Endothelin receptors are modulated in association with endogenous fluctuations in estrogen

DUSTAN A. BARBER, GARY C. SIECK, LORRAINE A. FITZPATRICK, AND VIRGINIA M. MILLER
Departments of Surgery, Anesthesiology, and Medicine, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Barber, Dustan A., Gary C. Sieck, Lorraine A. Fitzpatrick, and Virginia M. Miller. Endothelin receptors are modulated in association with endogenous fluctuations in estrogen. *Am. J. Physiol.* 271 (*Heart Circ. Physiol.* 40): H1999–H2006, 1996.—Experiments were designed to determine whether endogenous physiological fluctuations in sex steroid hormones affect expression or functional responses of endothelin receptors. Coronary arteries from sexually mature male, female, and ovariectomized pigs were prepared either for receptor binding or measurement of isometric force in organ chambers. Competitive binding of ^{125}I-labeled endothelin-1 was significant for a one-site model with unlabeled endothelin-1 and a two-site model with unlabeled endothelin-3 and sarafotoxin S6c in all pigs. The total number of binding sites for all endothelin ligands was not different between male and female pigs. Binding affinities for the high-affinity binding site for both endothelin-3 and sarafotoxin S6c were significantly greater (lower inhibition constant) in membranes prepared from female pigs with high endogenous estrogen. In organ chamber experiments, contractions to endothelin-1 but not endothelin-3 or sarafotoxin S6c were significantly greater in coronary arterial rings from female compared with male pigs and were not affected significantly by removal of the endothelium or by treatment of the rings with either indomethacin (10^{-5} mol/l) or the combination of indomethacin and N^{G}-monomethyl-L-arginine (10^{-4} mol/l). These results suggest endogenous fluctuations in estrogen are associated with an increase in affinity of a high-affinity endothelin receptor in coronary arterial smooth muscle of female pigs. In addition, independent of endogenous estrogen status, coronary arteries from female pigs generate significantly greater contractions to endothelin-1 compared with male pigs. This phenomenon occurs at the level of smooth muscle and is not dependent on the endothelium or synthesis of nitric oxide or prostaglandins.

17β-estradiol; sex steroids; sarafotoxin S6c; endothelin-3; coronary artery

SEX STEROID HORMONES regulate production and response to endothelium-derived factors, such as metabolites of arachidonic acid and nitric oxide (1, 5, 18, 19). Sex steroid hormones may regulate production of the endothelins. For example, plasma endothelin levels are decreased in male-to-female transsexuals and increased in female-to-male transsexuals (21). Little is known about how responses to endothelins are regulated by endogenous fluctuations in sex steroid hormones.

The endothelins may contribute to the development and/or progression of coronary artery disease. These peptides are endogenous coronary spasmogens (10), and their plasma levels are elevated in patients with symptomatic atherosclerosis (15). Furthermore, endothelin-1 acts synergistically with other mitogens (3, 9) to increase proliferation of smooth muscle.

Micromolar concentrations of 17β-estradiol added acutely to isolated coronary arteries of male and female rabbits reduce contractions to endothelin-1 (11). However, exogenous, acute administration of sex steroid hormones to isolated blood vessels does not address the question of whether there are changes in vascular function associated with the ovulatory cycle when estrogen and other sex steroid hormones fluctuate in a coordinated manner. Identifying differences in responses to endothelium-derived factors during the normal ovulatory cycle (i.e., premenopausal) is critical to understanding differences in the development of vascular disease between genders. Experiments were designed to determine whether gender and/or physiological fluctuations in endogenous sex steroid hormones affect expression of endothelin-receptors or functional responses to endothelins in porcine coronary arteries.

METHODS

Sexually mature pigs were chosen for study because the estrus cycle in pigs occurs monthly and the hormonal profile is analogous to women. At no time were either pigs or isolated preparations treated with exogenous sex steroid hormones. Animals were grouped according to gender and endogenous plasma sex steroid hormone levels when killed. Sexually mature, gonadally intact male (114 ± 7 kg, $n = 21$) and female (86 ± 3 kg, $n = 45$) or ovariectomized female (79 ± 2 kg, $n = 6$; 4 wk previously) (2) Yorkshire pigs were anesthetized with ketamine-xylazine-butorphanol (30:6:0.3 mg/kg, respectively, intramuscularly). Blood samples were collected from the femoral artery and analyzed for plasma 17β-estradiol, progesterone, and testosterone by radioimmunoassay at the Clinical Steroid Laboratory of Mayo Medical Laboratories. The detection limit for plasma sex steroid hormones was 0.04 pg/ml for progesterone and 10 pg/ml for estrogen and testosterone. Plasma lipid levels were assayed by Mayo Medical Laboratories. Plasma concentrations of endothelin-1 were determined by radioimmunoassay (Amersham International; Amersham, UK) as previously described (17). Hearts were removed and immediately placed in ice-cold modified Krebs-Ringer bicarbonate solution (control solution in mmol/l, 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 0.026 calcium disodium edetate, 11.1 glucose, pH 7.4) and aerated with 95% oxygen-5% carbon dioxide. The right and left circumflex and the left anterior descending coronary arteries were excised and prepared for study in organ chambers (right circumflex only) or frozen for isolation of membrane proteins. Animal care was conducted in accordance with both the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" [Department of Human Services Publication No. (NIH) 86–23, Revised 1985].

