$) in M (298% ± 52%) and in OvX-F (178 ± 57%).

mRNA Expression of COX-1, COX-2 and Tx Synthase 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 ± 0.1, $P = 0.046$) and VSM (0.450 ± 0.064, $P = 0.002$) compared to M (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. ERT of OvX-F rats restored COX-2 mRNA expression in both Endo (0.410 ± 0.071, $P = 0.705$) and VSM (0.333 ± 0.062, $P = 0.258$) in OvX+ER-F aortas, to levels similar to those in InT-F aortas (Figs. 5 and 7).
Similar to COX-2, TxS mRNA was expressed in both aortic Endo and VSM, and expression in InT-F aortas was significantly higher in both Endo (0.380 ± 0.123, \( P = 0.04 \)) and VSM (0.252 ± 0.015, \( P < 0.001 \)) than in M aortas (0.102 ± 0.063 and 0.090 ± 0.017, respectively). OvX markedly attenuated TxS mRNA expression by 78% in Endo (0.085 ± 0.072, \( P = 0.039 \)) and 72% in VSM (0.070 ± 0.010, \( P < 0.001 \)) of OvX-F aortas, to levels similar to those observed in M. ERT of OvX-F rats restored TxS mRNA expression in both Endo (0.387 ± 0.018, \( P = 0.960 \)) and VSM (0.363 ± 0.117, \( P = 0.316 \)) of OvX+ER-F aortas, to levels similar to those in InT-F aortas (Figs. 5 and 8).

*Expression of TP mRNA in Aortic VSM Cells and Effects of OvX and ERT on TP Expression*

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, \( P = 0.010 \)), while 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).
Immunohistochemistry of COX-1, COX-2 and Txs Enzymes and Effects of OvX and ERT on Enzyme Expression

Immunohistochemistry 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 and similar levels of staining for COX-1 were observed in the Endo of all four experimental groups (Fig. 10). The staining for COX-2 protein was more obvious in the Endo of InT-F and OvX+ER-F aortas than in M or OvX-F aortas. Similarly, staining for Txs was more pronounced in the Endo of InT-F and OvX+ER-F than in M or OvX-F aortas (Fig. 10). Since protein staining on the thin Endo layer could not be accurately scored, protein expression (staining) was quantitatively scored only in VSM. In VSM, there were no statistically significant differences in COX-1 staining between InT-F (3.6 ± 0.1) and M (3.5 ± 0.2), and OvX and ERT had no effects on COX-1 expression (Fig. 10, Table 2). In contrast, significantly more staining for COX-2 was observed in the VSM of InT-F (2.9 ± 0.5) than in M (1.6 ± 0.4, \( P = 0.041 \)). OvX markedly attenuated COX-2 staining in VSM of OvX-F (1.1 ± 0.1, \( P = 0.011 \)), while 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, Table 2). Similar to the pattern observed for COX-2 protein expression, significantly more staining for Txs was observed in the VSM of InT-F (2.8 ± 0.5) than in M (1.4 ± 0.2, \( P = 0.031 \)). OvX markedly attenuated Txs staining in VSM of OvX-F (1.2 ± 0.2, \( P = 0.017 \)), while ER restored the staining for Txs in OvX+ER-F (2.05 ± 0.166, \( P = 0.247 \)) (Fig. 10, 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 also were determined by measuring the expression of COX-1, COX-2, TxA S and TP mRNA by RT-PCR and by estimating COX-1, COX-2, and TxA S protein production by immunohistochemistry. The major findings of this study reveal that endogenous estrogen is an important regulator constrictor prostanoid function by the female rat aorta, which involves potentiation of COX-2 and TxA S message and protein expression in both endothelium 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 TxA 2 and PGI 2 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 PGI 2 in the systemic or TxA 2 in the pulmonary vasculatures. For example, estrogen treatment enhances PGI 2 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 PGI 2 and TxA 2, which reflects COX activity, varies during the course of the estrous cycle and peaks during the estrogen surge at proestrous (3). Similarly, pretreatment of isolated, blood-perfused lungs of juvenile
female sheep with estradiol enhances the production of TxA₂ (76). While 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 circulatory homeostasis (10,79) and an imbalance between these prostanoids is associated with the pathogenesis of cardiovascular diseases such as pulmonary hypertension and atherosclerosis (15,25,39,80). Indeed, injury-induced vascular proliferation and platelet activation are enhanced in mice that are genetically deficient in the PGI₂ receptor, but are depressed in mice genetically deficient in the TP receptor or treated with a TP receptor antagonist (14). The current dogma surrounding the literature states that PGI₂ is produced by the systemic vascular endothelium, while TxA₂ originates mainly from the platelets (10,12,55). Thus, physical and humoral interactions between the platelets and vascular wall are important determinants of the balance between TxA₂ and PGI₂ and the regulation of local vascular tone. However, the findings of this and recent companion studies of vascular function (51) now reveal the importance of the systemic vascular wall as a major source of constrictor prostanoids (TxA₂ and PGH₂) in the regulation of tone in the normal female vasculature.

