Gender and transcriptional regulation of NO synthase and ET-1 in porcine aortic endothelial cells

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Wang, Xiaofang, Dustin A. Barber, Debra A. Lewis, Christopher G. A. McGregor, Gary C. Sieck, Lorraine A. Fitzpatrick, and Virginia M. Miller. Gender and transcriptional regulation of NO synthase and ET-1 in porcine aortic endothelial cells. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H1962–H1967, 1997.—Experiments were designed to determine whether normal fluctuations in sex steroid hormones alter gene transcription for endothelial nitric oxide synthase (NOS) and preproendothelin-1 (prepro-ET-1). Aortic endothelial cells were removed from adult, gonadally intact male and female or ovariectomized Yorkshire pigs. Endothelial cells were prepared for Northern blot analysis, Western blot analysis or enzyme activity. Nitric oxide products (NOx) and endothelin-1 (ET-1) in plasma were measured by chemiluminescence and radioimmunoassay, respectively. Northern blot analysis identified single bands corresponding to endothelial NOS and prepro-ET-1. Quantification of the blots showed an increase in expression of mRNA for both endothelial NOS and prepro-ET-1 in ovariectomized pigs compared with gonadally intact male and female pigs. There were no differences in amount of endothelial NOS protein identified by Western blot analysis among groups. On the contrary, plasma concentrations of NOx were significantly decreased in ovariectomized pigs, and there were no differences either in the concentrations of ET-1 in the plasma or extracts from the coronary arteries. These results suggest that expression of endothelial NOS and prepro-ET-1 may be regulated at transcriptional level by ovarian hormones. In addition, the ovarian hormones may regulate production of these endothelial-derived factors at the posttranscriptional level.

Endothelial cells were scraped from aortas of adult, gonadally intact male and female Yorkshire pigs (80–120 kg) and female pigs that had been ovariectomized for 4 wk. Blood samples were obtained from the femoral artery for measurement of plasma 17β-estradiol (primary antibody, rabbit estradiol-6-CMV-bovine serum albumin; secondary antibody, goat anti-rabbit; both obtained from Pentax, Santa Monica, CA; protocol available from Clinical Steroid Laboratory of Mayo Medical Laboratories, Rochester, MN) and ET-1 by radioimmunoassay (human/porcine ET-1 antibody, Amersham International, Amersham, UK; Ref. 12) or oxidized products of NO (NOx) by chemiluminescence.

Northern blot analysis. Aortic endothelial cells from individual animals were placed directly into RNA STAT-60 (Tel- testB, Friendswood, TX). Poly(A)+ RNA was isolated following the protocols of a commercial kit (PolyATtract mRNA Isolation System, Promega, Madison, WI). Isolated mRNA was quantified by measuring the optical density at 260- and 280-nm wavelengths. mRNA from animals of the same gender or hormone status were combined, and 1.5 µg of mRNA were denatured by heating (65°C) in 50% formamide-4.4 M sodium citrate. After the transfer, membranes were baked in a vacuum oven at 80°C for 2 h. Membranes were prehybridized for 30 min at 65°C in Rapid-Hyb Solution (Amersham) and hybridized with herring sperm DNA and 32P-labeled bovine endothelial NOS probe (4 kb, gift from Dr. William Sessa, Yale University, New Haven, CT) or porcine prepro-ET-1 probe (1.8 kb) for 16–18 h at 42°C. After washing, nitric oxide synthase; endothelin-1
kb; gift from Dr. Masashi Yanagisawa, University of Texas, Southwestern Medical Center at Dallas, TX) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (600 bp, gift from Dr. Bruce Kline, Mayo Foundation, Rochester, MN) for 2.5 h at 65°C. After hybridization, membranes were washed in 2× SSC-0.1% sodium dodecyl sulfate (SDS) for 20 min at room temperature, followed by washing twice in 0.1× SSC-0.1% SDS for 15 min at 65°C. Membranes were dried and exposed to X-ray film at −70°C for 4 days.

