Role of aromatase in sex-specific cerebrovascular endothelial function in mice

Kristen L. Zuloaga, Catherine M. Davis, Wenri Zhang, and Nabil J. Alkayed

Department of Anesthesiology and Perioperative Medicine and The Knight Cardiovascular Institute, Oregon Health and Science University, Portland, Oregon

Submitted 10 September 2013; accepted in final form 3 February 2014

Zuloaga KL, Davis CM, Zhang W, Alkayed NJ. Role of aromatase in sex-specific cerebrovascular endothelial function in mice. Am J Physiol Heart Circ Physiol 306: H929–H937, 2014. First published February 7, 2014; doi:10.1152/ajpheart.00698.2013.—Stroke risk and outcome are strongly modified by estrogen. In addition to ovaries, estrogen is produced locally in peripheral tissue by the enzyme aromatase, and extragonadal synthesis becomes the major source of estrogen after menopause. Aromatase gene deletion in female mice exacerbates ischemic brain damage after stroke. However, it is not clear which cell type is responsible for this effect, since aromatase is expressed in multiple cell types, including cerebrovascular endothelium. We tested the hypothesis that cerebrovascular aromatase contributes to sex differences in cerebrovascular endothelial function. Cerebrocortical microvascular responses to the endothelium-dependent vasodilator ACh (acetylcholine) were compared between male and female wild-type (WT) and aromatase knockout (ArKO) mice by measuring laser-Doppler perfusion in vivo through a closed cranial window. Additional studies were performed in WT mice treated with the aromatase inhibitor fadrozole or vehicle. WT female mice had significantly greater responses to ACh compared with WT males (P < 0.001), which was associated with higher aromatase expression in female compared with male cerebral vessels (P < 0.05). ACh responses were significantly lower in ArKO compared with WT females (P < 0.05) and in WT females treated with fadrozole versus vehicle (P < 0.001). Conversely, ACh responses were significantly higher in ArKO versus WT males (P < 0.05). Levels of phosphorylated endothelial nitric oxide synthase (eNOS) were lower in ArKO versus WT female brains, but were not altered by aromatase deletion in males. We conclude that cerebrovascular endothelial aromatase plays an important and sexually dimorphic role in cerebrovascular function and that aromatase inhibitors in clinical use may have cardiovascular consequences in both males and females.

aromatase; CYP19; endothelial; sex differences; vascular

SEX DIFFERENCES IN STROKE RISK AND OUTCOME ARE IN PART LINKED TO SEX HORMONES. IN FEMALES, STROKE IS STRONGLY MODULATED BY THE FEMALE SEX HORMONES, YET DIFFERENCES BETWEEN MEN AND WOMEN PERSIST BEYOND MENOPAUSE, WHEN OVARIAN HORMONES ARE LOST (19, 45). FOR INSTANCE, DESPITE THE INCREASE IN STROKE RISK AFTER MENOPAUSE, WOMEN REMAIN AT LOWER RISK FOR STROKE COMPARED WITH AGE-MATCHED MEN EVEN AFTER MENOPAUSE (46). IN ADDITION, SEX DIFFERENCES HAVE BEEN OBSERVED EVEN BEFORE SEXUAL MATURITY (15). THE ENZYME RESPONSIBLE FOR ESTRADIOL SYNTHESIS FROM TESTOSTERONE IS P450 AROMATASE, WHICH IN ADDITION TO OVARIAN EXRESSION IS EXPRESSED IN OTHER TISSUES, INCLUDING BONE, BRAIN, ADIPOSE TISSUE, AND BLOOD VESSELS (4). EXTRAGONadal ESTROGEN SYNTHESIS BY LOCAL TISSUE AROMATASE BECOMES THE MAJOR SOURCE OF ESTROGEN IN POSTMENOPAUSAL WOMEN (47, 51). WITHIN THE BRAIN, AROMATASE HAS BEEN LOCALIZED TO MULTIPLE CELL TYPES, INCLUDING NEURONS, ASTROCYTES, SMOOTH MUSCLE CELLS, AND ENDOTHELIAL CELLS (6, 12, 16, 25, 31, 48).

