Gender differences in endothelium-dependent relaxations do not involve NO in porcine coronary arteries

DUSTAN A. BARBER AND VIRGINIA M. MILLER
Department of Surgery, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Barber, Dusdan A., and Virginia M. Miller. Gender differences in endothelium-dependent relaxations do not involve NO in porcine coronary arteries. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2325–H2332, 1997.—Experiments were designed to determine whether normal fluctuations in endogenous sex steroid hormones and/ or gender affect endothelium-dependent relaxations in coronary arteries, and, if so, to identify endothelium-derived factors contributing to these differences. Coronary arteries from sexually mature, gonadally intact male and female pigs or ovariolectomized pigs were prepared either for study of isometric force in organ chambers or for measurement of prostanoids and activity of nitric oxide (NO) synthase. In organ chamber studies, neither the magnitude nor the sensitivity of endothelium-dependent relaxations correlated with endogenous estrogen or progesterone in female pigs. However, relaxations to bradykinin and UK-14304 were significantly greater and/or shifted leftward in arterial rings from female compared with male pigs. Indomethacin (10−5 mol/l) increased endothelium-dependent relaxations only in arteries from male pigs. Nω-monomethyl-L-arginine reduced endothelium-dependent relaxations to a similar extent in coronary arteries from either sex. Neither production nor response to thromboxane A2 or prostacyclin differed in coronary arteries from male compared with female pigs. Activity for calcium-dependent or -independent NO synthase was similar in both sexes. These results suggest that normal fluctuations in endogenous sex steroid hormones do not affect endothelium-dependent relaxations in coronary arteries from female pigs. There are, however, gender differences in endothelium-dependent relaxations that are indomethacin sensitive and may be due to cyclooxygenase products other than thromboxane A2 or prostacyclin.

arachidonic acid; eicosanoids; nitric oxide synthase; prostacyclin; estrogen; thromboxane

BEFORE MENOPAUSE women have a lower incidence of coronary artery disease compared with men of the same age. After menopause this difference persists only if estrogen replacement therapy is initiated. Such data suggest that female sex steroid hormones prevent the development of coronary artery disease (see Refs. 7 and 23 for review). Mechanisms accounting for this cardioprotective action of estrogen may include differences in regulation and production of endothelium-derived factors. For example, estrogen treatment of whole animals after ovarioectomy enhances endothelium-dependent relaxations in isolated arteries to some, but not all, endothelium-dependent agonists (10, 18). Similarly, overnight incubation of isolated porcine coronary arteries with physiological concentrations of 17β-estradiol enhances relaxations to the Ca2+ ionophore A-23187 but not adenosine 5′-diphosphate or nitric oxide (4). Other studies suggest that progesterone and testosterone also alter endothelium-dependent relaxation of coronary arteries (1, 9, 19). All of these studies involve depletion of nearly all endogenous sex steroid hormones followed by replacement of one, or at best two, sex steroid hormones. Such an approach does not reflect potential interactions between the sex steroid hormones during the normal ovulatory cycle, when a milieu of sex steroid hormones is present and fluctuating in a coordinated manner. An understanding of the regulation of endothelium-derived factors and coronary vasomotion during the normal ovulatory cycle is critical because this is when developmental differences in coronary artery disease occur in humans. Experiments were therefore designed to determine whether normal endogenous fluctuations in sex steroid hormones and/or gender affect endothelium-dependent relaxations and, if so, to identify endothelium-derived factors accounting for the differences.

