Effect of puberty on coronary arteries from female pigs

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Chatrath, Ritu, Karen L. Ronningen, Peter LaBreche, Sandra R. Severson, Muthuvel Jayachandran, Margarita P. Bracamonte, and Virginia M. Miller. Effect of puberty on coronary arteries from female pigs. J Appl Physiol 95: 1672–1680, 2003. First published June 6, 2003; 10.1152/japplphysiol.00099.2003.—Vascular function changes following loss of ovarian hormones in women at menopause and in experimental animals following surgical ovariectomy. Little is known about changes in vascular function during hormonal transition from sexual immaturity (juvenile) to sexual maturity. Therefore, experiments were designed to determine effects of natural puberty on vascular function in female pigs. Tissue was studied from eight juvenile (2–3 mo) and eight adult (5–6 mo) female pigs. Plasma nitric oxide (NO) was measured, and mRNA for endothelium-derived NO synthase (eNOS) and eNOS protein were determined in aortic endothelial cells. Rings of coronary arteries were suspended for measurement of isometric force in organ chambers. Serum 17β-estradiol levels were comparable in the two groups, whereas the arithmetic mean of progesterone levels was about two-thirds lower in adults compared with juveniles. Plasma NO was significantly higher in juveniles compared with adults, but mRNA and protein for eNOS were comparable. In coronary arteries, an α2-adrenergic agonist caused greater endothelium-dependent relaxations in rings from juvenile compared with adult pigs. Relaxations to bradykinin were similar in arteries from both groups, but inhibition of NO reduced relaxations only in arteries from juvenile pigs. Relaxations from NO were greater in arteries from adult compared with juvenile female pigs. In conclusion, coronary arterial endothelial and smooth muscle responses are selectively modulated at puberty in female pigs. At maturity, plasma NO is reduced and sensitivity of the smooth muscle to exogenous NO is increased. Posttranscriptional regulation of eNOS protein may explain differences in NO bioavailability in juvenile pigs.

bradykinin; C-type natriuretic peptide; endothelin-1; estrogen; nitric oxide

CLINICAL OBSERVATIONS INDICATE that vascular disease characterized as accelerated atherogenesis begins in the teenage years (52). Prevention of vascular diseases in adulthood will require control of the risk factors early in life during childhood or adolescence (37, 38). The Task Force on Research in Pediatric Cardiovascular Disease identified the need for studies to determine the childhood antecedents of atherosclerotic vascular disease (35). However, there is little information about the physiology of the vascular wall in young individuals, including changes in vascular function that may result from normal puberty.

Puberty is a cascade of physiological changes in adolescent children associated with changes in production of hormones of the pituitary/gonadal axis. Hormonal changes at puberty lay a framework for biological differences in males and females that persist throughout life. With both hormonal and atherosclerotic changes beginning early in life, understanding the influence of natural puberty on vascular function is important if preventive strategies are to be developed specifically for the adolescent population.

Sex-steroid hormones modulate vascular function in adult humans and experimental animals (2, 4, 10, 12). For example, in women, observational and epidemiological studies suggest that estrogen replacement therapy provides primary prevention of coronary arterial disease when therapy is started in the perimenopausal stage but increases the risk of venous thrombosis (6, 19, 20, 25, 28, 46, 53). Estrogen has direct effects on the endothelium, smooth muscle, and interaction of platelets with the vascular wall (22, 30, 40, 41, 53). Mechanisms accounting for these effects of estrogen on the vascular wall include regulation of production of vasoactive factors from endothelial cells such as nitric oxide (NO), C-type natriuretic peptide (CNP), endothelin (ET)-1, prostacyclin, angiotsins converting enzyme, vascular adhesion molecules, and chemoattractants (40). Estrogen is known to regulate protein expression of endothelium-derived NO synthase (eNOS) by increasing the mRNA expression of eNOS (12). Much less is known about how other ovarian hormones, for example, progesterone, affect production of endothelium-derived factors, and controversies remain regarding whether progesterone counters effects of estrogen on the endothelium (34, 42, 47). However, plasma NO, skin blood flow, and baroreflex activity vary with the menstrual cycle in women (1, 17, 33, 43), suggesting that fluctuations in production of estrogen and progesterone in premenopausal women affect vascular functions. Little is known about the functions of...
the endothelium and smooth muscle during the hormonal transition to sexual maturity in females. Therefore, experiments were designed to determine endothelial and smooth muscle function before and after sexual maturity in female animals. Based on studies in postmenopausal women and ovariectomized animals (4, 6, 14, 39, 42, 44), loss of ovarian function decreased endothelium-dependent relaxations. If secretion of ovarian hormones increases with puberty, it can be hypothesized that endothelium-dependent relaxations would increase after the developmental transition to sexual maturity.