The present study also reveals the existence of a unique dynamic interrelationship between TxA₂ and PGI₂ synthesis within the prostanoid biosynthesis pathway. In the
presence of the TxS inhibitor DAZ, VP-stimulated release of TxA₂ was markedly attenuated, whereas the production of PGI₂ was increased. This finding reveals that inhibition of the TxS pathway and accumulation of the common upstream intermediate PGH₂ enhances the production of PGI₂. The dazoxiben-mediated reduction in TxA₂ production may itself enhance PGI₂ synthase activity and the production of PGI₂. 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, dazoxiben-mediated reductions in TxA₂ production would be expected to enhance PGI₂ synthase activity and the production of PGI₂, 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 constrictor prostanoid-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 male (298%) and OvX-F (178%) aortas, but only increased the release of this prostanoid by 66% in InT-F and by 42% in OvX+Est-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 involving regulation of PGI$_2$ synthase activity, so that in the presence of DAZ and loss of estrogen, the PGI$_2$ 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 prostanoid production (1,62); however, the present and recent companion studies (51), involving measurements of vascular reactivity, prostanoid 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 prostanoid-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 prostanoid-potentiated vasoconstriction (23), as discussed above, the inhibition of Txs will result in the accumulation of the upstream intermediate, PGH$_2$, which is also a vasoconstrictor and interacts with the same TP (PGH$_2$/TxA$_2$) receptor as TxA$_2$. Thus, the failure of the Txs inhibitor to attenuate vasoconstriction establishes the involvement of PGH$_2$ 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 endothelium 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 vascular endothelium (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 endothelium and VSM of intact female systemic blood vessels. Previous studies reported the production of TxA₂ from cultured vascular endothelium 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 endothelium 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 expression is higher in female than in male aorta. Further, 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 which 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. Further, an association has been reported between Raynaud’s Disease and these other afflictions, suggesting that a common mechanism of vasospasm may be involved (77). 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 (498), and Raynaud’s Disease (67). Animal studies also have revealed a similar relationship between TxA2 and excessive pulmonary vasoconstriction (11,22). Further, TxS inhibitors such as CGS13080 and Dazoxiben 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 (WHI) 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 (AMI) (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 TxA, as well as the COX-2 and TxA 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 (WHI; 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-29,548 into conscious normotensive rats reduces mean arterial
blood pressure (MAP) by 15% in female but has no effect in male; even more striking,
infusion of this antagonist into aortic coarctation-hypertensive rats reduces MAP by 34%
in female but only 15% in male (4). 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, 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 TxA_2. The results reveal that VP
stimulated a much greater release of TxA_2 from female than from male aorta, and that
estrogen potentiated the release of TxA_2 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/PGH₂ 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. 21,23,42) 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 and unexpected deleterious effect of estrogen to potentiate constrictor 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 NO and other local vasodilators such as PGI₂ (13,35,48,70,85). Clearly, the balance between vasodilation 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.
ACKNOWLEDGEMENTS

We gratefully acknowledge the excellent technical assistance of Ms. Feng Xu.

This study was supported by the National Institutes of Health (NHLBI, HL-47432) and by an American Heart Association–Texas Affiliate Grant-In-Aid (both to J.N. Stallone).

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REFERENCES


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FIGURE LEGENDS

Fig. 1. Basal and VP-stimulated (low-dose, $10^{-8}$ M, or high-dose, $10^{-6}$ M) release of TxB$_2$ by aortic rings from male, intact female (InT-F), ovariectomized (OvX)-F, and OvX+estrogen-replaced (ER)-F rats. Bars represent means ± SE (n, no. of animals).

$^a$$^d$Among the four groups (male vs. InT-F vs. OvX-F vs. OvX+ER-F) and three experimental treatments (Basal vs. Low vs. High), mean values for TxB$_2$ release without common script are significantly different ($0.0001 < P < 0.05$). The effects of all three experimental treatments were measured in rings from each aorta.

Fig. 2. Basal and VP-stimulated (Low-dose, $10^{-8}$ M, or high-dose, $10^{-6}$ M) release of 6-keto-PGF$_{1\alpha}$ by aortic rings from male, InT-F, OvX-F, and OvX+ER-F rats. Bars represent means ± SE (n, no. of animals).

$^a$$^d$Among the four groups (male vs. InT-F vs. OvX-F vs. OvX+ER-F) and three experimental treatments (Basal vs. Low vs. High), mean values for 6-keto-PGF$_{1\alpha}$ release without common script are significantly different ($0.0001 < P < 0.05$). The effects of all three experimental treatments were measured in rings from each aorta.