METHODS

Sexually mature pigs were chosen for this study because the estrous 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 concentrations of sex steroid hormone when they were killed. Sexually mature, gonadally intact Yorkshire male (130 ± 10 kg, n = 19) and female (87 ± 1 kg, n = 32) or laparoscopically ovarioctomized (6) pigs (79 ± 2 kg, n = 5) were anesthetized by an intramuscular injection of a ketamine-xylazine-butorphanol mixture (30, 6, and 0.3 mg/kg, respectively). Weight gain in maturing male and female pigs is not the same. Therefore, animals were age matched to assure groups of comparable sexual maturity. Ovariectomy was performed on sexually mature female pigs (5–6 mo of age), and experiments were performed after 4 wk. All other animals were studied between 5 and 7 mo of age. Blood samples were collected from the femoral artery and analyzed for plasma 17β-estradiol, progesterone, and testosterone 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 17β-estradiol and testosterone. Serum cholesterol and lipoprotein profiles were determined by Mayo Medical Laboratories. 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, 1.2 MgSO4, 1.2 KH2PO4, 25.0 NaHCO3, 2.0 CaCl2, 0.026 calcium disodium edetate, 1.11 glucose, pH 7.4, and aerated with 95% oxygen-5% carbon dioxide). The right circumflex coronary artery was excised and prepared for assessment of nitric oxide synthase (NOS) assay. 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 [DHHS Publication No. (NIH) 86–23, Revised 1985].
Organ chamber experiments. The right circumflex coronary artery was excised, cleaned of connective tissue, and cut into 4-mm rings for study in organ chambers. One-half of the arterial rings were denuded mechanically of endothelium by gently scraping the lumen with fine-tipped forceps. Removal of the endothelium was confirmed by the absence of relaxation to endothelium-dependent vasodilators described below. Pairs of rings with and without endothelium were suspended between a fixed stirrup and gossamer 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 was added to each bath to determine maximal response to KCl. To some of the baths either indomethacin (10⁻⁵ mol/l, dissolved in sodium carbonate, final bath concentration sodium carbonate 2 × 10⁻⁵ mol/l) or N⁶-monomethyl-L-arginine (L-NMMA, 10⁻⁴ mol/l), or both, was added 45 min before administration of the various agonists. Rings were contracted with prostaglandin F₂α (2 × 10⁻⁶ mol/l) or endothelin-1 (10⁻⁷ mol/l) and cumulative concentration responses to bradykinin (10⁻⁷ to 10⁻⁴ mol/l), UK-14304 (5-bromo-6-[2-imidazol-2-ylamino]-quinoxaline, 10⁻⁸ to 10⁻⁶ mol/l), the calcium ionophore A-23187 (10⁻⁹ to 10⁻⁶ mol/l, dissolved in dimethyl sulfoxide (DMSO)), final bath concentration DMSO 8.2 × 10⁻³ mol/l or nitric oxide (3 × 10⁻⁸ to 10⁻⁵ mol/l) determined. Once indomethacin and/or L-NMMA was added to an organ bath they remained in the baths for the duration of the experiment.

In a separate set of organ chamber experiments, rings with and without endothelium were set to their optimal tension as previously described and cumulative concentration-response curves to either the thromboxane A₂ mimetic (U-46619, 10⁻⁶ mol/l) or prostacyclin (10⁻⁶ mol/l, dissolved in dimethyl sulfoxide) were performed in rings set at their optimal tension developed to 20 mmol/l KCl at each level of stretch. Once the optimal tension was set, 60 mmol/l KCl was added to each bath to determine maximal response to KCl. To some of the baths either indomethacin (10⁻⁵ mol/l, dissolved in sodium carbonate, final bath concentration sodium carbonate 2 × 10⁻⁵ mol/l) or N⁶-monomethyl-L-arginine (L-NMMA, 10⁻⁴ mol/l), or both, was added 45 min before administration of the various agonists. Rings were contracted with prostaglandin F₂α (2 × 10⁻⁶ mol/l) or endothelin-1 (10⁻⁷ mol/l) and cumulative concentration responses to bradykinin (10⁻⁷ to 10⁻⁴ mol/l), UK-14304 (5-bromo-6-[2-imidazol-2-ylamino]-quinoxaline, 10⁻⁸ to 10⁻⁶ mol/l), the calcium ionophore A-23187 (10⁻⁹ to 10⁻⁶ mol/l, dissolved in dimethyl sulfoxide (DMSO)), final bath concentration DMSO 8.2 × 10⁻³ mol/l or nitric oxide (3 × 10⁻⁸ to 10⁻⁵ mol/l) determined. Once indomethacin and/or L-NMMA was added to an organ bath they remained in the baths for the duration of the experiment.