Aromatase inhibitors (AIs), which are used clinically for treatment of breast cancer, carry the potential risk of increased cardiovascular events (5, 22, 28), in part mediated by the favorable effects of estrogen on plasma lipids (61) and vascular endothelial function (28). Although the link between the use of AIs and cardiovascular disease risk is still under investigation (33), aromatase gene polymorphisms in the human population have been shown to influence stroke and cardiovascular disease risk in a sexually dimorphic manner. Specifically, the −81371 C>T allele, which is believed to increase aromatase activity, results in a 65% increase in death, myocardial infarction, or stroke in men and a 69% decrease in women (2).

In female mice, both aromatase gene deletion and pharmacological inhibition lead to worse outcome following cerebral ischemia (29, 34), suggesting that aromatase plays a protective role against ischemic brain injury in females. Together these data support the role of aromatase in cardiovascular function and the importance of assessing the potential cardiovascular side effects of AIs.

We have previously shown that one mechanism by which estrogen contributes to the protection enjoyed by young females relative to age-matched males is enhanced cerebrovascular response to ischemic brain injury (1, 64). Cerebrovascular endothelial functional integrity is essential in maintaining proper tissue perfusion and in determining cerebrovascular response to ischemic injury, and, consequently, preserved endothelial function could be partially responsible for the protection enjoyed by females. Therefore, we tested the hypothesis that female mice have enhanced endothelial function (endothelium-dependent vasodilation) compared with age-matched males, in part due to higher cerebrovascular aromatase expression. Cerebrocortical microvascular responses to the endothelium-dependent vasodilator ACh (1–100 μM) were compared between male and female mice using an in vivo closed cranial window preparation combined with laser Doppler perfusion monitoring. To determine the role of aromatase in the sex difference in cerebrovascular function, endothelium-dependent vascular responses were compared between ArKO and wild-type (WT) mice, and between WT mice treated with aromatase inhibitor and vehicle. We observed sexually dimorphic effects.
of aromatase deletion on cerebrovascular endothelial function, in part linked to higher aromatase expression and enhanced endothelial nitric oxide synthase (eNOS) activation in female cerebral vessels.

MATERIALS AND METHODS

This study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research, and protocols were approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University (Portland, OR).

Animal model. Animals were bred in the Oregon Health and Science University animal facilities or purchased from Charles River Laboratories (Wilmington, MA). Animals were weaned at 21 days old and group-housed. At weaning, PCR was used to determine which offspring lacked the aromatase gene, as previously described (32). Age-matched young adult (12–15 wk) male and female C57BL/6 mice WT and aromatase knockout (ArKO) mice were used. To minimize natural fluctuations in sex hormones, female mice were synchronized and used during proestrus (high estrogen). Mice are synchronized by taking advantage of the Whitten effect, whereby estrus is induced in group-housed female mice by exposure to bedding soaked with pheromone-containing male urine. Estrous cycle stage is then determined via vaginal cytology (9). For some studies, C57BL/6 WT mice were treated with the aromatase inhibitor fadrozole (10 mg/kg) or vehicle (20% β-cyclodextrin in 0.9% saline) i.p. for 3 days before cranial window studies.

Serum hormone concentrations. At the end of each experiment, blood was collected via cardiac puncture in anesthetized mice. Blood was centrifuged and serum collected. Serum levels of testosterone and estradiol were measured by the Endocrine Technology Support Laboratories (Wallingford, CT). Serum hormone concentrations were normalized to 18S. Results are expressed as means ± SE. Groups were compared by t-test for two groups (estradiol levels, uterine weight, mRNA expression, protein expression) or two-way ANOVA with post hoc Bonferroni tests for multiple measurements with multiple comparisons.