Membrane preparation. Membranes were isolated from each artery separately (right circumflex, left circumflex, and left anterior descending coronary arteries without endothelium). Frozen arteries were pulverized in liquid nitrogen and twice homogenized in 10 volumes of ice-cold membrane buffer [in mmol/l, 25 sucrose, 3 $MgCl_2$, 1 EDTA, 0.5 phenylmethylsulfonyl, 50 tris(hydroxymethyl)aminomethane (Tris)·HCl, pH 7.4] for 10 s using a Tekmar tissue homogenizer (Tekmar, Cincinnati, OH) at full speed. The homogenate was centrifuged at 2,000 *g* for 15 min at 4°C. The supernatant was collected and filtered through a 212-μm nylon screen and set aside on ice. The pellet was resuspended in 2-ml membrane buffer and homogenized with the Tekmar for 10 s at full speed. The homogenate was centrifuged at 2,000 *g* for 15 min at 4°C, and the resulting supernatant was added to the initial supernatant after passing through a 212-μm nylon screen. The pellet was discarded. The combined supernatant was then homogenized with the Tekmar at speed 60 for 30 s. The solution was centrifuged at 40,000 *g* for 30 min at 4°C, and the supernatant was discarded. The pellet was resuspended in 575-μl membrane buffer and disrupted using a sonic dismembrator (Fisher, model 300; Pittsburgh, PA) for ~15 s. A 50-μl aliquot was immediately collected for use in determining protein concentration using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as standard. The remaining membrane suspension was immediately aliquoted (5 × 100 μl) and stored at −76°C for up to 3 wk before diluting for use in binding experiments.

Receptor binding assay. Experiments were carried out in a final volume of 200 μl using binding buffer (50 mmol/l Tris·HCl, 0.4% bovine serum albumin, 0.1% bacitracin, 0.5 mmol/l phenylmethylsulfonyl, 5 mmol/l $MgCl_2$, 25 mmol/l $MnCl_2$, 1 mmol/l EDTA, pH 7.4). Bound and free ligands were separated using a Skatron cell harvester (Skatron Instruments; Sterling, VA). With this device, the contents of the reaction chamber are rapidly rinsed (50 mmol/l Tris·HCl buffer, pH 7.4) and immediately vacuum filtered through a 1-μm retention glass filter mat. Radioactivity retained on the filters was counted on a gamma counter (Beckman Gamma 4000; Fullerton, CA). The specific activity of label (^{125}I-labeled endothelin-1) was 2,000 Ci/nmol and was diluted so that 50 μl yielded 20,000 counts/min (23 pmol/l, final concentration). An incubation time of 3 h at 25°C was determined in preliminary studies to result in equilibrium for ^{125}I-endothelin-1 binding (data not shown). To determine a suitable membrane concentration for the binding studies, increasing concentrations of membranes (1–20 μg) were incubated with ^{125}I-endothelin-1. From these data 2 μg of membrane protein were selected for subsequent studies because this concentration was on the linear portion of the membrane concentration vs. maximal ^{125}I-endothelin-1 binding curve for membranes from both male and female pigs for all three types of coronary arteries.

Total ^{125}I-endothelin-1 binding was determined in saturation studies by incubating membranes (2 μg) with increasing concentrations of ^{125}I-endothelin-1 (0–200 pmol/l). Nonspecific binding was determined in parallel incubations containing excess unlabeled endothelin-1 (1 μmol/l). Specific binding was calculated as the difference between total binding and nonspecific binding.

Competition binding experiments were performed with membrane (2 μg) and ^{125}I-endothelin-1 (23 pmol/l) plus increasing concentrations (0–10^{-7} mol/l) of unlabeled endothelin-1, endothelin-3, or sarafotoxin S6c. Nonspecific binding was determined from nonlinear curve fitting as described in *Data analysis* but was originally estimated in parallel incubations containing the same without competitor plus 1 μmol/l unlabeled endothelin-1. Binding profiles from membranes of all three types of coronary arteries (i.e., left and right circumflex and left anterior descending) were the same. Therefore, results from three arteries from a single animal were averaged to yield a single binding profile.

Organ chamber studies. The right coronary artery was cut into rings, half of which were mechanically denuded of endothelium by gently rolling with watchman's forceps. Removal of the endothelium was confirmed functionally by the absence of relaxation to bradykinin (10^{-7} mol/l) or UK-14304 (10^{-6} mol/l). Pairs of rings with and without endothelium were suspended between a fixed stirrup and force transducer for measurement of isometric force in 25-ml organ chambers filled with control solution.

Each ring was stretched to the optimal point on its length-tension curve as determined by tension developed to 20 mmol/l KCl at each level of stretch. Once the optimal tension was set, 60 mmol/l KCl were added to each bath to determine maximal response to KCl. To some of the baths either indomethacin (10 μmol/l; dissolved in sodium carbonate; final bath concentration sodium carbonate 20 μmol/l), N^G-monomethyl-L-arginine (L-NMMA, 100 μmol/l), or both were added 45 min before administration of various endothelin agonists. All rings were contracted with prostaglandin $F_{2\alpha}$ (2×10^{-6} M) and relaxed with cumulative doses of either isoproterenol (10^{-9} to 10^{-7} mol/l), the α_2-agonist UK-14304 (10^{-9} to 10^{-6} mol/l), bradykinin (10^{-10} to 10^{-7} mol/l), or C-type natriuretic peptide (10^{-9} to 10^{-7} mol/l). Responses to these vasodilatory agents were part of a related study and are not reported here. Cumulative concentration-response curves to either endothelin-1, endothelin-3, or sarafotoxin S6c (10^{-11} to 10^{-7} mol/l) were obtained. In preliminary experiments ($n = 3$ each sex), contractions to endothelin-1 reached a maximum at 10^{-7} mol/l and diminished at higher concentrations of endothelin-1.

Histology. Coronary arteries were placed in 10% formaldehyde for a minimum of 24 h before paraffin embedding and sectioning (6 μm). Sections mounted on silanized slides were stained with hematoxylin and eosin and examined by light microscopy.