Activity of NOS. Activity of NOS was determined by measuring the conversion of L-[³H]arginine to L-[³H]citrulline in cellular homogenates according to previously described methods with minor modifications (20). Right and left circumflex and left anterior descending coronary arteries with endothelium were cleaned of fat and connective tissue, combined and pulverized in liquid nitrogen, and suspended in six volumes of ice-cold homogenization buffer of the following composition: 320 mmol/l sucrose, 50 mmol/l tris(hydroxymethyl)aminomethane-HCl, 0.1 mmol/l EDTA, 100 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 10 µg/ml antipain, pH 7.8. The suspension was homogenized three times for 10 s each using a Tekmar homogenizer (Tekmar, Cincinnati, OH). Homogenates were centrifuged at 2,000 g for 15 min at 4°C to remove cellular debris. The supernatant was passed through a 213-µm nylon sieve onto an equilibrated 10-DG desalting column (Bio-Rad, Hercules, CA) and eluted according to the manufacturer’s directions. A small aliquot was used to determine protein concentrations using biocinchoninic acid protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as standard. To quantify NOS activity, duplicate reactions were carried out in the presence of calcium (total activity), in the absence of calcium plus ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′′-tetraacetic acid (EGTA) (calcium-independent activity), and in the absence of calcium plus EGTA in the presence of L-NMMA (nonspecific activity). Reactions were started by adding 150 µl protein homogenate to 150 µl cofactor mix such that the final concentrations were as follows: 14.7 mmol/l L-[³H]arginine (0.3 µCi specific activity at 68 Ci/mmol), 54 mmol/l L-valine, 1.2 mmol/l MgCl₂, 1.0 mmol/l NADPH, 2 µmol/l FAD, 5 µmol/l L-arginine; 10 µmol/l tetrahydrobiopterin, 50 U/ml calmodulin, and, as described above, with or without 0.83 mmol/l CaCl₂, 1 mmol/l EGTA, and 2 mmol/l L-NMMA. The reaction was carried out in a shaker bath at 27°C for 1 h and terminated by the addition of 1.5 ml ice-cold stop buffer (20 mmol/l N/2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid and 8 mmol/l EDTA, pH 5.5). Separation of L-[³H]arginine from L-[³H]citrulline was accomplished using affinity columns containing a resin that retains the charged species of L-[³H]arginine while allowing L-[³H]citrulline to pass through. The assay mixture was then passed over Poly-Prep chromatography columns (Bio-Rad) loaded with 1 ml of equilibrated AG50 WX-8 Na⁺ form 200-400 mesh molecular biology grade Dowex resin (Bio-Rad), and the eluate was collected into 18-ml OptiFluor (Packard, Meriden, CT). The column was washed with 2 ml of water while continuing to collect into the scintillation fluid. L-[³H]citrulline activity was determined using a Beckman 6800 liquid scintillation counter. Blank incubations contained 150 µl protein-free homogenization buffer previously passed over a desalting column as described. Activity calculations account for scintillation counting efficiency and the ratio of L-[³H]arginine to nonradioactive L-argnine in the incubation mixture. Nitric oxide produced by NOS is presumably in a 1/1 molar ratio with L-citrulline, and thus NOS activity is expressed as picomoles of L-[³H]citrulline produced per milligram of protein per hour. Calcium-dependent activity equaled total activity minus calcium-independent activity after correcting for nonspecific activity.