Quantitative RT-PCR. RNA was isolated using a commercial kit (mirVana Isolation Kit; Ambion, Austin, TX), contaminant genomic DNA removed by DNase treatment, and RNA reverse-transcribed using a commercial high capacity cDNA archive kit (Applied Biosystems, Carlsbad, CA). Resulting cDNA was amplified using TaqMan Universal PCR amplification in a sequence detection system (ABI Prism 7000; Applied Biosystems). Quantitative PCR was performed in a 96-well plate in duplicate. PCR was concurrently run on controls without template to assess DNA contamination and primer dimer formation. Remaining RNA not reverse-transcribed was included as another negative control to rule out genomic DNA amplification. As an internal control, 18S was measured using an 18S rRNA control kit (FAM-TAMRA; Eurogentec, Seraing, Belgium). CYP19 primers were purchased as a CYP19 TaqMan Gene Expression Assay (Catalog No. 4331182; Invitrogen). All final CYP19 mRNA levels were normalized to 18S.

Western blot. Mice were perfused with ice-cold heparinized saline to remove blood from brains before tissue collection. For aromatase Western blotting, pial vessels were rapidly dissected from the brain surface and collected in ice-cold PBS. For ENOS Western blots, whole brain was used. Tissue was homogenized in lysis buffer containing sucrose (250 mM), potassium chloride (60 mM), tris-hydrochloride (15 mM), sodium chloride (15 mM), EDTA (5 mM), EGTA (1 mM), PMSF (0.5 mM), DTT (10 mM), 1 complete Mini-EDTA free protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN), and 10 µg/ml each of phosphatase inhibitor solution 1 and phosphatase inhibitor solution 2 (Sigma-Aldrich, St. Louis, MO). Lysates were then centrifuged at 2,000 g for 10 min at 4°C, and the supernatant was collected and centrifuged at 17,000 g for 20 min at 4°C. Protein samples (20 µg for pial vessels and 40 µg for brain) were separated by gel electrophoresis and then transferred to polyvinylidene difluoride membranes. Blots were blocked in 5% dry milk and incubated at 4°C overnight with a primary goat polyclonal CYP19 (C16) antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit total eNOS antibody (1:100; BD Biosciences), rabbit phospho-eNOS (pSer1177) (1:1,000; Thermo Scientific, Waltham, MA), or beta actin (1:2,000; Sigma-Aldrich). The signal was visualized using horseradish peroxidase-linked secondary antibodies against goat (1:5,000; Santa Cruz), rabbit (1:1,000; GE Healthcare, Salt Lake City, UT), or mouse (1:1,000; GE Healthcare) followed by detection using SuperSignal chemiluminescent reagents (Thermo Fisher Scientific) with a FluorChem FC2 (Protein Simple, Santa Clara, CA). For the aromatase antibody a peptide competition assay was performed (data not shown). The CYP19 (C16) antibody was pre-incubated in corresponding blocking peptide (sc-14245 P; Santa Cruz) at a 0·X, 2·X, or 5·X concentration relative to the antibody concentration before incubating the membrane with the primary antibody. Blots were stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and reblocked in milk after imaging each blot for CYP19 or phospho-eNOS and before incubation in a subsequent primary antibody for total eNOS or beta-actin. Densitometry was quantified with AlphaView software (Protein Simple); CYP19 was normalized relative to beta-actin, and phospho-eNOS was normalized to total eNOS. Data were normalized to the loading control beta-actin, and they were then expressed relative to levels in WT females.

Statistical analysis. Data are expressed as means ± SE. Groups were compared by t-test for two groups (estradiol levels, uterine weight, mRNA expression, protein expression) or two-way ANOVA with post hoc Bonferroni tests for multiple measurements with multiple comparisons.
groups (cranial window data). Differences were considered significant at $P < 0.05$. Data for WT mice in Fig. 1 are repeated in Figs. 3 and 4 so that a comparison could be made to the ArKO mice. Because the data points for the ArKO females and WT males are nearly identical, they could not be easily distinguished from one another if they were all presented on the same graph.