Data analysis. All data are expressed as means ± SE; n equals the number of pigs from which arteries were taken. Saturation and competition binding data were analyzed using the mean values for each group with the nonlinear curve-fitting program Prism (GraphPad Software; San Diego, CA). Saturation experiments were transformed into Scatchard plots for display but were analyzed with nonlinear regression. ^{125}I-endothelin-1 binding in the absence of competitor was normalized to 100% for all inhibition curves. Single vs. two-site models were selected with the residual variance of an F test, whereby a one-site model was deemed best unless the P value was less than the critical value of $P < 0.05$. The maximal number of receptors (B_{max}) and inhibition constants (K_i) were compared using the asymptotic 95% confidence intervals of the values determined from the nonlinear curve fit. Nonoverlapping 95% confidence intervals were taken to indicate a significant difference among groups.

For organ chamber studies, data are expressed as absolute increase in grams of tension. Maximal tensions, area under the tension curve, and concentrations producing half-maximal contraction (EC_{50}) were calculated for individual concentration-response curves, and the means of these values were compared with one-way analysis of variance (ANOVA). If a significant F value was obtained, a Bonferroni post hoc test was used to compare means while controlling for multiple comparisons. Plasma hormone levels were sometimes below detectable limits and, therefore, these data were analyzed by

Table 1. *Plasma sex steroid hormones, endothelin-1, and lipid levels in male and female pigs*

	High-Estrogen Females ($n = 15$)	Low-Estrogen Females ($n = 30$)	Ovariectomized ($n = 6$)	All Females ($n = 51$)	Males ($n = 21$)
Estrogen, pg/ml	19 ± 2*	5 ± 0*	5 ± 0*	9 ± 1*	31 ± 5
Progesterone, pg/ml	1.5 ± 1.5	4.5 ± 2.2	0.4 ± 0.3	3.4 ± 1.4	0.2 ± 0.1
Testosterone, pg/ml	7 ± 1*	6 ± 1*	NA	6 ± 1*	363 ± 120
Endothelin-1, pg/ml	13.3 ± 1.7	15.0 ± 0.9	NA	14.9 ± 1.0	12.6 ± 0.9
Cholesterol, mg/dl	85 ± 4*	78 ± 3*	78 ± 4*	79 ± 2*	59 ± 17
LDL, mg/dl	43 ± 5	43 ± 3	43 ± 7	43 ± 3	37 ± 4
HDL, mg/dl	35 ⊥ 4	30 ± 2	36 ± 2	32 ± 1	27 ± 1
Triglycerides, mg/dl	19 ± 3	16 ± 2	19 ± 3	17 ± 1	17 ± 4

Values are means ± SE; n = no. pigs. Means of sex steroids are reported by substituting the midpoint between the minimal detectable level and zero as the value for those animals with hormone levels below the detection limit. Sex steroid hormones were compared with nonparametric rank sum analysis with a Kruskal-Wallis 1-way analysis of variance (ANOVA). All other means were compared with a 1-way ANOVA followed by a Bonferroni's post hoc test. LDL and HDL, low- and high-density lipoprotein; NA, not available. *$P < 0.05$ compared with male pigs.

nonparametric rank sum analysis with a Kruskal-Wallis one-way ANOVA. The plasma steroid means are reported by substituting the midpoint between the minimal detectable level and zero as the value for those pigs with an undetectable level of a given hormone. Plasma lipids and endothelin-1 levels were compared by a one-way ANOVA followed by Bonferroni post hoc test. For all statistical tests, only those differences with a P value of <0.05 were considered significant.

Drugs and chemicals. Porcine endothelin-1 and porcine endothelin-3 were from Peptides International (Louisville, KY). Sarafotoxin S6c was from Phoenix Pharmaceuticals (Mountain View, CA). Porcine (3-[^{125}I]iodotyrosyl) endothelin-1 and radioimmunoassay kits for plasma endothelin-1 were from Amersham (Arlington Heights, IL). L-NMMA was from Calbiochem (La Jolla, CA). All other reagents were from Sigma Chemical (St. Louis, MO). All concentrations are expressed as the final molar (mol/l) concentration in the organ bath or incubation solution.

RESULTS

Blood chemistry and histology. Gonadally intact female pigs exhibited cyclic variation in hormonal status as observed by external examination of the genitalia and direct measure of plasma estrogen. Female pigs were grouped according to plasma 17β-estradiol as either low (<10 pg/ml) or high (>10 pg/ml) at the time of the experiments. 17β-Estradiol was below the detection limit of the assay in all ovariectomized pigs. Male pigs had significantly higher estrogen levels compared with all female groups. Progesterone levels were not significantly different among any group of pigs. Testosterone levels were significantly higher in males compared with each group of females. Total cholesterol was significantly lower in males compared with all female groups. There were no significant differences in plasma low-density lipoprotein, high-density lipoprotein, or triglycerides among groups. Plasma endothelin-1 levels were not significantly different among any of the pig groups (Table 1). There was no consistent evidence of atherosclerosis in histological sections of arteries from either sex.

Receptor binding assays. Binding of ^{125}I-endothelin-1 as a function of membrane protein was linear between 1 and 10 μg protein for all three coronary arteries from male and female pigs (Fig. 1*A*). Total ^{125}I-endothelin-1 binding as a function of membrane concentration was not different among sexes (Fig. 1*B*).

Nonlinear regression analysis of the saturation binding isotherms demonstrated that total ^{125}I-endothelin-1 binding sites (B_{max}) and affinities (dissociation constant, K_d) were not different between membranes prepared from coronary arteries from male and female pigs