Eicosanoid production. Rings of right coronary artery (4 mm) with endothelium from male (n = 8) or female (n = 7) pigs were placed in 1.5-ml tubes containing 1 ml control solution at 37°C continuously aerated with 95% oxygen-5% carbon dioxide. After a 30-min equilibration period, the control solution was replaced with fresh solution. Two minutes after prostaglandin F₂α (2 × 10⁻⁶ mol/l) and calcium ionophore A-23187 (10⁻⁹ to 10⁻⁶ mol/l, dissolved in dimethyl sulfoxide) were added to one of the rings, and solvent (water, 10 µl) was added to another rings. Ten minutes later UK-14304 (10⁻⁶ mol/l) was added to one of the tubes containing prostaglandin F₂α, and to one tube not containing prostaglandin F₂α. Exactly 3 min later the arterial rings were removed, and the test tube containing incubation solution was immediately frozen in liquid nitrogen for subsequent radioimmunoassay of 6-ketoprostaglandin F₁α (stable metabolite of prostacyclin) and thromboxane B₂ (stable metabolite of thromboxane A₂). The arterial rings were dabbed dry with tissue paper and weighed. Radioimmunoassays were performed according to the manufacturer’s instructions for the individual assay kit.

Histology. Right and left circumflex and left anterior descending 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. For organ chamber studies, data are analyzed as percent change in tension from contraction to prostaglandin F₂α or endothelin-1. Maximal relaxations and effective concentrations producing half-maximal relaxation (EC₅₀) were calculated for individual concentration-response curves.
NOS activities were normalized to protein concentration of cellular homogenates and are expressed as L-[3H]citrulline formed per unit time. Calcium-dependent activity was equal to total activity in the presence of calcium minus activity in the absence of calcium after correcting for nonspecific and background activity. The scintillation counter was preset to correct for counting efficiency to convert counts per minute to disintegrations per minute.

For comparisons among multiple groups, a one-way analysis of variance (ANOVA) was used. If a significant F value was obtained, a Bonferroni post hoc test was used to compare means while controlling for multiple comparisons. In instances in which results were grouped only by gender, a two-tailed, unpaired t-test was used. For data where responses were from arteries of the same animal, a two-tailed, paired t-test was used. For all statistical tests P < 0.05 was required for significance.

Drugs and chemicals. Nitric oxide was prepared by the method of Palmer et al. (21). Indomethacin was dissolved in an aqueous solution of sodium carbonate (bath concentration 20 µmol/l). A-23187 was dissolved in DMSO, final bath concentration 8.2 mmol/l, and diluted with distilled water. This concentration of DMSO has been shown to not affect smooth muscle (17). All other drugs used in organ chamber experiments were grouped according to plasma 17β-estradiol 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. Plasma levels of 17β-estradiol were significantly higher in high-estrogen females compared with ovariectomized and low-estrogen females (Table 1). Males had significantly higher plasma levels of 17β-estradiol than either the ovariectomized or low-estrogen females. Progesterone levels were not significantly different among all groups (Table 1). Plasma levels of testosterone were significantly higher in males compared with all female groups (Table 1). Total serum cholesterol was lower in males compared with either high- or low-estrogen females (Table 1). There was no significant difference in either high- or low-density lipoprotein among groups (Table 1). There was no consistent evidence of atherosclerosis in histological sections of arteries from either sex (data not shown).

Organ chamber experiments. In arteries from female pigs, there was no significant difference in relaxations to any of the drugs tested when the data were grouped according to endogenous plasma concentrations of 17β-estradiol, progesterone, or testosterone (Fig. 1). Neither were there significant correlations between maximal relaxations and plasma estrogen concentrations in

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**Table 1. Plasma sex steroid hormone and serum cholesterol and lipoproteins in sexually mature pigs**

<table>
<thead>
<tr>
<th></th>
<th>High-Estragen Females</th>
<th>Low-Estragen Females</th>
<th>Ovariectomized</th>
<th>Males</th>
</tr>
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<tbody>
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<td>No. of subjects</td>
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<td>18</td>
<td>5</td>
<td>19</td>
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<td>17β-Estradiol, pg/ml</td>
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<td>ND</td>
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<td>Progesterone, pg/ml</td>
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<td>Cholesterol, mg/dl</td>
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<td>LDL, mg/dl</td>
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<tr>
<td>HDL, mg/dl</td>
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<td>31.6 ± 2.1</td>
<td>26.1 ± 1.7</td>
</tr>
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Values are means ± SE. Data were compared using 1-way analysis of variance followed by Bonferroni's post hoc test. LDL, low-density lipoprotein; HDL, high-density lipoprotein; ND, not detected. *P < 0.05 compared with male pigs.