Fig. 3. High-dose ($10^{-6}$ M) VP-stimulated release of TxB$_2$ 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).

$^{a-c}$Among the four groups (male vs. InT-F vs. OvX-F vs. OvX+ER-F) and two experimental treatments (Veh. vs. DAZ), mean values for TxB$_2$ release without common script are significantly different ($0.0001 < P < 0.03$). The effects of the two experimental treatments were measured in rings from each aorta.
Fig. 4. High-dose \((10^{-6} \text{ M})\) VP-stimulated release of 6-keto-PGF\(_{1\alpha}\) by aortic rings from male, InT-F, OvX-F, and OvX+ER-F rats, in the presence of DAZ (50\(\mu\)M) or its Veh. Bars represent means ± SE (n, no. of animals). a-c Among the four groups (male vs. InT-F vs. OvX-F vs. OvX+ER-F) and two treatments (Veh vs. DAZ), mean values without common script are significantly different (0.0001 < \(P\) < 0.033).

Fig. 5. Representative RT-PCR gels for expression of COX-1, COX-2, TxS and TP mRNAs from aortic endothelium (Endo) and vascular smooth muscle (VSM) cells of male, InT-F, OvX-F and OvX+ER-F rats. The first lane on each gel was the standard DNA ladder. bp, = size of RT-PCR products. 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.

Fig. 6. COX-1 mRNA expression in aortic Endo and VSM cells from male, InT-F, OvX-F and OvX+ER-F rats. Values are expressed as the ratio of COX-1 mRNA to GAPDH mRNA level obtained from the same tissues. Bars represent means ± SE (n, no. of animals). No statistically significant differences exist among the four experimental groups (male vs. InT-F vs. OvX-F vs. OvX+ER-F) in either Endo (\(P\) > 0.39) or VSM (\(P\) ≥ 0.69).

Fig. 7. COX-2 mRNA expression in aortic Endo and VSM cells from male, InT-F, OvX-F and OvX+ER-F rats. Values are expressed as the ratio of COX-2 mRNA to GAPDH mRNA levels obtained from the same tissues. Bars represent means ± SE (n, no. of animals). a-b Among the four experimental groups (male vs. InT-F vs. OvX-F vs. OvX+ER-F) and the two tissues (Endo vs. VSM), mean values without common script are significantly different (0.002 ≤ \(P\) ≤ 0.03).
**Fig. 8.** Thromboxane synthase (TxS) mRNA expression in aortic Endo and VSM cells from male, InT-F, OvX-F and OvX+ER-F rats. Values are expressed as the ratio of TxS mRNA to GAPDH mRNA levels obtained from the same tissues. Bars represent means ± SE (n, no. of animals). a-b Among the four experimental groups (male vs. InT-F vs. OvX-F vs. OvX+ER-F) and two tissues (Endo vs. VSM), mean values without common script are significantly different (0.0003 < P ≤ 0.04).

**Fig. 9.** PGH₂/TxA₂ receptor (TP) mRNA expression in aortic VSM from male, InT-F, OvX-F and OvX+ER-F rats. Values are expressed as the ratio of TP mRNA to GAPDH mRNA levels from the same tissues. Bars represent means ± SE (n, no. of animals). a-b Among the four experimental groups (InT-F vs. M vs. OvX-F vs. OvX+Est-F), mean values without common script are significantly different (0.008 < P < 0.019).