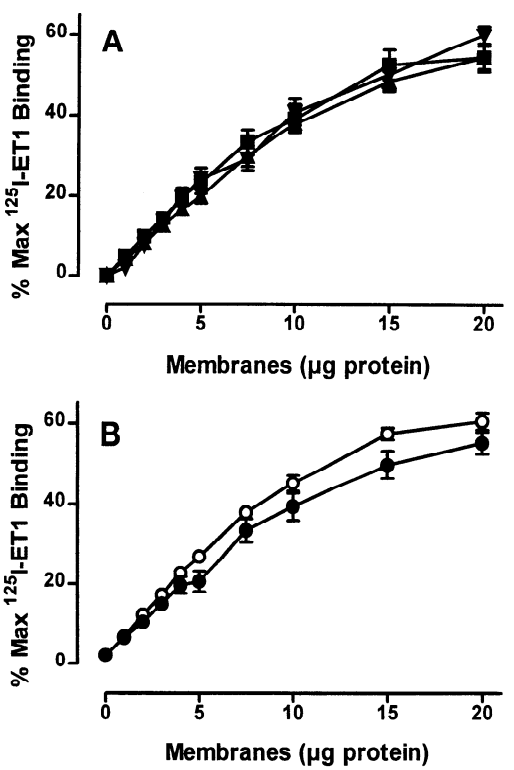

Fig. 1. *A*: binding of ^{125}I-endothelin-1 as a function of the protein concentration of membranes prepared from left circumflex arteries (■, $n = 9$), right circumflex arteries (▼, $n = 4$), or left anterior descending coronary arteries (▲, $n = 7$) without endothelium from gonadally intact male and female pigs. Binding was linear between 1 and 10 μg protein and was not significantly different among membranes prepared from the 3 different types of coronary arteries. *B*: binding from membranes prepared from the 3 different types of coronary arteries was averaged to yield a single value for each pig and divided according to sex: male (○, $n = 5$) and female (●, $n = 8$). Binding was not significantly different between sexes, particularly in the linear portion of the curve.

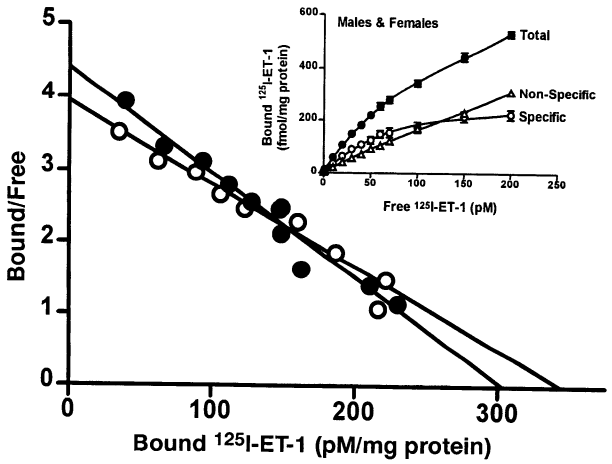

Fig. 2. Scatchard analysis of saturation binding isotherms (*inset*) of mean binding for membranes prepared from coronary arteries without endothelium of gonadally intact male (●, $n = 4$) and female (○, $n = 4$) pigs. Membranes were incubated with increasing concentrations of ^{125}I-endothelin-1 for 3 h in the absence (total binding) and presence (nonspecific binding) of 1 μmol/l unlabeled endothelin-1. Specific binding was defined as the difference between total binding and nonspecific binding. Nonlinear regression of the saturation data presented here in Scatchard form yielded a 1-site binding model with a maximal number of binding receptors (B_{max}) of 312 and 303 fmol/mg protein and a dissociation constant of 72 and 70 pmol/l for membranes prepared from male and female pig arteries, respectively. There was no statistically significant difference in these values between sexes.

(Fig. 2). Nonlinear regression of the saturation data for the statistically preferred one-site model produced a B_{max} of 312 and 303 fmol/mg protein and a K_d of 72 and 70 pmol/l for males and females, respectively.

Binding of ^{125}I-endothelin-1 was inhibited in a concentration-dependent manner by unlabeled endothelin-1, endothelin-3, and sarafotoxin S6c (Fig. 3). Nonlinear regression analysis of the curves was significant for a one-site model for endothelin-1, a two-site model for endothelin-3, and sarafotoxin S6c for all arteries. The total number of binding sites (B_{max}) for endothelin-1 did not significantly differ among groups (Table 2). Similarly, the percentage of receptors occupied by each of the two binding sites for both endothelin-3 and sarafotoxin S6c did not differ significantly among high-estrogen females, low-estrogen females, males, or ovariectomized females (Table 2). Affinity for inhibition of ^{125}I-endothelin-1 binding by unlabeled endothelin-1 (K_i) did not differ significantly among males, high-estrogen females, low-estrogen females, or ovariectomized females (Table 3). For both endothelin-3 and sarafotoxin S6c, the high-affinity K_i was significantly lower for membranes from high-estrogen females compared with low-estrogen females, males, or ovariectomized females (Table 3). However, the low-affinity K_i for both endothelin-3 and sarafotoxin S6c was not different among high-estrogen females, low-estrogen females, males, or ovariectomized females (Table 3).

Organ chamber experiments. Contractions to potassium chloride (60 mmol/l) in arteries with or without endothelium were not significantly different among arteries from males, high-estrogen females, low-estrogen females, or ovariectomized females (Fig. 4).

Endothelin-1 caused concentration-dependent increases in tension that did not differ significantly between rings with and without endothelium in arteries from male or female pigs. Maximal contractions to endothelin-1 were significantly greater in rings with and without endothelium from all female pigs, including ovariectomized, compared with arteries from male pigs (Table 4 and Fig. 5*A*). Contractions of rings with or without endothelium to endothelin-1 were not affected significantly by either indomethacin (10 μmol/l) or the combination of indomethacin (10 μmol/l) and L-NMMA (100 μmol/l) (Table 4).