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**Fig. 1. Concentration-response curves for α1-adrnergic agonist UK-14304 (A), bradykinin (B), and calcium ionophore A-23187 (C) in rings of right coronary arteries with endothelium from high-estrogen females, low-estrogen females, and ovariectomized pigs.**

For UK-14304 and bradykinin, data are expressed as percent change in tension from contraction to prostaglandin F2α (2 × 10−6 mol/l). For A-23187, data are expressed as percent change in tension from contraction to endothelin-1 (10−7 mol/l). All data represent means ± SE.
either male or female pigs. Therefore, all data from female pigs were combined for analysis by gender. Contractions to potassium chloride (60 mmol/l) in arteries with or without endothelium were not significantly different among arteries from all pigs (males, n = 8, with endothelium 24.9 ± 2.4 g and without endothelium 21.8 ± 3.3 g; females, n = 22, with endothelium 23.8 ± 1.2 g and without endothelium 21.1 ± 1.3 g). The addition of indomethacin to organ chambers did not cause a change in tension in rings with or without endothelium from pigs of either sex. However, the addition of indomethacin plus L-NMMA caused similar increases from baseline tension in arteries with endothelium in 5 of 8 (62.5%) arteries from male pigs (1.54 ± 0.73 g, n = 5) and in 14 of 22 (63.3%) arteries from female pigs (1.43 ± 0.31 g, n = 14). There were no statistically significant differences in contraction to prostaglandin F2α among groups (range 13–24 g in all groups). Contractions to endothelin-1 (10⁻⁷ M) were significantly greater in arteries from females (high estrogen 27.8 ± 2.2 g, n = 6; low estrogen 24.1 ± 1.6 g and ovariectomized 23 ± 1.9 g, n = 5) compared with male pigs (16.6 ± 2.6 g, n = 8).

UK-14304. UK-14304 (10⁻⁸ to 10⁻⁶ mol/l) caused concentration-dependent relaxations in rings with endothelium in arteries contracted with prostaglandin F2α (2 × 10⁻⁶ mol/l) from male (n = 8) and female (n = 22) pigs. Endothelium-dependent relaxations to UK-14304 in arteries from female pigs were significantly shifted leftward (EC₅₀ 6.93 ± 0.08 –log mol/l female vs. 6.33 ± 0.33 –log mol/l male) compared with rings from male pigs (Fig. 2A). Maximal relaxations to UK-14304 were not significantly different between genders. In arteries from male but not female pigs, indomethacin potentiated relaxations to UK-14304 (males, maximum 41.7 ± 11.2% control vs. -68.1 ± 9.2% + indomethacin, Fig. 3). In the presence of indomethacin (10⁻⁵ mol/l) relaxations were reduced by L-NMMA (10⁻⁴ mol/l) to the same extent in both sexes (Fig. 3). In the presence of indomethacin (10⁻⁵ mol/l) or indomethacin plus L-NMMA (10⁻⁴ mol/l), endothelium-dependent relaxations to UK-14304 were not different between arteries from male and female pigs (Fig. 3).