RESULTS

**Enhanced cerebrovascular endothelial function in female compared with male mice.** Cerebrocortical microvascular responses to the endothelium-dependent vasodilator ACh (1–100 $\mu$M) were compared between male and female WT mice by measuring laser-Doppler perfusion in vivo through a closed cranial window. Female mice were used during proestrus or estrus when estradiol levels are highest. *$P < 0.001$*. WT, wild-type.

**Aromatase gene deletion/inhibition impairs cerebrovascular endothelial function in female mice.** To determine whether aromatase was responsible for the enhanced endothelium-dependent response in female mice, cerebrocortical microvascular responses to ACh were compared between WT and ArKO female mice. We also compared responses to ACh between WT female mice treated for 3 days with vehicle or the aromatase inhibitor fadrozole. Successful inhibition of aromatase by fadrozole was confirmed by low serum estradiol and low uterine wet weight (Fig. 2), which were similar to what has been reported in ArKO mice (11). Female ArKO mice had impaired cerebrovascular endothelial function compared with WT female mice at both 10 and 100 $\mu$M ACh doses ($P < 0.05$; Fig. 3A). Responses in female ArKO mice (Fig. 3A) were not significantly different than responses in WT male mice (Fig. 1) at any dose tested. As expected, fadrozole-treated female mice showed reduced cerebrovascular endothelial response compared with vehicle-treated female mice at both 10 and 100 $\mu$M ACh doses ($P < 0.001$; Fig. 3B). To determine whether aromatase inhibition altered baseline perfusion, we used optical microangiography (OMAG) imaging (64), which allows us to measure vascular perfusion in the cerebral cortex noninvasively through the intact skull ($n = 9$ to 10 per group). In a previous study, we have validated OMAG against iodoantipyrine (IAP) autoradiography for measurement of cerebral blood flow in mice (64). We found no differences in baseline cerebrovascular cortical perfusion between vehicle- and fadrozole-treated WT female mice (mean pixel intensity 59.46 ± 4.16 in vehicle vs. 51.39 ± 2.73 in fadrozole group; $n = 9$ to 10 per group; $P = 0.11$). Furthermore, to determine whether aromatase alters vascular smooth muscle function, we administered the endothelium-independent dilator SNP (100 $\mu$M at a rate of 1 $\mu$L per min for 6 min) into the cistern magna and monitored cortical perfusion using OMAG. We found no differences in response to SNP between vehicle- and fadrozole-treated WT female mice (percent change in perfusion compared with baseline 112% ± 5.12% in vehicle vs. 109.1 ± 2.02% in fadrozole group; $n = 4$ to 5 per group; $P = 0.6$).

**Aromatase gene deletion/inhibition enhances cerebrovascular endothelial function in male mice.** To determine whether aromatase contributes to the observed sex difference in endothelial function by modulating vasodilator responses in male mice, cerebrocortical microvascular responses to ACh were compared between WT and ArKO male mice. In contrast with...
the impaired endothelial function observed in female ArKO mice, male ArKO mice exhibited enhanced cerebrovascular endothelial-dependent response to $100\mu M$ ACh compared with WT male mice ($P < 0.01$; Fig. 4A). Surprisingly, pharmacological inhibition of aromatase in WT males did not significantly alter endothelial function as observed in ArKO male mice, although there was a trend toward improved function ($P = 0.12$; Fig. 4B). Fadrozole-treated WT male mice had increased testosterone level compared with vehicle-treated WT male mice (619 ± 102.7 pg/ml vs. 351 ± 47.40 pg/ml, respectively; $P < 0.05$) and were similar to levels in ArKO males (793.8 ± 232.8 pg/ml).

