Aromatase-Deficient (ArKO) Mice Have Reduced Blood Pressure and Baroreflex Sensitivity

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Aromatase-deficient (ArKO) mice are deficient in estrogens due to deletion of the aromatase gene. We hypothesized that there may be changes in the cardiovascular system of ArKO mice because of evidence linking estrogens with improved cardiovascular outcomes and the induction of the glucocorticoid-metabolizing enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), gene in the kidney, which is important for the regulation of blood pressure (BP). BP and baroreflex sensitivity (BRS) in female conscious ArKO mice were compared with those in age- and weight-matched wild-type (WT) mice. Power spectral analysis was used to determine cardiovascular variability and BRS. Although systolic BP was similar in the two groups, diastolic and mean BPs were lower in the ArKO mice (−6.3 ± 1.9 and −4.6 ± 2.1 mm Hg, respectively).

Heart rate (HR) was greater in the ArKO mice (+36 ± 6 beats/min). The mean BP in WT mice was 105 mm Hg, and the HR was 481 beats/min. In the autonomic frequency range, BP variability was 74% greater, and HR variability was only 26% that in WT mice. The BRS of ArKO mice was 46% of the value observed in WT mice. 11βHSD2 levels were unaltered in ArKO mice, except in the kidney, where they were only 10% of WT levels. Estradiol administration to ArKO mice restored renal 11βHSD2 to WT levels. The results show that ArKO mice have lower diastolic BP, but increased BP variability, perhaps due to an impaired BRS. Thus, aromatase activity is critical for normal autonomic control of the heart and, hence, for reducing the deleterious effects of high BP variability. (Endocrinology 145: 4286–4291, 2004)

There has been much interest in the cardiovascular role of estrogens, with the consistent findings that men are at higher risk of developing cardiovascular disease than premenopausal women (1). One explanation for this may be that age-matched women have been shown to have lower blood pressure (BP) than males (1). However, the precise role of estrogen in mediating beneficial cardiovascular effects has been difficult to establish, perhaps due to its wide variety of actions. Furthermore, the beneficial effects of hormone replacement therapy on the cardiovascular system have been particularly controversial (2). There are a number of different mechanisms proposed by which estrogen may bring about its beneficial effects on the cardiovascular system. In humans and experimental animals, estrogen facilitates vasodilation by stimulating prostacyclin and nitric oxide synthesis as well as by decreasing the production of vasoconstrictor substances, such as cyclooxygenase-derived products, reactive oxygen species, angiotensin II, and endothelin-1 (3). Estrogen also reduces the number of adrenal angiotensin type I (AT1) receptors, but indirectly, by modulating adrenal angiotensin II (4). In estrogen receptor-deficient states, there is evidence of increased atherosclerosis, an attenuated response to vascular smooth muscle cell proliferation in response to injury and reduced endothelial nitric oxide production (5). In addition to classical genomic mechanisms, estrogens can act via nongenomic pathways involving membrane-bound estrogen receptor signaling through kinases (6).

Another way in which estrogen may be of cardiovascular benefit is by alteration of cardiovascular variability through changing baroreflex mechanisms. In humans, the cardiac baroreflex sensitivity is correlated positively with the sd of heart rate (HR) and negatively with the sd of BP (7). A diminished baroreflex and, therefore, a reduced HR variability have also been shown to be independent risk factors for sudden death after myocardial infarction (8). Recently, estrogen was found to augment the slope of the baroreflex bradycardia response to phenylephrine and angiotensin II, supporting previous studies that suggested it modulates baroreflex control of autonomic function (9).

The actions of estrogens may also be mediated via local effects on glucocorticoid-metabolizing enzymes, 11β-hydroxysteroid dehydrogenase types 1 and 2 (11βHSD1 and 11βHSD2), because glucocorticoids are known to be important in maintaining normal vascular tone and the baroreflex response (10). The metabolism of glucocorticoids is pivotal in the kidney, where 11βHSD2 prevents binding to the nonselective mineralocorticoid receptor in the renal distal tubule (11). Mutations in the HSD11B2 gene or inhibition of the enzyme with licorice leads to sodium retention and elevated BP (12). Gene reporter studies show that 11βHSD2 expression is estrogen dependent, suggesting that estrogen deficiency may elevate BP (13).