METHODS

This study was approved by the Mayo Foundation Institutional Animal Care and Use Committee, Mayo Clinic Rochester. Pigs were used as experimental animals for this study because their monthly estrous cycle is homologous to humans. Sixteen female pigs (8 juvenile and 8 adult) were included in this study. All pigs were received from the same vendor, and each was a four-cross breed of Yorkshire, Hampshire, Duroc, and Landrace strains. These pigs achieve puberty (sexually ready for breeding) at 3–4 mo of age. Therefore, juvenile pigs were 2–3 mo old, and the immediate postpubertal pigs were 5–6 mo old (adult) at the time of the experiments. Neither animals nor tissues were treated with exogenous sex steroids at any time before or during experiments. Female animals were housed within sight, sound, and smell of male pigs of comparable age.

General Methods

Animals were anesthetized with a mixture containing (in mg/kg) 1 ketamine HCl, 1 xylazaine HCl, and 2 telazol intramuscularly. Within 10 min of anesthetic administration, the carotid artery was exposed and cannulated. Blood was collected for measurement of 17β-estradiol, progesterone, NO, ET-1, and CNP. The cannula was removed, and pigs were exsanguinated via the carotid arteries. The heart was removed through a bilateral V-shaped cut through the ribs and placed in chilled modified Krebs-Ringer bicarbonate solution (control solution in mM: 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25.0 NaHCO3, 0.026 calcium disodium edetate, 11.1 glucose, pH 7.4). The uterus was removed and weighed. Aortic endothelial cells were scraped and stored for determination of eNOS mRNA and eNOS protein.

Measurement of Blood Parameters

For determination of NO, plasma was withdrawn into a syringe, transferred into siliconized vacuum tubes, and stored at −70°C. Oxidized products of NO were measured by chemiluminescence (Sievers NO analyzer model 270 B, Boulder, CO) by using a previously described technique (11). Plasma ET-1 (assay system from Nycomed Amersham, Bucks, UK) and CNP were measured by using radioimmunoassay (8, 51). Serum 17β-estradiol and progesterone were analyzed by the Clinical Steroid Laboratory of Mayo Clinic by using chemiluminescent technology (ACS-180 Bayer Diagnostics, East Walpole, MA). The detection limit with double-volume specimen extraction was 3.6 pg/ml for 17β-estradiol and 0.04 pg/ml for progesterone. Plasma ET-1 and CNP were measured by using previously established techniques of radioimmunoassay (8, 51).

Western Blotting for eNOS Protein Expression

Aortic endothelial cells from four adult and four juvenile pigs were used to determine eNOS protein expression by using a previously described method (31). The eNOS-specific primary antibody used was anti-eNOS monoclonal antibody isotype mouse IgG1 (1:1,000 dilution). Secondary antibodies were goat anti-mouse IgG-horseradish peroxidase conjugates. Colorimetric method using Opti-4CN Substrate kit (Bio-Rad) was used to determine protein expression on membranes, and the intensity of protein bands was analyzed by UN-SCAN-IT gel automated digitizing system through positive segmental analysis.

