Impaired Acetylcholine-Induced Release of Nitric Oxide in the Aorta of Male Aromatase-Knockout Mice

Regulation of Nitric Oxide Production by Endogenous Sex Hormones in Males

Masahiko Kimura, Krishnankutty Sudhir, Margaret Jones, Evan Simpson, Ann-Maree Jefferis, Jaye P.F. Chin-Dusting

Abstract—The consequences of estrogen deficiency on the cardiovascular system have been widely examined in females. The effects of endogenous estrogen deficiency in males are less clear. The aromatase-knockout (ArKO) mouse lacks a functional Cyp19 gene, which encodes aromatase, and is thus incapable of synthesizing endogenous estrogen. In the present study, we examined the effect of lack of endogenous estrogens on vascular function in aortic rings isolated from male ArKO mice and compared these effects to rings from wild-type (WT) littermates. Full concentration-response curves to norepinephrine, acetylcholine, isoprenaline, and sodium nitroprusside were obtained in the absence and presence of the nitric oxide synthase inhibitor N\textsuperscript{-}nitro-L-arginine in aortic segments set up in isometric myographs. Responses to norepinephrine were not different in aorta from ArKO compared with WT mice. Both N\textsuperscript{-}nitro-L-arginine and endothelium denudation significantly shifted the norepinephrine concentration-response curve to the left; however, this shift was not different in ArKO compared with WT. Responses to the endothelium-dependent vasodilator acetylcholine were significantly blunted in aortic rings from ArKO mice (Emax, 58.2 ± 0.9% and 34.0 ± 0.5% in wild-type and ArKO, respectively; P < 0.05), whereas responses to the endothelium-independent agonist sodium nitroprusside and to the partial endothelium-dependent agonist isoprenaline were not affected. These findings suggest that endogenous estrogen facilitates vasorelaxation in males. This may be via modulating endothelial function rather than vascular smooth muscle cell responsiveness. (Circ Res. 2003;93:1267-1271.)

Key Words: aromatase knockout ■ mice ■ nitric oxide ■ endothelium

It is well recognized that women are relatively protected against cardiovascular disease in comparison with men, suggesting a cardiovascular benefit of endogenous estrogen in women. Although in men estrogen are produced in significant quantities by local tissue aromatization of androgenic precursors from the testes and adrenal glands,\textsuperscript{1} there has been relatively limited study of the biological role of these hormones in males. There is now increasing evidence for a cardiovascular role of estrogen in men.\textsuperscript{2} Evidence from a young male with estrogen insensitivity caused by a disruptive mutation in the estrogen receptor gene suggests that estrogen may play an important role not only in bone metabolism\textsuperscript{3} but also in arterial health.\textsuperscript{4,5} The role of endogenous estrogen on vascular function in males was also addressed in a study in male homozygous estrogen receptor-α knockout mice (ERαKO) in which a diminished basal release of nitric oxide was observed.\textsuperscript{6}

Aromatase catalyzes the final step in the biosynthesis of C\textsubscript{18} estrogens from C\textsubscript{19} steroids; it is encoded by the Cyp19 gene.\textsuperscript{7} Recently, a number of mutations of the aromatase gene have been described in humans that give rise to complete estrogen deficiency. Such patients have low HDL, increased total and LDL cholesterol concentrations and triglycerides, and hyperinsulinemia,\textsuperscript{8} but it is unclear if they have other cardiovascular abnormalities. Mice lacking a functional aromatase enzyme (ArKO) have been generated by targeted disruption of the Cyp19 gene.\textsuperscript{9} The absence of detectable estradiol levels in the plasma of these mice\textsuperscript{9} make them a useful model for the study of the role of endogenous estrogen on vascular function. In the present study, we examined the effects of estrogen deficiency on vascular responses to endothelium-dependent and independent vasodilators in male ArKO mice. Our findings indicate that nitric oxide release stimulated by acetylcholine is diminished in estrogen deficiency in male ArKO mice.