The production of estrogen (17β-estradiol) is mediated via the cytochrome P450 enzyme aromatase. Mice with a targeted disruption of the aromatase (ArKO) are deficient in estrogen, and their phenotype has been well characterized. ArKO mice have greater adiposity, with altered fatty acid

Abbreviations: ArKO, Aromatase deficient; AT1, angiotensin type I; BP, blood pressure; BRS, baroreflex sensitivity; HR, heart rate, 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1; MAP, mean arterial pressure; WT, wild-type.

Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.
metabolism and elevated circulating levels of testosterone (14), gonadotropins (15), leptin, cholesterol, and insulin (16). Many of these changes are also present in humans with the metabolic syndrome, in which the cardiovascular system can be severely compromised. However, there are currently no reported studies of the cardiovascular system of ArKO mice. The availability of these mice offered us the opportunity to examine the contribution of aromatase to basal levels of BP and HR, to BP and HR variability, and to baroreflex sensitivity. In addition, we measured the effects of aromatase gene deletion and estrogen replacement on 11βHSD2 in various tissues. Importantly, these mice were kept on a soy-free diet to eliminate exposure to phytoestrogens.

**Statistical analysis**

Values are expressed as the mean ± SEM. For the cardiovascular measures, six 10-min consecutive periods from each of the ArKO and WT mice (10–14 wk old; n = 3/group) were killed in a CO2 chamber, and tissues were immediately removed and frozen in liquid nitrogen, followed by storage at −70°C. The MOH22 antibody (2 μg/ml) was used to detect 11βHSD2 in tissues of female mice by Western blot analysis (21). Bands on Western blots were quantitated using densitometry running Optimiz version 6.5 software. MOH22 is an immunoregulated rabbit polyclonal directed against residues A331–L346 of the mouse 11βHSD2 enzyme. The antibody was raised and purified according to a protocol previously used for the generation of the HUH23 antibody (21). For estrogen replacement, mice were implanted with 21-d release 17β-estradiol pellets (0.05 mg; Innovative Research of America, Toledo, OH) at 7 wk of age.

**Materials and Methods**

The experiments were performed in 6-month-old conscious ArKO (n = 6; 26 wk) and wild-type (WT; C57BL/6; n = 6; 27 wk) female mice in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The ArKO mice weighed 26.3 ± 1.2 g (range, 21–29 g), and the WT mice weighed 26.2 ± 2.1 g (range, 21–32 g) and were generated by disrupting the CYP19 gene as previously described (14). Heterozygote mice were bred to homozygosity at the Prince Henry’s Institute for Medical Research (Clayton, Australia). WT and ArKO animals were kept under pathogen-free conditions and maintained on a soy-free chow diet (Glenn Forrest Stock Feeders, Glenn Forrest, Australia).

A femoral arterial catheter was implanted under halothane anesthesia. The catheters were tunneled under the skin, the incision was closed, and the catheter was fixed in place with tissue glue. After surgery the mice were placed in a small holding box on a warmed experimental table and allowed to recover for 3 h, and then cardiovascular measurements were made for 1 h. The 3-h recovery period has been shown to be sufficient for the influence of halothane on cardiovascular reactivity (via nitric oxide pathways) to be undetectable (17). The arterial catheter was filled with heparinized saline (20 U/ml) and connected to a P23Dc pressure transducer (Statham, Hato Rey, Puerto Rico) for continuous measurements of arterial pressure. The pulsatile arterial pressure was digitized and systolic, mean (MAP), and diastolic arterial pressure and HR were calculated (18). The respiration rate was derived from the respiratory-induced change in pulse pressure.