Real-Time Polymerase Chain Reaction for eNOS mRNA

Aortic endothelial cells from juvenile (n = 8) and adult (n = 8) female pigs were used for determination of mRNA expression for eNOS. RNA was extracted from the aortic endothelial cells in 1 ml of RNA STAT-60 (TEL-TEST, Friendswood, TX). Total RNA was extracted with 0.2 ml of chloroform and precipitated with 0.5 ml of isopropanol. After the supernatant was removed, the RNA pellet was washed with 1 ml of 75% ethanol, air dried, and then reconstituted with diethyl pyrocarbonate-treated water. RNA concentration was measured in each sample by using absorbance at 260 nm in the Beckman DU640 spectrophotometer.

RNA was treated with DNase by using DNase-free (Ambion, Austin, TX). The reverse transcriptase (RT) reaction was performed by using 1 μg of total RNA, with a final reaction volume of 50 μl, which consisted of 5 μM random hexamers, 500 μM deoxynucleotide triphosphates (dNTP), 5.5 mM MgCl2, 0.4 U/μl RNase inhibitor, 1.25 U/μl Multi-Scribe RT, 1× TaqMan RT buffer, and RNAse-free water (TaqMan Gold RT-PCR kit). After this, the cycling parameters used were incubated for 10 min at 25°C, reverse transcribed for 30 min at 48°C, and RT inactivated for 5 min at 95°C. The resultant cDNA samples were stored at −20°C.

Real-time polymerase chain reaction (PCR) was performed in accordance with guidelines from Applied Biosystems. A PAGE-purified 66-base pair porcine eNOS oligonucleotide standard was used (Intergrated DNA Technologies). The forward primer CAAAGTGCACATTGTTGACCAT (anneals between residues 1,251 and 1,272 at 58°C), the reverse primer TGGCTGTTCTCAGGTTGCTT (anneals between residues 1,316 and 1,297 at 60°C), and the dual-labeled probe sequence 5'-FAM-CGCACACAGTTCTTCTCAGGTTGCTT-3' were synthesized by Intergrated DNA Technologies. The cDNA was then amplified. A 25-μl reaction was performed with 5 μl of cDNA, 300 nM forward primer, 300 nM reverse primer, 100 nM of the target probe, and 1× TaqMan Universal PCR Master Mix and RNAse-free water. The reaction mixture for each sample was placed in duplicate on a 96-well plate and incubated in the ABI Prism 7700 detection system. PCR was performed at 50°C for 2 min and at 95°C for 10 min and then run for 45 cycles at 95°C for 15 s and at 60°C for 1 min. The threshold cycle number at which the initial amplification becomes detectable by fluorescence was determined. A standard curve was obtained with the threshold cycle number (y-axis) and copy number of cDNA (x-axis, starting quantity). The copy number of cDNA was determined for each RT sample as an approximation of mRNA copies. These computations were done through the in-built programming of the ABI Prism 7700 detection system. 18S rRNA was detected after RT reaction in each sample with real-time PCR to provide a control for RNA input and efficacy of the RT reaction (TaqMan ribosomal control reagents, Applied Bio-
systems). Quantification of mRNA for eNOS was expressed as a ratio of eNOS to 18S rRNA.

Organ-Chamber Experiments

The right coronary artery was isolated from the heart and placed in cold modified Krebs-Ringer bicarbonate solution. After adventitia was removed, 3-mm rings were cut from the artery. In some rings, endothelium was removed by gently rubbing the luminal surface with the tip of a microsurgical forcep. Rings were suspended between the fixed clip and a force transducer (Gould Statham UC-2, Cleveland, OH) in organ chambers filled with 25 ml of Krebs solution for measurement of isometric tension. Organ chambers were bubbled with a 95% oxygen-5% carbon dioxide mixture at pH 7.4 at 37°C. Tension on each ring was increased stepwise, and each ring was stimulated with KCl (20 mM) at each level of stretch until maximal tension was achieved. This level of tension was designated as "basal tension." After a thorough washout and equilibration at basal tension for 30 min, maximal contraction to KCl (60 mM) was obtained for each ring. After a 30-min recovery period, response to various drugs was studied as per protocols defined below. The order of drugs was kept the same for every experiment, and rings incubated in the absence or presence of inhibitors were studied in parallel.