Western blot analysis. Aortic endothelial cells from individual animals were placed immediately into 250 µl of isolation buffer (0.5 M tris(hydroxymethyl)aminomethane (Tris)-10% SDS) and boiled for 5 min. Boiled endothelial cells were then passed through a 27-gauge needle multiple times to decrease viscosity. The sample then was centrifuged at 2,500 g for 5 min at 4°C. Protein in the detergent-extracted homogenate was measured by bicinchoninic acid protein assay (Pierce, Rockford, CA) with bovine serum albumin as a standard. Protein from the detergent-extracted homogenate (60 µg/lane) was separated on 7.5% SDS-polyacrylamide gel by electrophoresis and transferred to nitrocellulose. After blocking in Tris-buffered saline containing 5% nonfat dry milk for 1 h, the membrane was washed and immunoblotted with the monoclonal mouse anti-endothelial NOS antibody (American Cyanamid, Princeton, NJ) at a dilution of 1:1000. Membranes were then washed with 2 ml of water while continuing to collect into the scintillation fluid. [3H]citrulline activity was detected using a Beckman 6800 liquid scintillation counter. Incubations containing 150 µl protein-free homogenization buffer previously passed over a desalting column were used as “blank” controls. Activity calculations account for scintillation counting efficiency and the ratio of [3H]arginine to nonradioactive L-citrulline in the incubation mixture.

NO production by endothelial NOS is presumably in a 1:1 molar ratio with L-citrulline, and thus endothelial NOS activity is expressed as picomoles of L-[3H]citrulline produced per milligram of protein per hour. Ca2+-dependent activity equals total activity minus Ca2+-independent activity after correcting for nonspecific activity.

Measurement of NO. Plasma NOx was measured by chemiluminescence (Sievers NO analyzer, model 270B, Boulder, CO). NOx was added to NO by 0.1 M vanadium(III) (Aldrich Chemical, Milwaukee, WI) in 3 M hydrochloric acid. At 85°C, vanadium(III) reduces NOx to NO (1). Standard curves for sodium nitrite (50–2,000 pmol, Sigma) and potassium nitrate (50–2,000 pmol, Fisher Scientific) were obtained every day before analysis of the plasma samples. Plasma samples (100 µl) were injected into the reducing solution.

Table 1. Concentration of 17β-estradiol, progesterone, and testosterone in plasma of female and male pigs

<table>
<thead>
<tr>
<th>Gender</th>
<th>17β-Estradiol (pg/ml)</th>
<th>Progesterone (pg/ml)</th>
<th>Testosterone (pg/ml)</th>
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<tbody>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High estrogen</td>
<td>21.1 ± 2.7</td>
<td>1.3 ± 1.3</td>
<td>2.9 ± 1.6</td>
</tr>
<tr>
<td>Low estrogen</td>
<td>&lt; 10</td>
<td>5.2 ± 2.3</td>
<td>0.8 ± 0.8</td>
</tr>
<tr>
<td>OVX</td>
<td>4</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Males</td>
<td>21</td>
<td>44.9 ± 8.1</td>
<td>0.2 ± 0.1</td>
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<td></td>
<td></td>
<td></td>
<td>260.6 ± 39.4*</td>
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Values are means ± SE; n, no. of animals. Lower limit of assay is represented by < 10 pg/ml. OVX, ovariectomized. *Statistically greater than values for females, P < 0.05 (analysis of variance).

L-[3H]citrulline was accomplished by passing the assay mixture over Poly-Prep chromatography columns (Bio-Rad) loaded with 1 ml of equilibrated AG 50W-X8 Na+ form 200–400 mesh molecular biology grade resin (Bio-Rad), and the eluate was collected into 18 ml of Opti-Fluor (Packard, Meriden, CT). The eluate 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. Incubations containing 150 µl protein-free homogenization buffer previously passed over a desalting column were used as “blank” controls. Activity calculations account for scintillation counting efficiency and the ratio of L-[3H]arginine to nonradioactive L-citrulline in the incubation mixture.

NO produced by endothelial NOS is presumably in a 1:1 molar ratio with L-citrulline, and thus endothelial NOS activity is expressed as picomoles of L-[3H]citrulline produced per milligram of protein per hour. Ca2+-dependent activity equals total activity minus Ca2+-independent activity after correcting for nonspecific activity.

Male HE2 LE2 OVX

Fig. 1. Representative Northern blot of mRNA for endothelial (ec) nitric oxide synthase (NOS) and preproendothelin-1 (prepro-ET-1) derived from aortic endothelial cells of sexually mature male, high-estrogen (HE2), low-estrogen (LE2), and ovariectomized (OVX) female pigs. Each lane contained poly(A) RNA (1.5 µg/ lane) from 2 or 3 pigs. Endothelial cells were hybridized with bovine endothelial NOS or porcine prepro-ET-1 probes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.
Output from the NO analyzer was recorded on Shimadzu Chromatopac Integer (model CR601, Shimadzu, Japan). The calculated areas of the output signal were used for both standard curves and plasma samples.