The beat to beat data were analyzed for spectral analysis using a program written in Labview (19). The auto- and cross-power spectra were calculated for each segment using Fast Fourier transform (19). The average value of the transfer gain in the frequency band between 0.3 and 0.5 Hz was used as the estimate of the baroreflex sensitivity (Fig. 1) (20). Other frequency bands included in the analysis were low frequency (0.08–0.3 Hz) and high frequency (0.5–1 Hz), and the total power was calculated between 0 and 1 Hz (20). For further details, see supplemental methods.

**Western blot analysis**

ArKO and WT mice (10–14 wk old; n = 3/group) were killed in a CO2 chamber, and tissues were immediately removed and frozen in liquid nitrogen, followed by storage at −70°C. The MOH22 antibody (2 μg/ml) was used to detect 11βHSD2 in tissues of female mice by Western blot analysis (21). Bands on Western blots were quantitated using densitometry analysis with the aid of a Linotype-Hell Saphir Ultra 2 densitometer running Optimiz version 6.5 software. MOH22 is an immunoregulated rabbit polyclonal directed against residues A331–L346 of the mouse 11βHSD2 enzyme. The antibody was raised and purified according to a protocol previously used for the generation of the HUH23 antibody (21). For estrogen replacement, mice were implanted with 21-d release 17β-estradiol pellets (0.05 mg; Innovative Research of America, Toledo, OH) at 7 wk of age.

**Statistical analysis**

Values are expressed as the mean ± SEM. For the cardiovascular measures, six 10-min consecutive periods from each of the ArKO and WT mice were compared using a split plot, repeated measure ANOVA with factors of time, groups, and mice.

**Results**

**Cardiovascular measurements**

Comparison of the BP over the course of the hour-long recording showed that diastolic arterial pressure was lower by 6.1 mm Hg in the six ArKO mice compared with the six WT (86.5 ± 0.4 compared with 92.6 ± 0.5 mm Hg; F between groups = 10; P = 0.002). However, there was no difference in the systolic BP between the two groups (113.8 ± 0.4 and 115.0 ± 0.4 mm Hg for ArKO and WT, respectively; F between groups = 0.1; Fig. 2). MAP was 4.5 ± 2.0 mm Hg lower in ArKO compared with WT mice (P = 0.04). There was no trend with time over the 1-h period, suggesting that the animals measurements were stable (F between times < 0.3). The values of MAP and HR are very similar to those measured by telemetry in conscious female WT mice at the same time of day by Zhu and colleagues (22). The maximum rate of change in BP (dP/dt) that occurs during the systole was 40% greater in the ArKO mice compared with WT (1427 ± 9 compared with 1019 ± 14 mm Hg/sec; P < 0.05; Fig. 3). HR was also higher in the ArKO mice, but only by about 7% (514 ± 5 compared with 481 ± 5 beats/min; P < 0.05; Fig. 3). There were no significant differences in respiratory rate between the two groups (155 ± 2 breaths/min in ArKO compared with 147 ± 2 in WT; F between groups = 2.9; P = 0.1).

**Cardiovascular variability**

Blood pressure and HR variability was estimated with power spectral analysis. Although there was no significant
difference between overall BP variability between the ArKO and WT mice, there was a marked increase in variability within specific frequency ranges, namely the mid and high frequency bands. The mid frequency power corresponding to the autonomic range was 1.7 times greater in ArKO mice compared with WT mice \((P < 0.01)\) and 2.4 times greater in the high frequency region (Table 1). By contrast, HR variability was lower in the ArKO mice compared with WT mice over all frequency ranges. Thus, the total HR variability was only 43% of the value in WT mice (Table 1).