Coronary arterial rings with and without endothelium were studied in parallel in the absence and presence of either indomethacin (10−5 M) or indomethacin plus NOS-1 (0.10−4 M) to inhibit cyclooxygenase and NOS, respectively. These inhibitors were added to the organ chambers 45 min before addition of the agonists and remained in contact with the tissue for the duration of the experiment. Rings were contracted with prostaglandin F2α (PGF2α; 2 × 10−6 M), and cumulative concentration response curves to UK-14304 (an α-adrenergic agonist, 5-bromo-6-(2-imidoxolino-2-yl-amo) quinoxaline, 10−8 to 10−6 M) and bradykinin (10−10 to 10−7 M) were obtained. After a washout and a 30-min recovery period, rings without endothelium were contracted with ET-1 (10−7 M), and cumulative concentration response to NO (3 × 10−8 to 10−5 M) was obtained.

To evaluate responses to CNP, rings with and without endothelium were set to basal tension with KCl, as described above. Rings were incubated for 30 min with either HS-142-1 (10−5 M, an inhibitor of particulate guanylate cyclase) or C-type atrial natriuretic factor (C-ANF; 10−5 M), inhibitor of natriuretic peptide clearance receptors. After contraction with PGF2α (2 × 10−6 M), cumulative concentration-response curves to CNP (10−9 to 3 × 10−7 M) were obtained.

Drugs and Chemicals Used in Organ Chamber Experiments

Indomethacin was dissolved in an aqueous solution of sodium bicarbonate. All other drugs used in organ chamber experiments were prepared in distilled water. All drug concentrations were expressed as the final molar concentrations in the organ chamber and were based on efficacy described from the literature (3, 4). PGF2α, L-NMMA, bradykinin, indomethacin, and all components of Krebs solution were purchased from Sigma Chemical (St. Louis, MO). CNP and C-ANF were purchased from Phoenix Pharmaceuticals (Belmont, CA), UK-14304 from Pfizer Research Central (Sandwich, UK), ET-1 from Peptide Institute (Louisville, KY), and HS-142-1 from Kyowa Hakko Kogyo (Tokyo, Japan). NO was prepared by using the method previously described by Palmer et al. (45).

Data Analysis

For organ-chamber studies, results are expressed as percent change in tension from contraction to PGF2α or ET-1. All data are expressed as means ± SE. GraphPad Prism software version 3.00 (San Diego, CA) was used for statistical analysis. For every concentration response curve, maximal relaxations were computed, and for sigmoidal curves effective concentrations producing half-maximal relaxations were also computed. For analyzing responses of rings with and without endothelium or rings in the absence and presence of inhibitors from the same animal, a two-tailed paired t-test was used. For comparing responses of rings from juvenile and adult female pigs, a two-tailed unpaired t-test was used. A P value of <0.05 was considered statistically significant for all tests.

RESULTS

Mean body weight was 46.2 ± 2.1 kg for juvenile pigs and 108.6 ± 3.1 kg (n = 8/group) for adult pigs. Uterine weight increased significantly with maturity from 43.6 ± 5 to 174 ± 37 g, so that the ratio of uterine weight to body weight increased from 0.93 ± 0.08 to 1.7 ± 0.29 g/kg (n = 8/group).