Female cerebral vessels have increased aromatase protein expression compared with male vessels. Aromatase is expressed in several cell types within the brain, including neurons, astrocytes, endothelial cells, and vascular smooth muscle cells (6, 12, 16, 25, 31, 48). We have previously shown that aromatase expression in astrocytes is higher in females compared with males (31). It is not clear, however, if aromatase expression is sexually dimorphic in cerebral vessels. Therefore, CYP19 (the gene encoding aromatase) mRNA was measured in freshly isolated mouse brain microvascular endothelial cells via quantitative RT-PCR. Aromatase protein expression was also measured in freshly isolated cerebral vessels. As shown in Fig. 5, no sex differences were observed in cerebrovascular endothelial aromatase mRNA expression. Before our protein study, we validated the aromatase antibody using a peptide competition assay on ovary lysate (a positive control tissue with high aromatase expression) and found that the antibody was specific, since addition of the blocking peptide at either 2 or 5× concentration relative to the primary antibody was able to effectively compete for antigen binding (data not shown). Although we did not detect sex differences in endothelial aromatase mRNA, we did detect a significant difference in cerebrovascular aromatase protein expression. Male cerebral vessels had 29% less aromatase protein expression compared with female vessels ($P < 0.05$; Fig. 6).

**Female ArKO mice have decreased phosphorylated eNOS in brain.** Estradiol, the product of aromatase enzymatic activity, is known to elicit vasodilation by enhancing the phosphorylation/activation of eNOS, thus leading to increased production of nitric oxide (NO), a potent vasodilator. To determine whether the decreased ACh response in ArKO females was linked to decreased eNOS phosphorylation, we measured total and phosphorylated eNOS protein in WT and ArKO mouse brains. We found that total eNOS expression did not differ between sexes and was not altered by aromatase gene deletion. In contrast, the phosphorylated/active form of eNOS was decreased in ArKO compared with WT female mice ($P < 0.01$; Fig. 6).

**Fig. 3.** Aromatase gene deletion/inhibition impairs cerebrovascular endothelial function in female mice. A: cerebrocortical microvascular responses to ACh were compared between WT ($n = 7$) and aromatase knockout (ArKO; $n = 9$) female mice by measuring laser-Doppler perfusion in vivo through a closed cranial window. *$P < 0.05$. B: cerebrocortical microvascular responses to ACh were compared between WT female mice treated for 3 days with vehicle ($n = 8$) or fadrozole (10 mg/kg ip; $n = 7$) by measuring laser-Doppler perfusion in vivo through a closed cranial window. *$P < 0.001$.

**Fig. 4.** Effect of aromatase gene deletion/inhibition on cerebrovascular endothelial function in male mice. A: cerebrocortical microvascular responses to ACh were compared between WT ($n = 8$) and ArKO ($n = 7$) male mice by measuring laser-Doppler perfusion in vivo through a closed cranial window. *$P < 0.01$. B: cerebrocortical microvascular responses to ACh were compared between WT male mice treated for 3 days with vehicle ($n = 8$) or fadrozole (10 mg/kg ip; $n = 7$) by measuring laser-Doppler perfusion in vivo through a closed cranial window.
No sex differences in phosphorylated eNOS protein levels were detected in WT mice, and aromatase gene deletion had no effect in males.

**DISCUSSION**

The goal of this study was to determine whether there are sex differences in cerebrovascular endothelial function that are mediated by aromatase. Using an in vivo closed cranial window preparation combined with laser Doppler perfusion monitoring, we examined sex differences in cerebrocortical microvascular responses to the endothelium-dependent vasodilator ACh. Additionally, we used a pharmacological aromatase inhibitor and ArKO mice to determine the role of aromatase in the observed sex difference in endothelial-dependent cerebrovascular responses. Results show that aromatase gene deletion eliminates the sex difference in cerebrovascular endothelial function. Aromatase gene deletion/inhibition impaired cerebrovascular endothelial function in females, but elicited a smaller opposing effect in male cerebrovascular endothelial function. Aromatase gene deletion/inhibition was associated with decreased eNOS phosphorylation in females.