Baroreceptor reflex

The gain of the baroreceptor HR reflex was calculated from the cross-spectral analysis of the transfer function between MAP and HR in the frequency range between 0.3 and 0.5 Hz, which corresponds to the mid frequency and relates to autonomic function. Four estimates were made during the 1-h recording for each group of mice. At this frequency the variability (power) in MAP was greater, whereas HR variability was dramatically less in ArKO mice compared with WT. Thus, the cross-spectral gain was markedly less (46% of WT; \(P < 0.01\); Fig. 4). There was, however, good coherence between BP and HR of between 0.55 and 0.6 in both groups (Fig. 4).

Levels of 11βHSD2 enzyme

The following tissues were analyzed for 11βHSD2 by Western blot analysis: liver, heart, brain, visceral fat, kidney, lung, pancreas, spleen, skeletal muscle, uterus, adrenal gland, cerebellum, cortex, and hippocampus. Only 11βHSD2 in the kidney showed differences between female WT and ArKO mice. The antibody recognized a 40-kDa murine antigen in the kidney, but not the liver, consistent with the calculated molecular size and distribution of 11βHSD2. Male and female mice displayed equivalent levels of 11βHSD2, but in female ArKO kidneys 11βHSD2 was present at 10% of the levels in the WT. Estradiol administered to ArKO mice re-
stored 11βHSD2 to levels seen in WT animals, but did not further increase levels in WT mice (Fig. 5).

Discussion

The main findings of the current study were that ArKO mice had lower diastolic BP and higher HR than WT mice and that the gain (sensitivity) of the baroreceptor HR reflex was considerably impaired. As a result, the BP variability in the autonomic frequency bands was twice that in WT mice, and the variability in HR was markedly less at all frequencies examined. These studies suggest that aromatase activity is important for maintaining baroreflex sensitivity, which may be highly relevant to the cardiovascular protective actions of estrogen observed in females.

The lower diastolic BP of ArKO mice suggests a lower vascular resistance, which indicates that aromatase activity is necessary to maintenance vascular tone. Although there are a number of hormonal changes associated with a lack of aromatase activity, such as increased testosterone and leptin that occur in young mice, other changes, such as increased insulin, occur only in older animals (16). Thus, the major effect of a lack of aromatase activity appears to be the absence of estrogen. Whether this change could explain the lower diastolic BP is debatable, because the majority of effects of estrogen suggest that it has a mainly vasodilatory role on the vasculature. The estrogen receptor β-deficient mice develop sustained systolic and diastolic hypertension, but only as they age (>6 months) (22). Estrogen reverses acetylcholine-induced vasoconstriction of coronary vessels, possibly via facilitation of endothelium-dependent relaxation (23), which may be mediated by an enhancement of NO production via stimulation of NO synthase (24). There is also a rapid non-genomic effect on endothelial cells leading to vasodilatation when estrogens are applied transdermally (25). Hormone replacement therapy increases arterial compliance in postmenopausal women, and ambulatory studies show that it can reduce BP (26). In addition, there have been a number of actions of estrogen postulated that are secondary to its effects on the renin-angiotensin system (27). Estrogen has been shown to down-regulate AT1 receptors that play a major role in the regulation of BP and fluid balance and could account for the association of estrogen deficiency with hypertension, atherosclerosis, and arteriosclerosis (28). Contrasting with these actions, estrogen has also been shown to promote the production of angiotensinogen (29), and it is this dichotomy of estrogen’s action on the renin-angiotensin system that has led to the proposition by Fischer and colleagues (27) that the overall effects of estrogen on the vasculature may very much depend on a shift in the balance between the dilatory and constrictor mechanisms. In high renin models, for example, the mRen2Lewis rat, estrogen is as effective as an AT1 receptor antagonist in limiting the severity of hypertension (30). Thus, in the current study the mice would be expected to have a relatively normal renin-angiotensin system, such that the overall effect of lack of estrogen in the ArKO mice may be to cause a small degree of vasodilatation.