Differences in maximal tension elicited by endothelin-3 in arteries without endothelium did not reach statistical significance for arteries from male (8.39 ± 1.6 g), high-estrogen female (13.6 ± 2.3 g), low-estrogen female (10.4 ± 2.5 g), or all female pigs combined

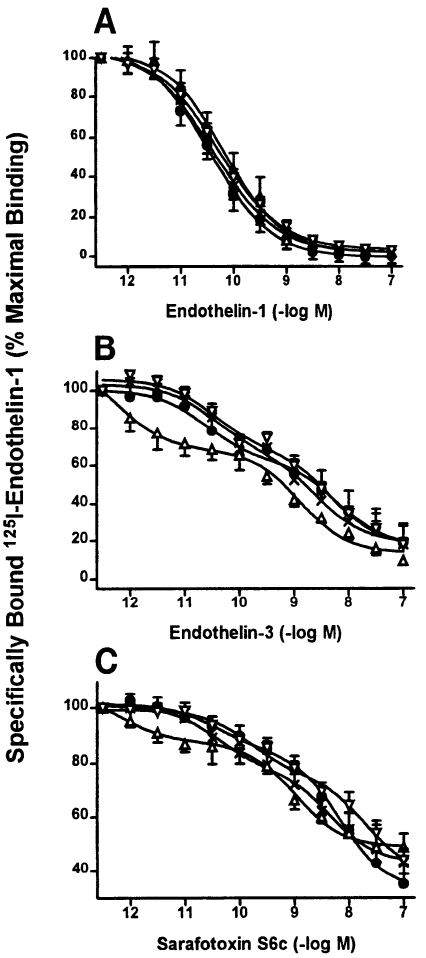

Fig. 3. Competitive inhibition of ^{125}I-endothelin-1 (23 pmol/l) binding by unlabeled ligands in membranes (2 μg) prepared from coronary arteries taken from male pigs (●, $n = 11$), low-estrogen female pigs (▽, $n = 16$), high-estrogen female pigs (△, $n = 5$), or ovariectomized pigs (×, $n = 5$). Nonlinear regression analysis for ^{125}I-endothelin-1 inhibition by endothelin-1 (*A*) was significant for a 1-site binding model. Inhibition of ^{125}I-endothelin-1 by either endothelin-3 (*B*) or sarafotoxin S6c (*C*) was significant for a 2-site binding model. In membranes from the high-estrogen females, the inhibition constant for the high-affinity binding site for both endothelin-3 and sarafotoxin S6c was significantly lower compared with all other groups. B_{max} was not different among any group for each of the 3 competing ligands.

Table 2. *Number of binding sites in membranes prepared from porcine coronary arterial smooth muscle*

	n	Endothelin-1, fmol/mg protein	Endothelin-3, High-Affinity Site, %Occupied	Sarafotoxin S6c, High-Affinity Site, %Occupied
Females				
High-estrogen females	5	310.8 ± 92.6	37.49 ± 7.81	25.44 ± 8.99
Low-estrogen females	16	341.5 ± 104.8	41.87 ± 14.17	35.40 ± 9.65
Ovariectomized	5	344.2 ± 89.0	42.08 ± 12.09	37.75 ± 7.14
Males	11	234.0 ± 68.5	45.67 ± 7.97	24.56 ± 6.40

Values were determined from nonlinear regression analysis of the mean values from competitive binding of ^{125}I-endothelin-1 with unlabeled endothelin-1, endothelin-3, and sarafotoxin S6c (Fig. 3) and are ± the 95% confidence interval of the regression fit; *n*, no. of animals used to construct the mean displacement curve for the analysis.

(11.6 ± 1.8 g; Fig. 5*B*). Increases in tension elicited by the endothelin B receptor-specific agonist sarafotoxin S6c were not significantly different among coronary arteries without endothelium obtained from male (10.1 ± 2.5 g), high-estrogen female (10.0 ± 2.0 g), low-estrogen female (12.4 ± 2.1 g), ovariectomized (9.5 ± 2.7 g), or all female pigs combined (11.7 ± 1.3 g; Fig. 5*C*). In all groups and treatments studied, EC_{50} did not differ significantly between arteries for all agonists (endothelin-1, endothelin-3, or sarafotoxin S6c) among all sex groups (Table 4).

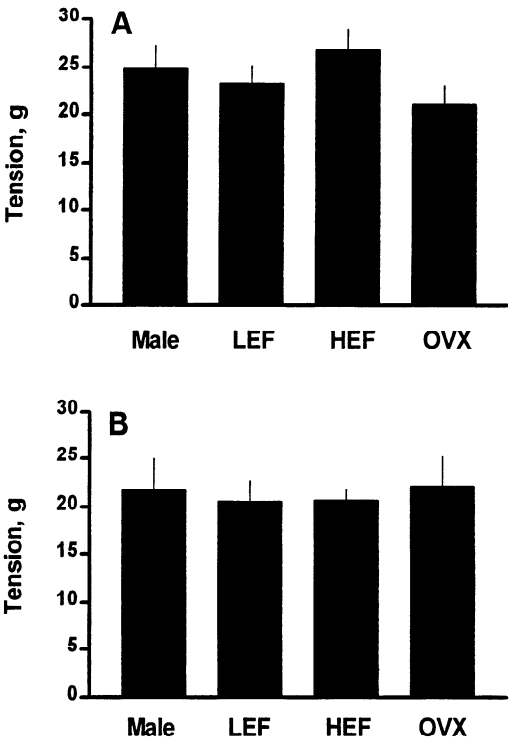

Fig. 4. Increases in tension to 60 mmol/l potassium chloride in coronary arterial rings with (*A*) or without (*B*) endothelium. For each animal, responses from 8 rings studied in parallel were averaged to yield a single value that was then used to calculate the group means. All values represent the group means ± SE. There was no significant difference among any of the groups. Males (n = 8), low-estrogen females (LEF, n = 11), high-estrogen females (HEF, n = 6), and ovariectomized (OVX, n = 5).