**Fig. 10.** Immunohistochemical staining for COX-1 (panel A), COX-2 (panel B) and TxS (panel C) protein expression in aortic Endo and VSM from male, InT-F, OvX-F and OvX+ER-F rats. Diaminobenzidine (DAB) stains the COX-1, COX-2, and TxS proteins brown. Control slides (panel D) were stained with same procedure but without primary antibodies for COX-1, COX-2, or TxS. Slides were counterstained with hematoxylin.
Table 1. Specific primers (sense and antisense) for COX-1, COX-2, Txs, TP and GAPDH were used for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide Primers</th>
<th>Size of PCR Product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>Sense: 5’CTCACAGTGCGGTCCAAC3’ Antisense: 5’CCAGCACCTGGTACTTAAG3’</td>
<td>424bp</td>
<td>8</td>
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<td></td>
<td>感性：5’CTCACAGTGCGGTCCAAC3’ 反义：5’CCAGCACCTGGTACTTAAG3’</td>
<td></td>
<td>45</td>
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<tr>
<td></td>
<td>Sense: 5’GAAATGGCTGCAGAGTTG3’ Antisense: 5’GGAATTCTCATCTAGTCTGGAGAGTTG3</td>
<td>356bp</td>
<td>8</td>
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<td>感性：5’GAAATGGCTGCAGAGTTG3’ 反义：5’GGAATTCTCATCTAGTCTGGAGAGTTG3</td>
<td></td>
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<tr>
<td>Txs</td>
<td>Sense: 5’TCCAGAGGTGTACTGCTGT3’ Antisense: 5’GAAGCATGACAAACATTTATTC3’</td>
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<td>感性：5’TCCAGAGGTGTACTGCTGT3’ 反义：5’GAAGCATGACAAACATTTATTC3’</td>
<td></td>
<td>78</td>
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<tr>
<td>TP</td>
<td>Sense: 5’TGCTGCAGACGCTACCTGTC3’ Antisense: 5’GATTGGCACCCTTCCTTCAGG3’</td>
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<tr>
<td>GAPDH</td>
<td>Sense: 5’GTGAAGGTCGTGTCACCGGATT3’ Antisense: 5’CACAGTCTTCTGAGTGCGAGTAT3’</td>
<td>558bp</td>
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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>Group</th>
<th>InT-F (4)</th>
<th>Male (5)</th>
<th>OvX-F (4)</th>
<th>OvX+ER-F (3)</th>
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<tbody>
<tr>
<td>COX-1</td>
<td>3.6±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.7±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>COX-2</td>
<td>2.9±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6±0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.1±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.1±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TxS</td>
<td>2.8±0.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.4±0.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.2±0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.1±0.2&lt;sup&gt;d&lt;/sup&gt;</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 naïve observer. <sup>a-e</sup>Within rows for each specific protein (COX-1, COX-2 or TxS), mean values for each group (male vs InT-F vs OvX-F vs OvX+ER-F) without common superscript are significantly different (0.011 < P < 0.041).
FIGURE 1

InT-F             Male             OvX-F        OvX+ER-F
(n=7)             (n=6)             (n=6)             (n=6)

Basal
Low-AVP
High-AVP

TxB2 (pg/mg ring wt./45 min)

abab a
b
bc
bd

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FIGURE 2

6-Keto-PGF₁α (pg/mg ring wt./45 min)

- Basal
- Low-AVP
- High-AVP

<table>
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<th>Condition</th>
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<th>Low-AVP</th>
<th>High-AVP</th>
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<tr>
<td>Male (n=6)</td>
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<tr>
<td>OvX-F (n=6)</td>
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<tr>
<td>OvX+ER-F (n=6)</td>
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</table>

a, b, c, d indicate statistically significant differences.
FIGURE 3

InT-F (n=7)  Male (n=5)  OvX-F (n=4)  OvX+ER-F (n=5)

Vehicle  DAZ (50 µM)

$T_xB_2$ (pg/mg ring wt./45 min)

- InT-F: 75
- Male: 50
- OvX-F: 25
- OvX+ER-F: 25

a, b, c indicate significant differences.
FIGURE 4

6-Keto-PGF1α (pg/mg ring wt./45 min)

- InT-F (n=7)
- Male (n=5)
- OvX-F (n=4)
- OvX+ER-F (n=5)

Vehicle
DAZ (50 µM)

Legend:
- a
- b
- c

Copyright Information
### FIGURE 5

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<th>Gene</th>
<th>Fem</th>
<th>Endo M</th>
<th>OvX</th>
<th>Est</th>
<th>SM Fem</th>
<th>M</th>
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</table>
FIGURE 6

% COX-1/GAPDH

- InT-F (n = 3)
- Male (n = 4)
- OvX-F (n = 4)
- OvX+ER-F (n = 3)

ENDO                      VSM
FIGURE 7

% COX-2/GAPDH

- InT-F (n = 3)
- Male (n = 4)
- OvX-F (n = 4)
- OvX+ER-F (n = 3)

ENDO VSM

Legend:
- a
- b

Copyright Information
FIGURE 8

- InT-F (n = 3)
- Male (n = 4)
- OvX-F (n = 4)
- OvX+ER-F (n = 3)

% TXS/GAPDH

ENDO

VSM

Copyright Information
FIGURE 9

- InT-F (n=3)
- Male (n=4)
- OvX-F (n=4)
- OvX+ER-F (n=3)

% TP/GAPDH

VSM
FIGURE 10

A

COX-1 Female

COX-1 Male

COX-1 Ovx-F

COX-1 Ovx+Est-F

B

COX-2 Female

COX-2 Male

COX-2 Ovx-F

COX-2 Ovx+Est-F
The graph illustrates the concentration of TxB2 (pg/mg ring wt./45 min) under different conditions and groups:

- **InT-F** (n=7)
- **Male** (n=6)
- **OvX-F** (n=6)
- **OvX+ER-F** (n=6)

The y-axis represents TxB2 concentration, and the x-axis represents the different groups. The bars indicate the mean concentration with standard error of the mean (SEM). Different letters above the bars indicate significant differences between groups. The legend specifies the conditions:

- **Basal**
- **Low-AVP**
- **High-AVP**

The graph shows that the concentration of TxB2 varies significantly across these conditions and groups.