Materials and Methods

Tissues were obtained from animals in studies approved by the Animal Ethics Committee of the Monash Medical Centre, which...
adheres to the Declaration of Helsinki with regards to animal experimentation.

**Mice**

Development of the ArKO mouse was accomplished by disrupting the Cyp19 gene as previously described. Ovaries from the female ArKO mouse were demonstrated to not have any aromatase activity and to not produce estrogen. Estrogen levels in male ArKO mice are nondetectable. Heterozygous males and females were bred to produce wild-type (wt) and homozygous-null offspring (ArKO); these were genotyped by PCR as described. To date, no polymorphisms have been reported in the mouse Cyp19 gene, first sequenced in 1991. Although it is recognized that aromatase polymorphisms may influence the cardiovascular system, we believe that in this instance it is highly unlikely. All animals were maintained under pathogen-free conditions and had drinking water and soy-free mouse chow (Glen Forrest Stockfeeders, Western Australia) ad libitum.

**Isolated Aortic Rings: Preparation of Vessels**

Mice (11 to 24 weeks old) were euthanized by detrusion. The aorta was quickly removed and placed into ice-cold Krebs solution (composition in mmol/L: NaCl 119, KCl 4.7, KH2PO4, 1.18, MgSO4 1.17, NaHCO3 25, CaCl2 2.5, EDTA 0.026, and glucose 5.5) where it was freed of all fat and connective tissue. Aortic ring segments (2 mm in length) were then cut and each segment mounted into separate Mulvany myograph chambers. This involved placing each ring onto two stainless steel wires (40 µm in diameter) in an isometric myograph (Myograph Model 610 mol/L, JP Trading, Denmark) filled with Krebs solution (5 mL) oxygenated with 95% O2 and 5% CO2 mixture and maintained at 37°C. Changes in isometric force were recorded online using a MacLab/8e (AD Instruments Inc) data acquisition system linked to an Apple Macintosh computer. After a 30-minute equilibration period, each vessel was subjected to a passive length-tension stretch. This procedure enabled each vessel to be normalized to an internal circumference equivalent to 90% the transmural pressure of 100 mm Hg. A further equilibration period of 30 minutes was observed before the application of any drugs.

**Study Protocol**

Full concentration-response curves to noradrenaline (1 nmol/L to 100 µmol/L) were obtained (13 vessels from 13 different WT mice). Of these, 9 were obtained in the absence and presence (9 vessels from the same 9 mice) of the nitric oxide synthase inhibitor, nitro-L-arginine (10 µmol/L) and 4 were matched with a separate ring (denuded of the endothelium by gentle rubbing) from the same animals. A similar protocol was used for ArKO mice (n=16 vessels from 16 different ArKO mice; 11 ± nitro-L-arginine; 5 ± denudation).

Full concentration-response curves to acetylcholine (1 nmol/L to 100 µmol/L), isoprenaline (1 nmol/L to 100 µmol/L), and sodium nitroprusside (1 nmol/L to 100 µmol/L) were constructed using vessels preconstricted with noradrenaline (100 nmol/L) in the absence and presence of the nitro-L-arginine. Again, only one concentration-response curve (with or without inhibitor) was constructed on any one vessel from any one animal.

Full concentration-response curves to acetylcholine were also constructed in the absence and presence of β-estradiol (10 µmol/L; n=4 in wild-type; n=6 in ArKO).

**Data Analysis**

The tension of each vessel at rest (ie, that during the equilibration period post normalization) was defined as 100% relaxation. The tension augmented by norepinephrine (100 nmol/L) was defined as 0% relaxation. The proportion of relaxation induced by vasorelaxative agents was calculated for each concentration. In the case of the concentration-constriction curve to norepinephrine, the tension developed by each concentration of norepinephrine was normalized by the diameter of the vessel obtained by the normalization procedure.

**Results**

Mean diameters of aortic ring segments were not different in the two groups (wild-type versus ArKO, 999.45±18.49 versus 1006.81±17.00 µm; P>0.05).

