Estrogen modulation of baroreflex function in conscious mice

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Epidemiological studies have shown that cardiovascular diseases such as hypertension and coronary heart disease are less common in premenopausal women compared with age-matched men (16, 18, 23, 50). This innate protection in women against cardiovascular disease disappears with reproductive senescence or with removal of endogenous ovarian steroids (4, 45, 49).

It is thought that estrogen may affect cardiovascular function at numerous levels including the vasculature, heart, and brain (36, 46). A potential mechanism by which estrogen provides cardioprotection in women is by acting at central cardiovascular regulatory centers to modulate autonomic regulation of the cardiovascular system. Hormone replacement therapy appears to have favorable effects on the cardiovascular autonomic regulation in postmenopausal women by improving baroreflex sensitivity and overall heart rate (HR) variability (19). Similar observations have also been made in male and ovariectomized female rats where estrogen replacement appears to improve baroreflex sensitivity via central mechanisms (32, 43). However, to date, there have been no studies about the role of estrogen or gender in baroreflex control of HR in mice.

ANG II is a potent circulatory peptide implicated in pathogenesis of hypertension. In addition to its peripheral vasoconstrictor effects, this peptide has been known to modulate reflex regulation of HR and sympathetic activity through circumventricular organs such as the area postrema (13, 37). In rabbits, dogs, and rats, acute increases in circulating ANG II blunt baroreflex regulation of HR (1, 31, 38, 39). It is interesting to know if centrally mediated effects of ANG II on baroreflex function are affected by differences in circulating estrogen levels and gender.

The purpose of the present study was to investigate the effects of estrogen on baroreflex regulation of HR to increases in pressure with phenylephrine (PE) and ANG II and decreases in pressure with sodium nitroprusside (SNP) in conscious freely moving ovariectomized females of C57/BL6J strain. In addition, these responses were compared with those of intact male mice. This strain of mouse is the most widely used for studies on genetically modified animals. It was hypothesized that estrogen would facilitate the PE- and ANG II-mediated baroreflex inhibition of HR in female ovariectomized mice.

METHODS

Animals

Male and female mice of C57/BL6J strain were obtained from a breeding colony maintained in the animal care facility at University of Missouri. The mice were 28–32 wk old and on an average weighed 20–24 g. The mice were fed a soy-based Purina 5001 lab chow (PMI feeds, St. Louis, MO) that is reported to contain high levels of phytoestrogens. Recent

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studies suggest that dietary phytoestrogens may influence cardiovascular homeostasis (5, 12). In the present study, the observed effects of estrogen on baroreflex function may include contributions from phytoestrogens in the diet. However, this contribution of dietary phytoestrogens to estrogenic activity should be comparable in all three groups as all the mice were fed the same diet. All the protocols were approved by the University of Missouri Animal Care and Use Committee. The experimental groups consisted of 1) gonadally intact male mice (n = 12), 2) female mice, ovariecotomized and implanted with silastic capsules containing 17β-estradiol (E) in corn oil (OvxE+; n = 7), and 3) female mice, ovariecotomized and implanted with silastic capsules containing corn oil alone (OvxE−; n = 6).

Surgical Procedures

All the surgical procedures were carried out in mice anesthetized with a mixture of ketamine (100 mg/kg i.p) and xylazine (10 mg/kg i.p) and supplemented with isoflurane when necessary.

For the purpose of making silastic capsules, a 14-mm length of silastic tubing (0.062-in. ID, 0.125-in. OD, Dow Corning) was filled with 20-µl solution of estradiol dissolved in tocopherol-sterilised corn oil (0.5 µg/µl). Both ends were sealed with silastic adhesive (Konigsberg Instruments) with the final length of the tubing containing estradiol (E) or the vehicle being 10 mm. The capsules were incubated in 0.9% NaCl overnight at room temperature before implanting in mice to provide a stable plasma estradiol concentration and to prevent a transitory peak in the plasma estrogen that would otherwise occur.

Serum estradiol was measured by radioimmunoassay as described by vom Saal et al. (48). Radiolabeled estradiol and estradiol antibody were obtained from ICN (Costa Mesa, CA). Blood samples were collected at 14 days (n = 3 in each group) after capsule implantation. Serum was separated by centrifugation and stored at −80°C until the time of measurement. Samples from all the animals were run in duplicate in a single assay following extraction with ethyl acetate:chloroform (80:20, Fisher Scientific); the intra-assay coefficient of variation was 4.2%, and assay sensitivity was 0.5 pg. In OvxE+ mice, serum concentrations were −8.6 ± 0.6 pg/ml. In OvxE− mice, the serum concentration of estradiol was 21 ± 2 pg/ml.

Before ovariecotomy, female mice weighed an average of 20 ± 0.5 g and the males weighed 23 ± 0.5 g. Ten days after ovariecotomy, the body weights of OvxE+ and OvxE− females were 24 ± 0.5 and 25 ± 0.8 g, respectively, indicating a comparable increase in body weight in ovariecotomized females with (4.0 ± 0.1 g) or without (4.5 ± 0.4 g) estrogen replacement. The effectiveness of the estrogen replacement was also confirmed by comparing uterine weights of ovariecotomized females with and without estrogen replacement. The uterus was collected from the mice after transecting from the oviduct at the uterotubal junction bilaterally and at the junction of the uterine body and cervix. The entire uterus was wet-weighed on a microbalance and the weight was expressed as milligrams per gram of body weight to correct for differences in body weight. Uterine weight in ovariecotomized mice with 17β-estradiol replacement was 1.58 ± 0.5 mg/g and with corn oil replacement alone was 0.36 ± 0.04 mg/g. These uterine responses are within the physiological range of responses described previously in bioassays for estrogen implants (7).