In the current study, we demonstrate that female mice have enhanced cerebrovascular endothelial function compared with males in vivo. This observation is supported by clinical data showing that premenopausal women have improved endothelial function compared with age-matched men (23, 59). Furthermore, cerebral blood flow measurements using transcranial laser Doppler show that mean arterial blood flow velocity in brain is higher in healthy women compared with age-matched men (57). A similar observation has also been made in healthy children using MRI, with girls showing enhanced cerebral perfusion compared with boys (56). Taken together, these findings indicate that females have enhanced cerebrovascular endothelial function and enhanced cerebral perfusion compared with males.

Sex differences in vascular function and tissue perfusion are in part mediated by sex steroids and their synthetic enzymes, such as aromatase. Both estrogens and androgens are known to...
H934  AROMATASE AND CEREBROVASCULAR FUNCTION

a protective role, in part by improving cerebrovascular endothelial vasodilator function.

We show for the first time that aromatase modulates cerebrovascular endothelial function in vivo and that this effect is sex-specific. Aromatase gene deletion/inhibition drastically decreased ACh-induced increases in microvascular laser Doppler perfusion in female mice, but caused a slight improvement in male mice. Because ACh is an endothelium-dependent vasodilator, we conclude that aromatase gene deletion and inhibition impair endothelial function. However, it is also possible that aromatase alters vascular smooth muscle vasodilator function. Therefore, in a subset of vehicle- and fadrozole-treated WT female mice, we also examined responses to the endothelium-independent dilator SNP. We found that aromatase inhibition did not alter the response to SNP. Furthermore, aromatase inhibition in these mice did not alter their baseline perfusion, suggesting that the differences in response to ACh between vehicle- and fadrozole-treated mice were not attributed to differences in vascular smooth muscle function or differences in baseline blood flow but rather to impairment of endothelial function. It is also important to note that although the effects observed in ArKO and fadrozole-treated mice were in the same direction, they varied in magnitude, with aromatase inhibition having greater effects on ACh responses in both male and female WT mice. This may in part be due to developmental changes caused by loss of aromatase in ArKO mice, which would not occur with short aromatase inhibition in adult WT mice. Developmental compensation of endothelial function has been reported for other knockout mice. For example, developmental compensation to maintain ACh-induced vasodilatory responses has been reported in the coronary circulation of eNOS knockout (eNOS−/−) mice. In these mice, coronary vasodilation to ACh was partially mediated by NO in both eNOS−/− and eNOS+/+ (WT) mice. The authors proposed that the NO produced by neuronal NOS (nNOS) could be compensating for loss of NO signaling due to eNOS deficiency (7).

Second possibility for the difference in effects between ArKO and fadrozole-treated mice is that fadrozole had off-target effects that enhanced the magnitude of changes observed with aromatase inhibition compared with aromatase gene deletion. Fadrozole (CGS 16949A) is a second-generation nonsteroidal aromatase inhibitor, which has higher selectivity to aromatase than first generation aromatase inhibitors, such as aminogluthethimide, which in addition to aromatase, inhibited biosynthesis of cortisol, aldosterone, and thyroid hormone. Although fadrozole is superior to aminogluthethimide in terms of potency, selectivity, and safety, at higher doses, it inhibits aldosterone synthesis (3).