Blood Parameters

Serum 17β-estradiol was within the detection limits of the assay in seven of eight animals in both groups. The mean of measurable values was similar in juveniles and adult animals (Table 1). Progesterone was measurable in five of eight juvenile animals and three of eight adult animals. The mean of measurable values was not different between groups. However, the arithmetic mean for all values, when the animals with values below the detection limit of the assay were considered as zero, was about two-thirds lower in adult compared with juvenile animals (Table 1). Plasma NO was significantly higher in juvenile compared with adult pigs (Table 1, Fig. 1), whereas plasma CNP and ET-1 were comparable (Table 1).

Table 1. Circulating levels of hormones in serum and plasma of juvenile and adult female pigs

<table>
<thead>
<tr>
<th></th>
<th>Juvenile</th>
<th>Adult</th>
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<tr>
<td>17β-Estradiol, pg/ml</td>
<td>5.4 ± 1.2 (n = 7)</td>
<td>6.2 ± 3.5 (n = 7)</td>
</tr>
<tr>
<td>Progesterone, pg/ml</td>
<td>0.98 ± 0.16 (n = 5)</td>
<td>0.67 ± 0.15 (n = 3)</td>
</tr>
<tr>
<td>Plasma NO, pmol/l</td>
<td>33.8 ± 6.1 (n = 8)</td>
<td>19.9 ± 2.6 (n = 8)</td>
</tr>
<tr>
<td>CNP, pg/ml</td>
<td>65.52 ± 4.8 (n = 8)</td>
<td>80.59 ± 11.7 (n = 8)</td>
</tr>
<tr>
<td>Endothelin-1, pg/ml</td>
<td>4.57 ± 1.0 (n = 8)</td>
<td>4.08 ± 1.36 (n = 8)</td>
</tr>
</tbody>
</table>

Values are means ± SE. 17β-Estradiol and progesterone levels were measured in all animals (n = 8/group); however, means of sex-steroid hormones are reported for those animals in which values were above the detection limit of the assay. Number of pigs in which values were above the detection limit of the assay is shown in parentheses. For all juvenile pigs, progesterone ranged from 0 to 1.4 pg/ml with an arithmetic mean of 0.61 pg/ml; for all adult pigs, progesterone ranged from 0 to 0.8 pg/ml with an arithmetic mean of 0.25 pg/ml. CNP, C-type natriuretic peptide; NO, nitric oxide.
Western Blotting for eNOS Protein and Real-Time PCR for eNOS mRNA

Western blotting for eNOS protein identified a single band of protein with an estimated molecular weight of 140 in aortic endothelial cells in juvenile (\( n = 4 \)) and adult (\( n = 4 \)) pigs (Fig. 1B). Expression of eNOS protein was similar in both groups (Fig. 1B). Expression of mRNA for eNOS was also comparable in the two groups: the mean eNOS-to-18S ratio was 0.0037 ± 0.003 in juvenile vs. 0.0032 ± 0.002 in adult female pigs (\( n = 8 \) / group; Fig. 1C).

Organ Chamber Experiments

Contractions to KCl, PGF\(_{2\alpha}\), and ET-1. Contractions to KCl (60 mM) were significantly greater in rings from adult compared with juvenile pigs. No statistically significant differences were observed in contractions to PGF\(_{2\alpha}\) (\( 2 \times 10^{-6} \) M) or ET-1 (Table 2).

Relaxations

\( \alpha \)-Adrenergic agonist UK-14304. In juvenile and adult female pigs, UK-14304 (\( 10^{-8} \) to \( 10^{-6} \) M) caused concentration- and endothelium-dependent relaxations in coronary arterial rings contracted with PGF\(_{2\alpha}\), which were greater in rings with endothelium compared with those without endothelium (Fig. 2A). Maximal relaxations to UK-14304 were significantly greater in arteries from juvenile compared with adult pigs (Fig. 2A). In arteries from both juvenile and adult pigs, maximal relaxations to UK-14304 were not significantly inhibited by indomethacin but were reduced by indomethacin in combination with L-NMMA (Fig. 2, B and C).