Assay for endothelin. Segments of right and left coronary arteries were blotted on filter paper and frozen in liquid nitrogen. The frozen tissues were pulverized and the fragments placed in 15 vol/wt solution of 1 M acetic acid and 2 mM HCl at 25°C. The suspensions were placed in a 100°C water bath for 3 min and then homogenized. The homogenates were centrifuged for 30 min at 4°C. The supernatant was frozen and subsequently extracted for the measurement of ET-1 by radioimmunoassay (Amersham), as previously described (12). The lower level of detection for this assay is 0.5 pg endothelin/ml.

Data analysis. All data are expressed as means ± SE. Autoradiograms for Northern and Western blots were analyzed by spectrophotometry (Beckman Spectrophotometer 640). Densitometry values for Northern blots were normalized to the GAPDH to serve as an internal control for gel loading. Analysis of variance was employed to access statistical significance. If a significant F value resulted from an analysis of variance, Scheffe’s test for multiple comparisons was used to identify differences among groups. *P < 0.05 was considered to indicate either a significant correlation or difference among means.

RESULTS

Plasma concentrations of 17β-estradiol ranged from undetectable in gonadally intact females to 50 pg/ml. Based on plasma 17β-estradiol, the gonadally intact females were grouped as having either high (10–50 pg/ml)- or low (<10 pg/ml)-estrogen status (Table 1). In ovariectomized pigs, 17β-estradiol was <10 pg/ml. Plasma 17β-estradiol concentrations were similar among males and high-estrogen female pigs. Plasma testosterone was significantly greater in males compared with female pigs (Table 1).

Northern blot analysis. Northern blot analysis revealed bands at 4.4 kb for endothelial NOS and 2.3 kb for prepro-ET-1 mRNA in all groups. Representative Northern blots are shown in Fig. 1. Expressions of both endothelial NOS and prepro-ET-1 was increased significantly in OVX compared with male and female pigs. *P < 0.05 (analysis of variance, ANOVA).

Fig. 2. Quantification of Northern blots of mRNA for ecNOS (A) and prepro-ET-1 (B) derived from aortic endothelial cells of sexually mature and ovariectomized pigs. Each bar represents mean ± SE of band densities relative to GAPDH: male, n = 11 lanes with mRNA from 2 to 3 pigs/lane (total 26 pigs); HE, female, n = 8 lanes with mRNA from 2 to 3 pigs/lane (total 19 pigs); LE, female, n = 11 lanes with mRNA from 2 to 3 pigs/lane (total 22 pigs); and OVX, n = 3 lanes with mRNA from 1 to 2 pigs/lane (total 5 pigs). Expression of mRNA for endothelial NOS and prepro-ET-1 was increased significantly in OVX compared with male and female pigs. *P < 0.05 (analysis of variance, ANOVA).

Fig. 3. A: quantification of Western blot for endothelial NOS from aortic endothelial cells of sexually mature male and female and OVX pigs. Protein from detergent-extracted homogenate (60 µg/lane) of aortic endothelial cells was separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Immunoblot was performed with mouse-anti-ecNOS (Signal Transduction Laboratories, Lexington, KY) monoclonal antibody and detected with chemiluminescence. Each bar represents mean ± SE of spectrophotometric analysis of blots from 3 pigs within each group. B: Western blot identifies a single band with an estimated molecular mass (biotinylated standards, Kaleidoscope Perstained Standards, Bio-Rad) of 140 kDa for endothelial NOS in aortic endothelial cells from male, female, and OVX pigs.
low-estrogen female pigs (Fig. 2). The expression of GAPDH mRNA did not differ significantly among the groups.

Western blot analysis. Western blotting identified a single band of protein from the detergent-extracted homogenate with an estimated molecular mass of 140 kDa for endothelial NOS in all groups (Fig. 3B). There were no significant differences among groups (Fig. 3).

Activity of NOS. There were no significant differences in total activity when corrected for nonspecific activity or Ca\(^{2+}\)-dependent (endothelial NOS) activity in enzymes isolated from a soluble homogenate fraction of aortic endothelial cells of male, high- and low-estrogen female, and ovariectomized pigs (Fig. 4). Ca\(^{2+}\)-independent activity ranged from <1 to 28% total activity and did not differ among groups.