Bradykinin. In coronary arterial rings contracted with prostaglandin F2α (2 × 10⁻⁶ mol/l), bradykinin (10⁻¹⁰ to 10⁻⁷ mol/l) caused concentration-dependent relaxations only in rings with endothelium (Fig. 2B). Endothelium-dependent relaxations to bradykinin in arteries from female (n = 22) pigs were shifted significantly leftward (EC₅₀ 8.27 ± 0.07 –log mol/l female vs. 7.74 ± 0.13 –log mol/l male) with significantly greater maximal relaxation (maximum -98.9 ± 0.8% female vs. -83.9 ± 8.9% male) compared with rings from male (n = 8) pigs. In the presence of indomethacin (10⁻⁵ mol/l) or indomethacin plus L-NMMA (10⁻⁴ mol/l), endothelium-dependent relaxations to bradykinin were not statistically different between male and female pigs (Fig. 4). In arteries from male pigs, indomethacin did not significantly change relaxations to bradykinin (Fig. 4).

![Figure 2. Concentration-response curves for α₂-adrenergic agonist UK-14304 (A), bradykinin (B), and calcium ionophore A-23187 (C) in rings of right coronary arteries with endothelium from male (n = 8) and female (n = 22) pigs. For UK-14304 and bradykinin, data are expressed as percent change in tension from contraction to prostaglandin F2α (2 × 10⁻⁶ mol/l). For A-23187, data are expressed as percent change in tension from contraction to endothelin-1 (10⁻⁷ mol/l). All data represent means ± SE. *Significant gender difference in half-maximal relaxations for UK-14304 and bradykinin and maximal relaxation for bradykinin (unpaired t-test, P < 0.05).](http://ajpheart.physiology.org/)

A-23187. A-23187 (10⁻⁹ to 10⁻⁶ mol/l) caused concentration-dependent relaxations in coronary arterial rings with endothelium contracted with endothelin-1 (10⁻⁷ mol/l) from male (n = 8) and female (n = 22) pigs (Fig. 2C). Relaxations to A-23187 were not statistically different between arteries from male and female pigs (EC₅₀ 6.77 ± 0.07 –log mol/l female vs. 6.52 ± 0.13 –log mol/l male; maximum -54.2 ± 5.4% female vs. -44.3 ± 11.2% male, Fig. 2C). In the presence of indomethacin
(10^{-5} \text{ mol/l}) or indomethacin plus L-NMMA (10^{-4} \text{ mol/l}), endothelium-dependent relaxations to A-23187 were the same for arteries from male and female pigs. In arteries from male but not female pigs, indomethacin significantly potentiated maximal relaxations to A-23187 (males maximum 244.3 \pm 11.2\% \text{ control vs. } 266.3 \pm 12.5\% \text{ indomethacin, Fig. 5}).

Nitric oxide. In coronary arterial rings without endothelium contracted with endothelin-1 (10^{-7} \text{ mol/l}), nitric oxide (3 \times 10^{-8} \text{ to } 10^{-5} \text{ mol/l}) produced concentration-dependent relaxations in rings from male (n = 8) and female (n = 22) pigs. Relaxations to nitric oxide were not different in arteries without endothelium between male and female pigs (maximum 65.7 \pm 12.5\% control vs. 67.2 \pm 13.4\% \text{ indomethacin + LNMA, Fig. 5}).
compared with rings with endothelium from both male and female pigs. There were no gender differences in the responses to prostacyclin in rings with or without endothelium. In rings without endothelium, the lower concentrations of prostacyclin (3 × 10^{-9} to 10^{-7} mol/l) elicited a small relaxation (maximum relaxation, −2.50 ± 0.69 g female, −2.74 ± 1.14 g male, P = not significant) before increasing tensions at higher concentrations.

NOS activity. There were no significant correlations between endogenous concentrations of sex steroid hormones and activity of NOS derived from membrane homogenates of coronary arteries with endothelium from female pigs. There were no gender differences in either calcium-dependent (male 47 ± 6 and female 44 ± 7 pmol L-[^3]H]citrulline·mg protein^{-1}·h^{-1}) or -independent (male 7 ± 2 and female 11 ± 2 pmol L-[^3]H]citrulline·mg protein^{-1}·h^{-1}) NOS activity of coronary artery homogenates.