It is possible that the decrease in cerebrovascular responses in ArKO/fadrozole-treated females was due to a decrease in eNOS activity/NO production, since estradiol increases eNOS activation and NO production (35, 36, 38, 52–54). In support of this idea, serum estradiol levels and phosphorylated eNOS protein in brain were decreased in ArKO compared with WT females. Likewise, a local increase in testosterone in male vessels may have led to increased eNOS-mediated NO production (14), which could be partially responsible for the preserved/enhanced endothelial function in vessels of male ArKO mice. WT male mice treated with fadrozole had increased T levels compared with vehicle-treated WT males, an effect which is also observed in ArKO male mice (11). Furthermore,

Fig. 7. Female ArKO mice have decreased phosphorylated endothelial nitric oxide synthase (eNOS) in brain. Phosphorylated eNOS and total eNOS protein expression were measured via Western blot in freshly isolated brains from WT female, ArKO female, WT male, and ArKO male mice (n = 4 per group). Phosphorylated eNOS protein expression was normalized to total eNOS expression. β-Actin protein expression is also shown as a loading control. *P < 0.01 vs. WT female via t-test.

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00698.2013 • www.ajpheart.org
phosphorylated eNOS expression was preserved in brains of ArKO male mice. One limitation of the current study is that only a correlational link between eNOS and aromatase was established. Future studies examining endothelial function in vessels isolated from eNOS knockout mice treated with aromatase inhibitor would allow us to determine a mechanistic link between eNOS and aromatase. Although preserved phosphorylated eNOS expression could explain why the ArKO male mice did not have impaired ACh responses like the ArKO female mice, it does not account for the enhanced ACh responses seen in ArKO versus WT male mice. This effect may not be linked to eNOS, but rather attributed to other endothelium-dependent vasodilator pathways not evaluated in the current study, such as prostacyclin or endothelium-derived hyperpolarizing factor (EDHF). Future studies, beyond the scope of the current study, will determine the mechanism behind the enhanced ACh response in male ArKO mice.

Some of our data in the cerebral circulation vary from observations in other vascular beds. For example, it has been reported that male ArKO mice have reduced endothelium-mediated dilation in aorta (26). Furthermore, reduced flow-mediated dilation has also been observed in brachial arteries of men treated with aromatase inhibitors (28). Taken together, these data suggest that the effect of aromatase on endothelial function, at least in males, may be tissue specific. This is not surprising considering that responses to a variety of stimuli are known to vary between different vascular beds and even along the same bed as the vessels become smaller (20). One limitation of the current study is that we only assessed ACh-mediated vasodilation as our readout for cerebrovascular endothelial function. It is likely that aromatase also affects other aspects of vascular function. For instance, aromatase gene knockdown ex vivo in gracilis arteries has also been shown to decrease production of vasodilatory and anti-inflammatory EETs (55). Responses to ACh in vivo, especially in our closed cranial window preparation, are different than responses of isolated vessels or responses using an open cranial window where the agent is directly applied to the vessel. In our preparation the ACh is diluted in the cerebral spinal fluid within the subdural space; therefore, we observe a slower response than in other preparations and the magnitude, at least in males, is smaller. However, the responses we observed are not much different than those reported by others using other in vivo methods (27, 37, 44).

Our current data show for the first time that cerebral vessels from female mice have increased aromatase protein expression compared with male vessels. Therefore, aromatase may play a larger role in the cerebral vasculature in females compared with males. This idea is supported by our cerebrovascular function studies, which showed that aromatase gene deletion/inhibition had a greater effect on endothelial function in females compared with males. In addition, we have previously shown that female astrocytes have greater aromatase expression compared with male astrocytes (31). Furthermore, human studies also indicate that females have greater expression of aromatase in aorta compared with males (39). Surprisingly, despite the observed sex difference in aromatase protein expression in cerebral vessels, we did not detect any sex differences in aromatase mRNA expression in microvascular endothelial cells. A lack of sex difference in aromatase expression has also been reported in rat coronary artery endothelial cells (50). Our data suggests that the sex difference in aromatase in cerebral vessels may be due to either increased expression in vascular smooth muscle cells from female mice or due to sex differences in post-transcriptional regulation of aromatase. For instance, it is possible that males have increased cerebrovascular expression of micro RNAs that post-transcriptionally downregulate aromatase, such as miR-378 (60), let-7f (49), or miR-98 (43). Sexually dimorphic expression has been reported for several micro RNAs (42). Although it is currently unknown if expression of miR-378, let-7f, or miR-98 is sexually dimorphic in brain, if expression was higher in males this would explain why male brain endothelial cells have similar CYP19 mRNA levels as females, but decreased cerebral vessel protein expression.