Norepinephrine caused a concentration-dependent vasoconstriction in aortic rings from both strains of mice (Figure 1). Maximal force obtained was 0.63±0.05 g/mm (n=13) and 0.77±0.08 g/mm (n=14) in wild-type and ArKO mice, respectively. Although there was a trend toward an increased response in ArKO to noradrenaline, this was not statistically significant (P>0.05). The potency of norepinephrine was not different in the two groups (negative log EC_{50} wild-type versus ArKO, 7.10±0.14 versus 7.27±0.11 mol/L; P>0.05). N^6-nitro-L-arginine augmented the contractile responses to norepinephrine and increased potency significantly in both groups (Table 1). However, the degree of augmentation was not different in the transgenic mice compared with wild-type. Similarly endothelium denudation augmented responses to norepinephrine in both groups but the degree of augmentation was not different in transgenic animals compared with wild-type (Table 1).

Acetylcholine produced concentration-dependent aortic vasorelaxation in both groups. Maximal dilatation achieved for acetylcholine was significantly diminished in ArKO mice compared with wild-type (E_{max}, 51.44±4.55% and
TABLE 1. Responses to Noradrenaline in Aorta Obtained From Wild-Type and ArKO Mice

<table>
<thead>
<tr>
<th>Noradrenaline</th>
<th>−log EC50</th>
<th>Emax, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type before NOLA (10 μmol/L; n=9)</td>
<td>7.44±0.11</td>
<td>0.60±0.06</td>
</tr>
<tr>
<td>Wild-type after NOLA (10 μmol/L; n=9)</td>
<td>7.92±0.28</td>
<td>0.85±0.09*</td>
</tr>
<tr>
<td>ArKO before NOLA (10 μmol/L; n=11)</td>
<td>7.28±0.15</td>
<td>0.85±0.10</td>
</tr>
<tr>
<td>ArKO after NOLA (10 μmol/L; n=11)</td>
<td>8.29±0.20*</td>
<td>0.99±0.09*</td>
</tr>
<tr>
<td>Wild-type intact endothelium (n=5)</td>
<td>7.01±0.11</td>
<td>0.51±0.07</td>
</tr>
<tr>
<td>Wild-type denuded (n=5)</td>
<td>7.66±0.13*</td>
<td>0.52±0.11</td>
</tr>
<tr>
<td>ArKO intact endothelium (n=7)</td>
<td>7.20±0.11</td>
<td>0.60±0.1</td>
</tr>
<tr>
<td>ArKO denuded (n=7)</td>
<td>7.85±0.16*</td>
<td>0.56±0.07</td>
</tr>
</tbody>
</table>

*P<0.05 before vs after intervention. Emax indicates maximal response; −log EC50, negative log concentration producing 50% maximal response.

36.63±4.06% in wild-type versus ArKO, respectively; P<0.01; Table 2, Figure 2). Nω-nitro-L-arginine abolished the endothelium-dependent vasorelaxation induced by acetylcholine in both groups, the degree of blockade was not significantly different in ArKO compared with wild-type (Table 2, Figure 2). Nω-nitro-L-arginine at both 10 and 100 μmol/L produced a similar degree of inhibition confirming that maximal antagonism had been achieved (Figure 2). Incubation with β-estradiol (10 μmol/L) had no influence on the concentration-response curve to acetylcholine (Table 2).

Both isoprenaline and sodium nitroprusside also produced concentration-dependent vasorelaxation (Table 3). The deficiency of aromatase had no effect on the response of either of these agonists either in the absence or presence of nitro-L-arginine. Responses to isoprenaline were significantly blunted by Nω-nitro-L-arginine (Table 3) although there was a large dilatory residue effect following blockade with the nitric oxide synthase inhibitor, which suggests that isoprenaline acts both via releasing nitric oxide as well as by direct smooth muscle vasodilatation. Responses to sodium nitroprusside were not affected by Nω-nitro-L-arginine.