Table 2. Comparison of contractions to KCl and PGF\(_{2\alpha}\) and endothelin-1 in coronary arteries of juvenile and adult female pigs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Endothelium</th>
<th>Juvenile Tension, g (( n = 8 ))</th>
<th>Adult Tension, g (( n = 8 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>With</td>
<td>8.0 ± 1.6</td>
<td>15 ± 2.6*</td>
</tr>
<tr>
<td></td>
<td>Without</td>
<td>5.3 ± 1.3</td>
<td>10.4 ± 1.7*</td>
</tr>
<tr>
<td>PGF(_{2\alpha})</td>
<td>With</td>
<td>5.6 ± 1.5</td>
<td>8.4 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Without</td>
<td>5.3 ± 0.7</td>
<td>7.7 ± 0.98</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>With</td>
<td>10.7 ± 1.3</td>
<td>13.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Without</td>
<td>10.5 ± 0.8</td>
<td>12.7 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Statistically significant differences (\( P < 0.05 \)) between rings from juvenile and adult female pigs.
Bradykinin. In rings contracted with PGF$_{2\alpha}$, bradykinin ($10^{-10}$ to $10^{-7}$ M) caused comparable concentration- and endothelium-dependent relaxations in coronary arterial rings from both juvenile and adult female pigs (Fig. 3A). In arteries from juvenile pigs, indomethacin alone did not affect relaxations to bradykinin, but addition of L-NMMA inhibited relaxations to bradykinin significantly more than indomethacin alone (Fig. 3B). In arteries from adult female pigs, no significant change in relaxations to bradykinin was observed with indomethacin alone or in combination with L-NMMA (Fig. 3C).

Fig. 2. Cumulative concentration-response curves to $\alpha_2$-adrenergic agonist (UK-14304) in coronary arterial rings with and without endothelium from juvenile ($n = 8$) and adult ($n = 8$) female pigs. Relaxations are shown as means $\pm$ SE of percent change in tension from contraction to PGF$_{2\alpha}$, which were similar between groups (Table 2) and not affected significantly by addition of antagonists. Relaxations to UK-14304 were greater in rings with endothelium compared with those without endothelium. In addition, relaxations in rings with endothelium were greater in arteries from juvenile compared with those from adult animals (controls; A). *Statistically significant difference in maximal relaxations to UK-14304 between rings with endothelium from juvenile and adult pigs ($P < 0.05$). Indomethacin ($10^{-5}$ M) did not significantly affect relaxations in juvenile (B) or adult pigs (C). Relaxations were significantly inhibited by indomethacin plus $N^G$-monomethyl-L-arginine (L-NMMA; $10^{-4}$ M) in both groups. *Statistically significant differences in maximal relaxations to UK-14304 in the absence and presence of indomethacin plus L-NMMA ($P < 0.05$; B and C).

Fig. 3. Concentration-response curves to bradykinin (BK) in coronary arterial rings with endothelium from juvenile ($n = 8$) and adult ($n = 8$) female pigs (controls; A). Relaxations are shown as means $\pm$ SE of percent change in tension from contraction to PGF$_{2\alpha}$, which were similar between groups (Table 2) and were not affected significantly by addition of antagonists. Relaxations to BK were significantly greater (denoted by asterisk) in rings with endothelium compared with those without endothelium in juvenile and adult pigs. In juvenile pigs (B), indomethacin plus L-NMMA caused a rightward shift of the curve (denoted by asterisk), whereas in adult pigs (C) no such change was observed. Statistical significance was accepted at $P < 0.05$. 

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NO. Concentration-dependent relaxations to NO (3 × 10⁻⁸ to 10⁻⁵ M) were observed in coronary arterial rings without endothelium from both juvenile and adult female pigs during contraction with ET-1 (10⁻⁷ M; Fig. 4A). These relaxations were greater in arteries from adult compared with those from juvenile female pigs (Fig. 4A). Relaxations of the smooth muscle to exogenous NO were not significantly altered by indomethacin, or indomethacin plus L-NMMA, in arteries from either juvenile (Fig. 4B) or adult (Fig. 4C) pigs.