Given the protective effects of estrogen on the vasculature (30) and our data showing a negative effect of aromatase inhibition/gene deletion on cerebrovascular endothelial function in females, it is of concern that aromatase inhibitors are used clinically to treat hormone-sensitive breast cancer because aromatase inhibition may have adverse cardiovascular consequences in females. In support of this idea, aromatase inhibitors have been reported to have negative cardiovascular effects when compared with the estrogen receptor antagonist tamoxifen (22, 28), including reduced flow-mediated vasodilation, suggesting a protective role for aromatase in the human vascular endothelium. However, the potential link between aromatase inhibitors and cardiovascular risk is still under investigation (33). Nonetheless, aromatase gene polymorphisms in the human population have been shown to influence stroke and cardiovascular disease risk in a sexually dimorphic manner. For instance, the −81371 C>T allele, which is thought to increase aromatase activity, results in a 65% increase in death, myocardial infarction, or stroke in men and a 69% decrease in women (2). Alternatively, the sex difference in vascular function could also be mediated by high testosterone levels, rather than decreased estradiol, as aromatase inhibition or gene deletion drastically increases testosterone levels. Like estradiol, testosterone has been shown to affect vascular function. However, effects of testosterone on vascular function are not well defined, since testosterone has been reported to have both vasodilatory effects (8, 10, 14, 24, 40, 58, 62) and vasoconstrictor effects (13, 17, 18).

Rodent studies also indicate that aromatase plays a role in ischemia. Female ArKO mice have increased infarcts, reduced neurogenesis, and prolonged behavioral deficits following cerebral ischemia compared with WT mice (29, 34). Furthermore, female ArKO mice sustain greater injury than do ovariec-tomized WT females, providing evidence that extragonadal local estradiol plays a critical role in ischemic outcome in females (34). Although not tested in the current study, we predict that ACh response in ovariectomized WT females to be higher than ArKO females, but lower than intact WT females, because the ovariectomized WT mice would still have vascular aromatase that may be able to partially compensate for reduction in circulating estradiol levels. A similar effect would also be expected in reproductively senescent female mice, which also have low circulating estradiol levels, yet they presumably retain their endothelial aromatase. Now that we have established that aromatase plays a role in endothelial function in young mice, future studies will determine whether aromatase inhibition also alters endothelial function in aged female mice.

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00698.2013 • www.ajpheart.org

Downloaded from http://ajpheart.physiology.org/ by 10.220.33.4 on October 15, 2017
which already have low circulating estradiol levels but unknown concentrations of vascular estradiol.

In summary, we have shown that female mice have enhanced cerebrovascular endothelial function and cerebrovascular aromatase expression compared with male mice and that aromatase inhibition or gene deletion eliminates the sex difference in endothelial function. This impairment of function in ArKO females appears to be mediated, in part, by decreased eNOS phosphorylation. Interestingly, aromatase inhibition or gene deletion in male mice did not impair endothelial function, but rather caused a slight improvement in endothelial function. ArKO males also had preserved eNOS phosphorylation. These data show that aromatase plays an important role in enhancing cerebrovascular endothelial function in females, but plays a smaller, opposing role in male vessels. Understanding the role of aromatase in cerebrovascular function may lead to therapeutic strategies for cerebrovascular diseases aimed at enhancing local estradiol production in females specifically within the vasculature, thus avoiding the negative side effects associated with global estrogen administration, but maintaining the protective effects of estron on the female vasculature. Conversely, our data indicate that such a therapy may be ineffective or even potentially counterproductive in males.

AUTHOR CONTRIBUTIONS


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

AROMATASE AND CEREBROVASCULAR FUNCTION