Eicosanoid production. Prostaglandin F_2α, but not UK-14304, significantly increased both thromboxane B_2 and 6-ketoprostaglandin F_{1α} in the incubation medium of coronary arteries from male (n = 8) and female (n = 7) pigs (Fig. 7). The amounts of both thromboxane B_2 and 6-ketoprostaglandin F_{1α} detected in the incubation medium of unstimulated (solvent control), UK-14304-stimulated, or prostaglandin F_2α-stimulated coronary arterial rings were not statistically greater in arteries

Fig. 6. Concentration-response curves for thromboxane A_2 mimetic U-46619 (A) or prostacyclin (PGI_2; B) in coronary arteries with or without endothelium from female (n = 7 U-46619, n = 5 PGI_2) or male (n = 6 U-46619, n = 4 PGI_2) pigs. Removal of endothelium significantly increased maximal responses to U-46619 and PGI_2. There were no significant gender differences in response to either U-46619 or PGI_2 with or without endothelium. All data represent means ± SE.

Fig. 7. Thromboxane B_2 (stable metabolite of thromboxane A_2; A) and 6-ketoprostaglandin F_{1α} (stable metabolite of prostacyclin; B) detected by radioimmunoassay in incubation medium of right coronary arterial rings with endothelium from either male (n = 8) or female (n = 7) pigs. Rings were either unstimulated (control) or treated with UK-14304 (10^{-6} mol/l), prostaglandin F_{2α} (2 × 10^{-6} mol/l), or both as described in METHODS. Prostaglandin F_{2α} significantly increased both thromboxane B_2 and 6-ketoprostaglandin F_{1α} in incubation media of coronary arteries from either sex. For all treatment groups thromboxane B_2 and 6-ketoprostaglandin F_{1α} levels were not statistically different in arteries from male compared with female pigs. Results are normalized to tissue wet weight, and all data represent means ± SE.
from male compared with female pigs. The amounts of thromboxane B2 and 6-ketoprostaglandin F1α in rings stimulated with UK-14304 plus prostaglandin F2α were the same for arteries from male and female pigs. Endogenous concentrations of sex steroid hormones of female pigs did not correlate with either thromboxane B2 or 6-ketoprostaglandin F1α production.

**DISCUSSION**

Results of the present study suggest that physiological fluctuations in estrogen in the presence of other endogenous sex steroid hormones do not significantly affect agonist-stimulated endothelium-dependent relaxations of isolated coronary arteries from female pigs. This seems to vary with other studies when acute or chronic estrogen treatment potentiates endothelium-dependent relaxations (4, 10, 15, 22). However, studies of estrogen replacement do not address changes in estrogen that occur in conjunction with fluctuations in other endogenous sex steroid hormones during the estrous cycle. Interactions among the sex hormones are unclear (9, 19). A limitation of the present study is that estrogen grouping was based on measurable estrogen as related to the sensitivity of the assay. It could not be determined whether a given estrogen value represented a rising or falling phase of the estrous cycle. The lack of difference in responses between intact females and ovariectomized females is surprising and may relate to the age at which ovariectomy was performed and the time from ovariectomy to study of the arteries.

In healthy women flow-mediated endothelium-dependent vasodilatation of the brachial artery is enhanced in the follicular and luteal phases of menstruation when endogenous estrogen is elevated (12). In the present study no estrogen was present in the incubation medium of the organ baths as would be in the blood, and there is an absence of mechanical stimulation by flow. Flow-mediated coupling for release of endothelium-derived relaxing factors may differ from that of direct agonist stimulation and may be more sensitive to regulation by endogenous hormones.

An important finding of the present study is that gender differences in agonist-stimulated endothelium-dependent relaxations may not be related to estrogen levels alone. This conclusion is supported by the observation that plasma concentrations of estrogen in male pigs were greater than those of female pigs, probably as a result of metabolism of testosterone by aromatase in the adipose tissue. Therefore, it is not possible from the present study design to determine whether testosterone alone or estrogen-to-testosterone ratio is important in affecting production of endothelium-derived substances.