DISCUSSION

The major finding of this study is that the affinity of a subpopulation of high-affinity endothelin receptors changes in coronary arterial smooth muscle in association with fluctuations in endogenous estrogen in gonadally intact female pigs. This change in affinity of the endothelin receptor may not be due only to fluctuations in plasma 17β-estradiol because *1*) male pigs in this study had significantly higher endogenous plasma estrogen levels compared with all female groups (a phenomenon likely due to aromatase within the adipose tissue of male pigs) and *2*) affinity of endothelin-binding in coronary arterial smooth muscle from male pigs mirrored that of low-estrogen female pigs. Coronary arteries from male pigs may lack the ability to respond to estrogen in a manner similar to the response in arteries from female pigs. For example, total uptake of [^{3}H]17β-estradiol is significantly higher in coronary arteries from female compared with male pigs (22). Although there is a clear demonstration of the existence of estrogen receptors in vascular tissue (12), the possible differences in number, function, and/or distribution of estrogen receptors between sexes remain unknown. Therefore, estrogen-mediated effects in vascular tissue may differ and/or require considerably higher threshold levels of estrogen in males compared with females. Gender differences in concentrations of other sex steroid hormones (i.e., progesterone and testosterone; Table 1) may also influence the actions of estrogen.

Table 3. *Binding inhibition constants in membranes prepared from porcine coronary arteries from male and female pigs*

	n	Endothelin-1, −log mol/l	Endothelin-3, High-Affinity Site, −log mol/l	Sarafotoxin S6c, High-Affinity Site, −log mol/l	Endothelin-3, Low-Affinity Site, −log mol/l
Females					
High estrogen	5	10.43 ± 0.13	12.05 ± 0.48*	12.07 ± 0.74*	8.96 ± 0.23
Low estrogen	16	10.37 ± 0.14	10.56 ± 0.58	10.00 ± 0.49	8.40 ± 0.49
Ovariectomized	5	10.59 ± 0.13	10.61 ± 0.43	10.59 ± 0.32	8.68 ± 0.34
Males	11	10.46 ± 0.12	10.80 ± 0.33	10.49 ± 0.45	8.41 ± 0.33

All values are −log mol/l ± the 95% confidence interval of the regression fit. Values were determined from nonlinear regression analysis of the mean values from competitive binding of ^{125}I-endothelin-1 with unlabeled endothelin-1, endothelin-3, and sarafotoxin S6c (Fig. 3). *Nonoverlapping confidence intervals between high-estrogen females and all other groups.

Table 4. *Maximal increases in tension and EC_{50} for endothelin-1 in isolated porcine coronary arteries untreated (control) or treated with indomethacin or both indomethacin and L-NMMA*

	High-Estrogen Females ($n = 6$)		Low-Estrogen Females ($n = 11$)		Ovariectomized ($n = 5$)		Males ($n = 8$)	
	Max, g	EC_{50}, −log mol/l	Max, g	EC_{50}, −log mol/l	Max, g	EC_{50}, −log mol/l	Max, g	EC_{50}, −log mol/l
With endothelium								
Control	27.8 ± 2.2*	7.28 ± 0.36	24.1 ± 1.6	7.67 ± 0.09	23.0 ± 2.0	7.54 ± 0.17	16.6 ± 2.6	7.66 ± 0.11
+Indomethacin	25.8 ± 4.2*	6.63 ± 0.48	18.6 ± 2.1	6.84 ± 0.27	23.4 ± 3.9	7.33 ± 0.07	12.8 ± 2.3	7.00 ± 0.26
+Indomethacin and L-NMMA	22.3 ± 2.3*	7.35 ± 0.13	21.4 ± 1.8*	7.33 ± 0.22	23.8 ± 2.9*	7.61 + 0.12	13.0 ± 2.3	7.33 ± 0.11
Without endothelium								
Control	22.5 ± 2.8	7.93 ± 0.13	21.2 ± 2.0	7.69 ± 0.09	26.8 ± 3.3*	7.55 ± 0.40	16.0 ± 2.4	7.67 ± 0.08
+Indomethacin	19.5 + 2.8	7.13 ± 0.16	18.9 ± 2.3*	7.12 ± 0.25	25.6 ± 3.5*	7.52 ± 0.09	10.3 ± 1.1	7.29 ± 0.10
+ Indomethacin and L-NMMA	22.2 ± 2.0*	7.10 ± 0.25	21.2 ± 2.1*	7.13 ± 0.30	26.4 ± 1.8*	7.33 ± 0.17	11.8 ± 2.3	7.04 + 0.22

Values are means ± SE; n = no. of pigs. Where indicated, indomethacin (10 μmol/l) and/or N^G-monomethyl-L-arginine (L-NMMA; 100 μmol/l) was added to the organ chambers 45 min before the cumulative addition of endothelin-1. EC_{50}, concentration producing half-maximal response. *Significantly different compared with male pigs ($P < 0.05$ by ANOVA). Removal of the endothelium did not significantly affect contractions to endothelin-1 for all groups of pigs. Max, maximal tension.

The present study demonstrates that contractions to endothelin-1 are significantly greater in coronary arteries from female compared with male pigs. Differences in contractions to endothelin-1 appear related to gender rather than endogenous estrogen status because the greater contractions to endothelin-1 in coronary arteries from high-estrogen compared with low-estrogen female pigs were not statistically significant. In arteries without endothelium from female pigs, there was no apparent relationship among the magnitude of endothelin-mediated contraction and the estrogen, progesterone, or testosterone status of the animal. These results are not in conflict with other observations that acute, exogenous estrogen may inhibit contractions to endothelin-1 (11). Rather, they point to the importance of differences in design for studies examining the effects of sex steroid hormones on the vasculature. In the present study, no estrogen or other sex steroid was added exogenously to the incubation media of the arteries. Rather, hormonal status of the animals was assessed based on endogenous concentrations of hormones in plasma before the arteries were removed.

The results of the present study also suggest that the sex differences in endothelin-evoked contractions occur at the level of smooth muscle because neither removal of the endothelium nor pretreatment with indomethacin or the combination of both indomethacin and L-NMMA abolished the gender-related differences. It is important to note that differences in contractions of the smooth muscle to endothelin-1 between sexes did not represent nonspecific changes to all agonists because contractions elicited by receptor-independent depolarization of the smooth muscle (KCl) did not differ between sexes.