Another possibility would be that other hormonal changes known to occur in ArKO mice, such as increased testosterone (14), may contribute to the effects observed. The levels of testosterone are about 7 times those seen in WT females, but are still very much lower than levels in males. At very high pharmacological doses (i.e. well above those measured in male mice), testosterone has been shown to cause vasodilation (31). Thus, it is possible that testosterone may contribute. Our studies also show that estrogens are important...
for maintaining the renal 11βHSD2 enzyme at normal levels in female mice. This is consistent with the results of 11βHSD2 gene reporter studies, which showed an 8-fold stimulation in the presence of estradiol (13). Our observation that the administration of estradiol to WT mice did not further increase 11βHSD2 levels suggests that the levels of endogenous estrogens maximally stimulate production of the enzyme. Although decreased 11βHSD2 activity results in apparent mineralocorticoid excess and leads to increases in BP, we did not observe any elevation in BP in ArKO mice. The levels of renal 11βHSD2 observed, although very low, may have been sufficient to protect the mineralocorticoid receptor from occupation by glucocorticoids and prevent hypertension. This is in keeping with the normal BPs seen in heterozygote relatives of patients with apparent mineralocorticoid excess who also display decreased 11βHSD2 activity (32). However, there is evidence that certain 11βHSD2 alleles are associated with increased salt sensitivity in humans (33), and it would be interesting to examine the BP of ArKO mice on a high salt diet. Studies in hypertensive rats show that a high salt diet does indeed increase MAP in ovariectomized rats and that this increase can be attenuated by phytoestrogens (34). The administration of sympathetic blockade restored arterial BPs to control levels in this study, suggesting that estrogen acts by attenuating sympathetic overactivity in response to a high salt diet. The tachycardia and greater dp/dt, which is an index of cardiac contractility, suggest that there may also be a chronic activation of cardiac sympathetic activity in ArKO mice. This may also be accompanied by lesser vagal tone, as indicated by the reduced HR variability over all frequencies. The withdrawal of cardiac sympathetic tone can only affect the low frequencies of HR variability (<0.2 Hz) due to the slow neuroeffector mechanisms involving β-adrenoceptors and cAMP. Thus, the decreased variability of the higher frequency bands up to 1Hz must be due to decreased vagal activity (35).

Also associated with these changes is a marked reduction in the gain of the baroreceptor HR reflex. This study is the first to demonstrate that deletion of the aromatase gene results in impaired baroreflex regulation of HR. Other studies have documented the effects of estrogen and testosterone on the baroreflex activity of intact and ovariectomized mice and rats (9, 36, 37). Estrogen significantly increased the slope of the baroreflex bradycardic response to phenylephrine and angiotensin II, but not to sodium nitroprusside, in ovariectomized mice. In an earlier study using anesthetized rats, ovariectomy lowered baroreflex sensitivity, whereas the administration of estradiol enhanced sensitivity to the level in sham-operated animals (37). Thus, the weight of evidence from these and other studies suggests that estrogen acts through the central nervous system to modulate processing of baroreceptor information. In support of this, estrogen receptors are found in the nucleus of the solitary tract and caudal ventral medulla, areas involved in processing baroreceptor feedback (38). Importantly, estrogen administration into hindbrain nuclei also augments phenylephrine-induced changes in HR (39), consistent with a role for estrogen mediated via the central nervous system. Thus, the lack of estrogen in ArKO mice may explain the reduced baroreflex sensitivity. In contrast, the higher levels of testosterone would be expected to increase the baroreflex gain according to studies in conscious rats (36) that suggests that in the ArKO mice, the effect of a lack of estrogen predominates.

In conclusion, the present study in ArKO mice maintained on a phytoestrogen-free diet suggests that aromatase plays an important role in maintaining normal vasoconstrictor activity. However, one of the most important findings in relation to the cardio-protective action of aromatase may be its ability to facilitate cardiac vagal activity, inhibit cardiac sympathetic activity, and increase the sensitivity of the cardiac baroreflex by converting androgens to estrogens.

**Acknowledgments**

Received April 1, 2004. Accepted May 21, 2004.

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This work was supported by a Block Institute Grant from the National Health and Medical Research Council of Australia.

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