Systemic evaluation of effects of testosterone on vascular responses in male animals warrants further study. However, such studies should take into consideration the timing of castration, because castration before puberty (as would be the case in the studies of male arteries obtained from the abattoir) may not represent the same developmental conditions as removal of gonads from animals that experienced puberty.

Metabolism of arachidonic acid by cyclooxygenase may be responsible for gender difference in agonist-stimulated endothelium-dependent relaxations because indomethacin potentiates relaxations in arteries from male but not female pigs. One metabolite of arachidonic acid is thromboxane A2, which elicits contractions that are sexually dimorphic in some animals (5, 8). Prostacyclin is another potential mediator of contractions, because male pigs used in this study had relatively high 17β-estradiol plasma levels and chronic estrogen treatment may increase sensitivity of smooth muscle to prostacyclin contractions (18). In the present study there was a tendency for isolated arteries of male pigs to have a greater sensitivity to both thromboxane A2 (U-46619) and prostacyclin (Fig. 5). There was also a trend toward greater basal and agonist-stimulated release of both thromboxane B2 and 6-ketoprostaglandin F1α from arteries of male compared with female pigs (Fig. 6). Neither of these observations alone was statistically significant; however, it is possible that the combination of these two effects in the arterial ring may account for the indomethacin-sensitive gender differences in endothelium-dependent relaxation.

Any of a number of endothelium-derived factors other than eicosanoids may contribute to gender differences in relaxations, including nitric oxide, hyperpolarizing factor(s), locally produced peptides (2), cytochrome P-450 products (11, 16), and free radicals (13). Several studies suggest that endothelium-derived nitric oxide is a mediator accounting for gender differences in endothelium-dependent relaxations. For example, relaxations to acetylcholine are greater in the aorta of female compared with male rats (13, 14). However, unlike the current study, indomethacin does not eliminate gender differences in acetylcholine-mediated relaxations in rat aorta (14). This suggests that mechanisms of gender differences in endothelium-dependent relaxations may differ by agonist, species, and/or anatomic origin of the artery. Results of the present study do not eliminate the possibility of gender differences in production of nitric oxide. The lack of gender differences in the biochemical assessment of NOS activity is limited because this assay reflects the enzyme under optimized conditions with all cofactors present. Therefore, potential gender-hormonal regulation of NOS cofactors and/or their availability or even endogenous pathways of NOS activation are not accounted for in this assay. However, the results confirm that the response of the smooth muscle to nitric oxide is the same in coronary arteries of male and female because both relaxations to exogenously administered nitric oxide and/or relaxations to endothelium-dependent agonists in the presence of indomethacin plus L-NMMA were similar between sexes.

Basal myogenic tone was not determined in these experiments. Therefore, it is not possible to evaluate differences in contractions or relaxations relative to contribution of myogenic tone. Differences in relaxations cannot be attributed to the level of contraction of arteries with prostaglandin F2α, because these were
similar among groups. Contractions to endothelin-1 are greater in females than males (3). However, a greater level of contraction would be expected to decrease relaxations in females relative to males, a result opposite to what was observed.

In summary, the present study demonstrates that normal fluctuations in endogenous sex steroid hormones do not change endothelium-dependent relaxations of isolated porcine coronary arteries from female pigs. There are, however, gender differences in endothelium-dependent relaxations that are indomethacin sensitive and not due to differences in NOS activity. This indomethacin-sensitive component of the relaxations is not due solely to either differences in sensitivity of coronary arteries to thromboxane A2 and/or prostacyclin or to differences in production and/or release of thromboxane A2 and/or prostacyclin.

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Address for reprint requests: V. M. Miller, Medical Sciences, 4–57, Mayo Clinic & Foundation, 200 First St., S. W., Rochester, MN 55905.

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