Gender differences in contractions of coronary arteries to endothelin-1 are only partially explained by the estrogen-associated change in endothelin-receptor affinity because EC_{50} values for a given agonist were comparable among all pig groups and the gender differences in response to endothelin-1 were present in all female groups, including female animals with low endogenous levels of estrogen. Therefore, gender differences in endothelin-mediated contractions may be due to differences in pathways downstream of the endothelin receptor that ultimately activate contractile proteins, such as hydrolysis of plasma membrane phospholipids, inositol trisphosphate-mediated calcium mobilization, or diacylglycerol activation of protein kinase C (7, 13, 14, 20).

The increase in affinity for a high-affinity binding site identified with both endothelin-3 and sarafotoxin S6c may represent changes in the endothelin-B receptors. The physiological functions activated by these receptors are unclear and could include both activation of contraction and differentiation and/or proliferation of the smooth muscle (3, 4, 8, 9). In other cell types, activation of the endothelin-B receptor inhibits cellular growth (16). Increased affinity of endothelin-B receptors may result in increased sensitivity or contraction to sarafotoxin S6c. Although this is suggested in high-estrogen pigs, the shift in the concentration response curves was not statistically significant. It is unclear as to whether there are multiple subtypes of the endothelin-B receptor in vascular smooth muscle (6). Indeed, competitive inhibition of ^{125}I-endothelin-1 binding sites by sarafotoxin S6c was significant for a two-site model. Additional evidence for two populations of endothelin-B receptors and their associated functions remains to be defined.

In summary, results from this study provide evidence that endogenous elevations in 17β-estradiol during the normal ovulatory cycle are associated with an increase in affinity (i.e., lower K_i) of a high-affinity endothelin-receptor in coronary arterial smooth muscle of female pigs. However, plasma levels of estrogen even greater than those associated with the female pig ovulatory cycle do not have the same effect on endothelin receptor affinities in coronary arteries taken from male pigs. Effects of testosterone may be predominant in males and override the effect of estrogen in the males. Additionally, this study is the first to document gender differences in the responses of coronary smooth muscle

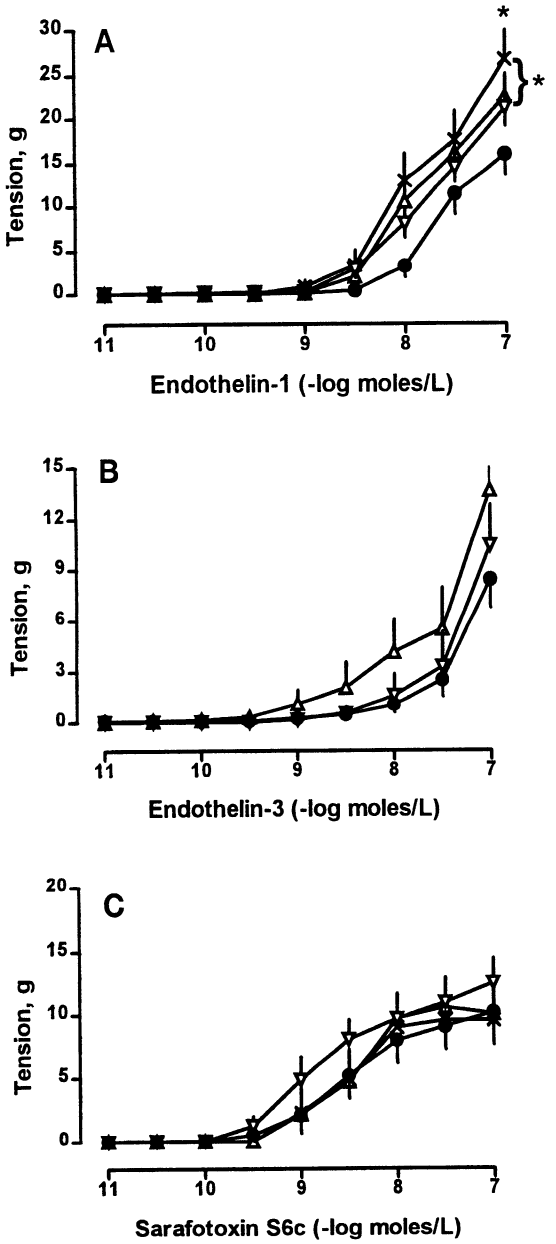

Fig. 5. Cumulative concentration-response curves for endothelin-1 (*A*), endothelin-3 (*B*), and sarafotoxin S6c (*C*) in porcine coronary arteries without endothelium. Experiments were performed on 2 separate groups of pigs. *A* and *C*: male (●, $n = 8$), low-estrogen female (▽, $n = 11$), high-estrogen female (△, $n = 6$), and ovariectomized (×, $n = 5$). *B*: male (●, $n = 11$), low-estrogen female (▽, $n = 8$), and high-estrogen female (△, $n = 5$). There was no ovariectomized group for endothelin-3 concentration-response curves. *Statistically significant difference in maximal tension compared with arteries from male pigs ($P < 0.05$, ANOVA, Bonferroni). Bracket indicates all female groups combined. All points represent the means ± SE.

to endothelin-1. Independent of sex steroid hormone status, coronary arteries in vitro from female pigs generate significantly greater contractions to endothelin-1 compared with male pigs. This gender difference in response to endothelin is not dependent on either the endothelium, synthesis of nitric oxide, or prostaglandins.

We thank Marylou Stewart, Kevin Rud, and Sandra Michener for dedicated technical support.

This work was supported by the National Heart, Lung, and Blood Institute Grants HL-51736 and HL-07111 and grants from the Mayo Clinic and Mayo Foundation.

Address for reprint requests: V. M. Miller, Medical Sciences, 4–54, Mayo Clinic and Foundation, 200 First St. SW, Rochester, MN 55905.

Received 1 November 1995; accepted in final form 1 April 1996.