CNP. Relaxations to CNP were significantly greater in rings without endothelium from juvenile compared with adult pigs (Fig. 5A). In both groups of pigs, relaxations to CNP were significantly attenuated by HS-142-1 but not by C-ANF (Fig. 5B and C). There were no statistically significant differences between responses of arteries from juvenile and adult animals in A and between relaxations in the absence and presence of HS-142-1 in B and C.
DISCUSSION

Results of the present study suggest that natural puberty in female pigs affects vascular functions such that endothelium-dependent relaxations mediated by NO are reduced whereas sensitivity of the smooth muscle cells to exogenous NO is increased.

Female pigs transition to sexual maturity at 6 mo of age (16). The pattern of hormones associated with ovarian function and ovulation (estrogen, progesterone, luteinizing hormone, and follicle-stimulating hormone) follow distinct patterns (16). In the present study, concentrations of estrogen and progesterone, when within the detection limit of the assay, were similar in juvenile and adult female pigs. However, progesterone levels were below the detection limit of the assay in over half of the adult animals. This observation is consistent with values reported in pubertal cycling female pigs 6 days before ovulation (16). Variation in progesterone between juvenile and adult animals suggests differences in ovarian function. Periodic sampling of blood from both groups of animals would have established patterns of cyclic variation in secretion of ovarian hormones and perhaps provided a more consistent representation of plasma concentrations of hormones. Large variability in 17β-estradiol in adult animals is most likely related to sampling the adult female pigs at different times related to ovulation, which can be influenced by environmental factors such as exposure to male pigs (16). Female pigs used in this study were housed in a common facility within sight, sound, and smell of male animals. Estrogen levels in adult female pigs housed under similar conditions show variability based on ovulation as evidenced by changes in external genitalia (4, 5). Physiological evidence that there were developmental differences in animals used in the present study is provided, albeit indirectly, by the significant increase in the ratio of uterine weight to body weight. Developmental transition to sexual maturity in female pigs may represent changes in pattern of secretion of gonadal hormones. Therefore, in adult females, the pattern of secretion of estrogen and progesterone in combination may be more important than the absolute concentration of either hormone as animals transition to sexual maturity. This concept could be tested in future experiments. Clinically, controversies exist regarding continuous compared with cyclic administration of progesterone as hormone therapy in postmenopausal women (21).

In a previous study (4), neither magnitude nor sensitivity of endothelium-dependent relaxations was different in animals with intact ovaries having low or high estrogen levels. Therefore, differences in endothelium-dependent responses observed between juvenile and adult animals in the present study may be due to changes in hormones other than estrogen or progesterone, such as those of the gonadal/pituitary axis, which were not measured in either study.

The ovarian hormone estrogen is known to affect the production of many endothelium-derived vasoactive factors such as NO, ET-1, CNP, and metabolites of arachidonic acid (8, 14, 18, 40). In the present study, plasma NO changed with puberty in female pigs. On the basis of observations in male-to-female transsexuals, androgen-deplete men, and female-male transsexuals, it has been suggested that estrogen upregulates the production of NO (23, 36, 44). In cultured endothelial cells, estrogen regulates NOS protein expression by genomic mechanism resulting in increased gene transcription (mRNA) (12) and nongenomic mechanisms involving release of NO from caveolin (50). Because estrogen levels were similar in both groups, as was mRNA and protein for eNOS, higher plasma NO observed in the juvenile compared with the adult female pigs may have resulted from differences in reactive oxygen species that would reduce bioavailable NO in adult female pigs. However, other posttranslational mechanisms for regulation of eNOS cannot be ruled out at this time. Increased sensitivity of rings without endothelium to exogenous NO is consistent with the observation that endogenous production of NO may be reduced in adult pigs. This increased sensitivity would maintain relaxations mediated by NO even when production is compromised.