TABLE 2. Responses to Acetylcholine in Aorta Obtained From Wild-Type and ArKO Mice

<table>
<thead>
<tr>
<th>Acetylcholine</th>
<th>−log EC50</th>
<th>Emax, g/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (n=17)</td>
<td>7.13±0.16</td>
<td>51.44±4.55</td>
</tr>
<tr>
<td>Wild-type after NOLA (10 μmol/L; n=10)</td>
<td>6.35±0.48*</td>
<td>16.28±11.67*</td>
</tr>
<tr>
<td>Wild-type after NOLA (100 μmol/L; n=5)</td>
<td>ND</td>
<td>−4.26±1.86*</td>
</tr>
<tr>
<td>ArKO (n=25)</td>
<td>6.80±0.13</td>
<td>36.63±4.06#</td>
</tr>
<tr>
<td>ArKO after NOLA (10 μmol/L; n=10)</td>
<td>6.39±0.42</td>
<td>1.47±6.92*</td>
</tr>
<tr>
<td>ArKO after NOLA (100 μmol/L; n=5)</td>
<td>6.54±0.80</td>
<td>−23.03±16.95*</td>
</tr>
<tr>
<td>Wild-type before β-estradiol (n=4)</td>
<td>7.24±0.18</td>
<td>53.05±7.33</td>
</tr>
<tr>
<td>Wild-type after β-estradiol (10 μmol/L; n=6)</td>
<td>7.28±0.14</td>
<td>49.89±9.99</td>
</tr>
<tr>
<td>ArKO before β-estradiol (n=6)</td>
<td>7.02±0.18</td>
<td>58.83±4.85</td>
</tr>
<tr>
<td>ArKO after β-estradiol (10 μmol/L; n=7)</td>
<td>7.34±0.08</td>
<td>48.71±0.06</td>
</tr>
</tbody>
</table>

#P<0.05 wild-type vs ArKO; *P<0.05 before vs after intervention.

Discussion

The present work represents the first study to examine cardiovascular function in mice in which the activity of aromatase, the enzyme responsible for estrogen biosynthesis, is eliminated by targeted disruption of the cyp19 gene.⁹ In these mice, there is an absence of detectable levels of estradiol in the plasma, as well as marked elevation in levels of testosterone and the gonadotropins, LH, and FSH. The high testosterone levels in the male presumably reflect stimulation of the interstitial cells of the testes by the high circulating LH levels.⁹

The present study demonstrates clearly that endogenous estrogen is necessary for normal vascular function in male mice. Estrogen deficiency, as observed in the male ArKO mouse, results in a diminished response to the endothelium-dependent agonist acetylcholine, a response shown to be totally dependent on nitric oxide production in this model. In
addition, we observed that the potency of the vasoconstrictor response to noradrenaline tended toward being enhanced in the ArKO mouse after inhibition, although this was not statistically significant. The responses to isoprenaline and sodium nitroprusside were not altered by endogenous estrogen deficiency. Our observations parallel similar findings in postmenopausal women and confirm a regulatory vascular role for endogenous estrogen in males.

The present findings are also in accordance to those previously reported for both the estrogen receptor-deficient male and for the ERα KO mice in which the estrogen receptor is inactivated by targeted disruption albeit with some differences. In the estrogen α-receptor-deficient male, Sudhir et al. reported the presence of early coronary artery disease, and an absence of flow-mediated endothelium-dependent vasodilation in a peripheral conduit artery, a response that is known to be nitric-oxide mediated. These observations suggest that estrogen deficiency in males is a cardiovascular risk factor. In aortic rings isolated from male homozygous estrogen receptor α knockout (ERα KO) mice, Rubanyi and colleagues showed that basal release of EDNO (determined by endothelium-dependent contraction caused by NOS-nitroarginine) was significantly lower in the aorta of male ERα KO compared with wild-types. Acetylcholine-induced endothelium-dependent relaxation was similar in all groups studied, unlike the impaired response to acetylcholine observed in ArKO mice in the present study. It should be noted however that the ERα KO mice used were incomplete knockouts and have been reported to have preserved vascular function. As well, it is debatable whether estrogen receptors α or β play the responsible role with regards the role of estrogen on the vasculature.