REFERENCES

1. **Bell, D. R., H. J. Rensberger, D. R. Koritnik, and A. Koshy.** Estrogen pretreatment directly potentiates endothelium-dependent vasorelaxation of porcine coronary arteries. *Am. J. Physiol.* 268 (*Heart Circ. Physiol.* 37): H377–H383, 1995.
2. **Cuschieri, A.** *Operative Manual of Endoscopic Surgery.* Berlin: Springer-Verlag, 1992, p. 1–353.
3. **Dubin, D., R. E. Pratt, J. P. Cooke, and V. J. Dzau.** Endothelin, a potent vasoconstrictor, is a vascular smooth muscle mitogen. *J. Vasc. Med. Biol.* 1: 150–154, 1989.
4. **Eguchi, S., Y. Hirata, T. Imai, K. Kanno, and F. Marumo.** Phenotypic changes of endothelin receptor subtype in cultured rat vascular smooth muscle cells. *Endocrinology* 134: 222–228, 1994.
5. **Gisclard, V., V. M. Miller, and P. M. Vanhoutte.** Effect of 17β-estradiol on endothelium-dependent responses in the rabbit. *J. Pharmacol. Exp. Ther.* 244: 19–22, 1988.
6. **Gray, G. A., B. Löffler, and M. Clozel.** Characterization of endothelin receptors mediating contraction of rabbit saphenous vein. *Am. J. Physiol.* 266 (*Heart Circ. Physiol.* 35): H959–H966, 1994.
7. **Griendling, K. K., T. Tsuda, and R. W. Alexander.** Endothelin stimulates diacylglycerol accumulation and activates protein kinase C in cultured vascular smooth muscle cells. *J. Biol. Chem.* 264: 8237–8240, 1989.
8. **Hahn, A. W., S. Regenass, T. J. Resink, F. Kern, and F. R. Buhler.** Morphogenic effects of endothelin-1 on vascular smooth muscle cells. *J. Vasc. Res.* 30: 192–201, 1993.
9. **Hirata, Y., Y. Takagi, Y. Fukuda, and F. Marumo.** Endothelin is a potent mitogen for rat vascular smooth muscle cells. *Arteriosclerosis* 78: 225–228, 1989.
10. **Igarashi, Y., Y. Aizawa, M. Tamura, K. Ebe, T. Yamaguchi, and A. Shibata.** Vasoconstrictor effect of endothelin on the canine coronary artery: is a novel endogenous peptide involved in regulating myocardial blood flow and coronary spasm? *Am. Heart J.* 118: 674–678, 1989.
11. **Jiang, C., P. M. Sarrel, P. A. Poole-Wilson, and P. Collins.** Acute effect of 17β-estradiol on rabbit coronary artery contractile responses to endothelin-1. *Am. J. Physiol.* 263 (*Heart Circ. Physiol.* 32): H271–H275, 1992.
12. **Karas, R. H., B. L. Patterson, and M. E. Mendelsohn.** Human vascular smooth muscle cells contain functional estrogen receptor. *Circulation* 89: 1943–1950, 1994.
13. **Kasuya, Y., Y. Takuwa, M. Yanagisawa, S. Kimura, S. Goto, and T. Masaki.** Endothelin-1 induces vasoconstriction through two functionally distinct pathways in porcine coronary artery: contribution of phosphoinositide turnover. *Biochem. Biophys. Res. Commun.* 161: 1049–1055, 1989.
14. **Lee, T. S., T. Chao, K. Hu, and G. L. King.** Endothelin stimulates a sustained 1,2-diacylglycerol increase and protein kinase C activation in bovine aortic smooth muscle cells. *Biochem. Biophys. Res. Commun.* 162: 381–386, 1989.
15. **Lerman, A., B. S. Edwards, J. W. Hallett, D. M. Heublein, S. M. Sandberg, and J. C. Burnett, Jr.** Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. *N. Engl. J. Med.* 325: 997–1001, 1991.
16. **Mallat, A., L. Fouassier, A. M. Preaux, C. S. Gal, D. Raufaste, J. Rosenbaum, D. Dhumeaux, C. Jouneaux, P. Mavier, and S. Lotersztajn.** Growth inhibitory properties of endothelin-1 in human hepatic myofibroblastic Ito cells. An endothelin B receptor-mediated pathway. *J. Clin. Invest.* 96: 42–49, 1995.
17. **Margulies, K. B., F. L. Hildebrand, Jr., A. Lerman, M. A. Perrella, and J. C. Burnett, Jr.** Increased endothelin in experimental heart failure. *Circulation* 82: 2226–2230, 1990.

18. **Miller, V. M., and P. M. Vanhoutte.** 17β-Estradiol augments endothelium-dependent contractions to arachidonic acid in rabbit aorta. *Am. J. Physiol.* 258 (*Regulatory Integrative Comp. Physiol.* 27): R1502–R1507, 1990.
19. **Miller, V. M., and P. M. Vanhoutte.** Progesterone and modulation of endothelium-dependent responses in canine coronary arteries. *Am. J. Physiol.* 261 (*Regulatory Integrative Comp. Physiol.* 30): R1022–R1027, 1991.
20. **Muldoon, L. L., K. D. Rodland, M. L. Forsythe, and B. E. Magun.** Stimulation of phosphatidylinositol hydrolysis, diacylglycerol release, and gene expression in response to endothelin, a potent new agonist for fibroblasts and smooth muscle cells. *J. Biol. Chem.* 264: 8529–8536, 1989.
21. **Polderman, K. H., C. D. A. Stehouwer, G. J. van Kamp, G. A. Dekker, F. W. A. Verheugt, and L. J. G. Gooren.** Influence of sex hormones on plasma endothelin levels. *Ann. Intern. Med.* 118: 429–432, 1993.
22. **Vargas, R., B. Wroblewska, A. Rego, J. Hatch, and P. W. Ramwell.** Oestradiol inhibits smooth muscle cell proliferation of pig coronary artery. *Br. J. Pharmacol.* 109: 612–617, 1993.