Relaxations to UK-14304 are mediated by NO. Age differences in this response probably relate to receptor-coupled release of NO and not to differences in level of contractions because contractions to PGF2α represented ~70 and 60% of contractions to KCl in arteries from juveniles and adults, respectively.

Greater contractions to KCl may relate to greater smooth muscle mass of arteries as animals increase in size. However, contractions to PGF2α or ET-1 did not show proportional increases. An alternative explanation is that hormonal changes at puberty may alter regulation of membrane ion channels (22, 49, 58).

An endothelium-derived factor that shares a common mechanism of action with NO via activation of cyclic guanosine 5′-monophosphate is CNP. CNP causes vasodilation through activation of a receptor-bound guanylate cyclase and by activation of potassium channels leading to hyperpolarization of vascular smooth muscle (15, 29, 57). Expression of CNP is known to vary with the stage of estrous cycle (27). However, in the present study, no difference in plasma CNP was found between juvenile and adult female pigs. Consistent with another study (3), relaxations of the smooth muscle (rings without endothelium) to exogenous CNP were attenuated by inhibition of receptor-mediated particulate guanylate cyclase, but not by inhibition of natriuretic peptide clearance receptors in both groups of pigs. CNP also hyperpolarizes vascular smooth muscle (3, 7). Contraction to KCl also require changes in membrane potential, thus providing additional support to the idea that regulation or activation of potassium channels may change with sexual development.

ET-1 is an endothelium-derived factor that is a potent vasoconstrictor and functionally antagonizes relaxations to NO and CNP. Previous studies have suggested that, in the absence of ovarian hormones, mRNA expression for ET-1, as well as the protein per
se, increases (48, 56), and estrogen replacement reduces ET-1 (8). In the present study, no differences were found in plasma ET-1 levels between juvenile and adult females. However, plasma ET-1 may not be representative of total production because ET-1 is released abluminally (55).

Arachidonic acid is metabolized by cyclooxygenase into thromboxane, a vasoconstrictor, and prostacyclin, a vasorelaxant. Estrogenic regulation of cyclooxygenase increases production of prostacyclin (39). An important observation of the present study is that inhibition of cyclooxygenase did not affect agonist-stimulated endothelium-dependent relaxations in either arteries from juvenile or adult female pigs. These results suggest that eicosanoids are not the major mediator of agonist-stimulated relaxations of coronary arteries from juvenile female pigs contrary to what has been observed in prepubertal and adult males (4, 13).

Coronary arteries from both juvenile and adult pigs responded to bradykinin in an endothelium-dependent manner, consistent with the previously published reports (4, 32). Inhibition of NO synthase reduced this response in juvenile but not in adult female pigs, thereby suggesting that NO may be an important mediator of bradykinin-induced relaxations in juvenile but not adult female pigs. Like previous reports (4, 32), relaxations to bradykinin were not affected by inhibition of cyclooxygenase. Bradykinin releases a hyperpolarizing factor from the endothelium (9, 24, 26).

In conclusion, coronary endothelial and smooth muscle responses are selectively modulated during the transition to sexual maturity in female pigs. With transition to sexual maturity, plasma NO is reduced; endothelium-dependent relaxations mediated by NO are reduced, whereas sensitivity of the smooth muscle to exogenous NO is increased. These results at puberty are contrary to what might be expected based on changes in endothelium function after loss of ovarian hormones in adult animals. Therefore, an important conclusion from this study is that changes in endothelium function with maturity in female pigs may result from hormones other than estrogen alone, e.g., changes in the cyclic pattern of release of estrogen and progesterone or hormones of the pituitary–gonadal axis. This study provides the first systematic comparison of vascular function in female pigs transitioning to sexual maturity (i.e., puberty) and can form the basis for future studies directed toward understanding development of cardiovascular disease in young females.

DISCLOSURES

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REFERENCES