Measurement of NO and ET-1. Concentrations of NO\(_{x}\) were significantly lower in plasma of ovariectomized female compared with gonadally intact, high-estrogen female pigs (Fig. 5). ET-1 in plasma and extracts of coronary arteries did not differ among male, female, and ovariectomized pigs (Table 2). ET-1 and endothelial NOS is increased, suggesting that both endothelium-derived factors may be regulated at the transcriptional level by sex steroid hormones. It was not possible in this study to determine whether the transcriptional regulation of the gene was due to the direct action of the hormones binding to the promoter regions or indirect modulation of another second messenger system. In support of an indirect action of estrogen affecting transcription of endothelial NOS are observations that the 5'-promoter region of the human endothelial NOS gene does not contain the full palindrome for the estrogen receptor (13).

Alternations in prepro-ET-1 and endothelial NOS mRNA may reflect changes in the rate of degradation of the mRNA or negative feedback of the final product NO or ET-1 on other regulatory factors. The lack of increase in mRNA for endothelial NOS in pigs with high estrogen levels is not in agreement with other studies in which exogenous estrogen treatment increased mRNA for endothelial NOS in extracts of brain and skeletal muscle or cultured endothelial cells (8, 28). The present study used isolated endothelial cells from aorta of animals exposed to endogenous levels of hormones rather than whole tissue homogenates, which contain many cell types. In addition, interactions among endogenous hormones may not represent conditions in which a single hormone is replaced to animals or cultured cells. The molecular mechanisms that regulate prepro-ET-1 and endothelial NOS expression may also differ among species and vascular beds.

Because there is a change in mRNA for endothelial NOS after ovariectomy but no change in the amount of endothelial NOS protein, sex steroid hormones may also regulate endothelial NOS at the posttranscriptional level. Indeed, plasma concentrations of NO\(_{x}\) were less in ovariectomized pigs compared with females with high estrogen levels, even though NOS protein and activity were the same. Some caution should be exercised in interpretation of activity of isolated enzymes as the assay is optimized for substrate and cofactors that may not represent intracellular conditions in which substrate, cofactors and myristylation of the enzyme may be limiting (4, 5, 16, 21, 25). However, the greater plasma NO\(_{x}\) in high-estrogen pigs compared with ovariectomized pigs is consistent with other studies which show a significant positive correlation between 17β-estradiol and NO\(_{2}/\text{NO}_3\) levels in women (6, 9, 22). In vivo, shear stress-induced release of NO and antioxidant effects of estrogen may also act to influence NO products measured in plasma at posttranscriptional regulatory sites (7). Differences in plasma NO\(_{x}\) probably do not reflect additive activity.

### Table 2. Concentrations of endothelin-1 in plasma and extracts from porcine coronary arteries

<table>
<thead>
<tr>
<th></th>
<th>Male (pg/ml)</th>
<th>Female (pg/ml)</th>
<th>OVX (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>18.9 ± 3 (8)</td>
<td>18.4 ± 6 (12)</td>
<td>17.4 ± 0.1 (9)</td>
</tr>
<tr>
<td>Arterial extracts</td>
<td>13.7 ± 4.9 (6)</td>
<td>14.0 ± 2.1 (10)</td>
<td>10.3 ± 6.7 (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE for no. of animals given in parentheses. ET-1, endothelin-1.
of endothelial and inducible NOS, since Ca\(^{2+}\)-independent NO activity, a measure of inducible NOS activity, was minimal and did not differ among groups.

Another unexpected finding is that plasma NOx was not different between male and female pigs. This finding is inconsistent with observations in isolated perfused aorta in male and female rats. This difference may reflect the high concentrations of estrogen in plasma of male pigs, which are probably the result of metabolism of testosterone by aromatase in fat tissue.

Contrary to findings in human transsexuals (20), circulating concentrations of ET-1 did not change significantly with ovariectomy or in the presence of testosterone. These differences could reflect the concentrations and treatment regimen of hormone replacement in the humans compared with an endogenous source of hormones or high endogenous concentrations of estrogen in the male pig. An alternative interpretation of the data based on the similarity in mRNA for prepro-ET-1. It is not clear as to how circulating concentrations of ET-1 correlate with local production, since ET-1 is released preferentially to the abluminal side of the blood vessel (26). However, ET-1 in homogenates of the coronary arteries was also similar between genders and females regardless of their estrogen status. Sensitivity of the assay to detect ET-1 bound to extracellular matrix may limit the value of these extraction methods, since positive immunostaining for endothelin-1 can be detected in adventia of arteries (10). Because mRNA for prepro-ET-1 was elevated with ovariectomy and changes in protein (ET-1) concentrations were not evident, sex steroid hormones may also regulate other enzymes of the endothelin cascade.

In summary, the present study suggests that ovarian steroid hormones regulate production of endothelium-derived factors, ET-1, and endothelin NOS at both gene transcriptional and posttranscriptional sites.

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