In women, the association between estrogen administration and nitric oxide release has been well studied. Physiological levels of estrogen enhances acetylcholine-induced vasorelaxation in the forearm and coronary beds in postmenopausal women. Short intravenous and intracoronary infusions of estrogen in women attenuate acetylcholine-induced vasoconstriction in vivo in atherosclerotic coronary arteries. Studies in perimenopausal women and elderly men have shown greater basal nitric oxide release in the forearm vasculature, after eight weeks of estrogen supplementation. Consistent with these observations, studies in human umbilical vein endothelial cells demonstrate upregulation of endothelial nitric oxide synthase after exposure to estrogen. Conversely, estrogen deficiency is associated with endothelial dysfunction. These observations are consistent with our findings of impaired stimulated release of nitric oxide in the aorta of mice that are deficient in aromatase, and thus incapable of synthesizing estrogens from their precursors. In the present study, incubation of aortic rings from either wild-type or ArKO in 17β-estradiol 10 μmol/L had no effect on the response to acetylcholine. This may be because the effects of estrogen in the current protocol require a longer time line than that allowed by in vitro incubation.

The high levels of testosterone to which the vasculature of ArKO mice are exposed may have contributed to the endothelial dysfunction observed in these animals. Testosterone has been reported to enhance apoptosis-related damage in endothelial cells in culture, and impair endothelial function in animals and humans. In a rabbit model of atherosclerosis, testosterone has been shown to augment endothelial dysfunction induced by hypercholesterolemia in males. On the other hand, testosterone was shown to inhibit early atherogenesis at the aortic root of male LDL receptor-deficient mice, an effect that was blocked by an aromatase inhibitor, suggesting that the beneficial action of testosterone was due to local conversion to estradiol, the presence of aromatase having been demonstrated in the vasculature.

It is unclear whether any of the human subjects reported to have aromatase deficiency exhibit abnormalities of cardiovascular function because vascular consequences are likely to be masked in these individuals by the estrogen therapy they receive. However, there is some evidence that pharmacological modulation of aromatase in men has vascular effects. In a study in men taking testolactone, an aromatase inhibitor, hormonal changes resulting from pharmacological manipulation of aromatase induced significant effects on endothelial function; acetylcholine-induced vasodilation improved after cessation of testolactone administration. These observations are consistent with the present report of impaired basal and acetylcholine-induced vasorelaxation in the aorta of ArKO mice, compared with wild-types.

In conclusion, this study confirms the close association between sex hormones and nitric oxide release in the vasculature in males. Further studies in this model of estrogen insufficiency may throw light on the role of estrogens in both male and female cardiovascular physiology.

| TABLE 3. Maximum Effects of Vasorelaxative Agents |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
| Agonist                        | Genotype       | E_{max} (%)     | E_{max} (%)     | −log EC_{50}   | −log EC_{50}   |
|                                |                | Before Nitro-L-Arginine | After Nitro-L-Arginine | Before Nitro-L-Arginine | After Nitro-L-Arginine |
| Isoprenaline                   | Wild-type      | 93.7 ± 3.7      | 71.0 ± 3.6*     | 6.45 ± 0.25    | 5.87 ± 1.7*    |
|                               | ArKO           | 88.9 ± 5.2      | 63.5 ± 13.1     | 6.51 ± 0.16    | 6.00 ± 0.31*   |
| Sodium nitroprusside           | Wild-type      | 100.8 ± 1.6     | 96.3 ± 1.0      | 7.50 ± 0.20    | 7.36 ± 0.11    |
|                               | ArKO           | 106.0 ± 2.1     | 100.5 ± 0.8     | 7.48 ± 0.11    | 7.62 ± 0.08    |
|                                |                |                 |                 | −log EC_{50}   |                |

*P<0.01 before vs after nitro-L-arginine.

E_{max} indicates maximal response; −log EC_{50}, negative log concentration producing 50% maximal response.
Acknowledgments
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References
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