Chronic catheterization. At least 4 days before the experiments, the mice were again anesthetized with ketamine-xylazine mixture and surgically instrumented with intracardiac catheters for direct measurement of pulsatile and mean arterial pressure (MAP) and HR. Intravenous catheters were inserted for administration of drugs. Catheters made of microrenethane tubing (MRE25, Braintree Sci., Bos- ton, MA) were inserted into the left femoral artery and right femoral vein. The catheters were then tunneled subcutaneously, exteriorized, and placed in a plastic protective tube sutured in place at the back of the neck. The exterior catheters were heat-sealed until use. The mice were housed individually in autoclaved filter top cages and allowed to recover from the surgery (3–4 days) before testing baroreflex function. In the interim, the catheters were flushed daily with dilute sterile heparinized saline (25 U/ml) to maintain patency and resealed. The mice were conscious and unrestrained in their own cages, while the exteriorized catheters were being connected to polyethylene tubing filled with heparinized saline for flushing. For blood pressure recordings, the arterial catheter was connected via polyethylene tubing filled with heparinized saline to a pressure transducer (MLT0698, AD instruments) placed at the level of the heart. Drugs were infused intravenously by a calibrated Razel pump modified for infusion of small volumes. The mice remained in individual home cages throughout the study and were handled only while replacing the bedding (twice/wk). All the protocols were carried out between 0900 and 1500.

Experimental Protocol

Resting MAPs and HRs. Resting blood pressures and HRs were measured in conscious, freely moving mice for a week. Within this week, baroreflex testing was in general carried out 3–4 days after surgery when the blood pressure readings were stable.

Evaluation of cardiac baroreflexes. On the day of the experiment, before any experimental intervention the mice were allowed to stabilize for at least 60 min after which a baseline recording of blood pressure was obtained. Arterial pressure was elevated with increasing doses of either intravenously administered PE (6 µg/min) or ANG II (0.6 µg/min) for 30- to 45-s periods, and baroreflex curves were constructed relating MAP and HR (Fig. 1). Infusion of the pressor agent was terminated immediately after a 30-mmHg increase in blood pressure was achieved. After the blood pressure and HR returned to baseline, the venous catheter was flushed with a small volume of saline before attaching another line with a different pressor agent. The mice were allowed to recover for 45–60 min before subsequent drug administration, taking care that the basal blood pressures and HRs were within ±5% of the resting values. Infusions of the pressor agents were in random order. Reflex tachycardic responses to decreases in blood pressure with SNP (6.0 µg/ min) were measured at the end of the experiments.

A PowerLab data-acquisition system (AD instruments) with a sampling rate of 4,000/s was used to record blood pressures and HRs during the entire baroreflex function curve.

Data Analysis

Control values for arterial pressure and HR were taken as the 2-min average before the drug infusions. Data were
Baroreflex function relating increases in arterial pressure with PE and ANG II to decreases in HR was analyzed with linear regression analysis. Baroreflex function relating decreases in arterial pressure with SNP to increases in HR was analyzed with linear regression analysis. The reflex HR responses were compared using two-way analysis of variance. Newman-Keuls test was used to determine significance when indicated by analysis of variance. The slopes of baroreflex curves for PE and ANG II within a group were compared with paired t-test and between groups were compared with unpaired t-test. The slopes of baroreflex curves for SNP between the groups were compared with unpaired t-test. The probability level of \( P < 0.05 \) was considered to be statistically significant.

RESULTS

The basal values of arterial pressure and HR before evaluation of baroreflex function were comparable in the males (47 ± 4 mmHg, 613 ± 44 beats/min), OvxE+ (105 ± 3 mmHg, 644 ± 36 beats/min), and OvxE− (104 ± 7 mmHg, 663 ± 40 beats/min) females. The high basal HRs could be an indication that mice are operating either at high sympathetic activity or low vagal activity (9, 21). However, in the absence of direct measurements of peripheral sympathetic nerve activity, it is difficult to determine the level of sympathetic activity in the mouse.

Baroreflex Responses to PE

Baroreflex curves relating MAP to inhibition of HR during intravenous infusions of PE in ovariectomized female mice with (OvxE+) and without (OvxE−) estrogen replacement are presented in Fig. 1. In OvxE−, progressive increases in MAP with PE resulted in a linearly related reflex decrease in HR, with a slope of \( -4.5 ± 0.4 \) beats⋅min\(^{-1}\)⋅mmHg\(^{-1}\) and regresion coefficient of \( -0.95 ± 0.02 \) (Table 1). In OvxE+ mice, the reflex inhibition of HR in response to increases in arterial pressure with PE was greater than that observed in the OvxE− mice. This is reflected in a significantly greater slope of the baroreflex relation in OvxE+ female mice (\( -7.65 ± 1.37 \) beats⋅min\(^{-1}\)⋅mmHg\(^{-1}\), \( r = -0.91 ± 0.03, P = 0.026; \) Table 1). In intact male mice, progressive increases in MAP with PE resulted in a linearly related related reflex decrease in HR, with a slope of \( -8.50 ± 0.92 \) beats⋅min\(^{-1}\)⋅mmHg\(^{-1}\) (\( r = -0.88 ± 0.02; \) Table 1).

Baroreflex Responses to ANG II

Baroreflex curves relating MAP to inhibition of HR during intravenous infusions of ANG II in OvxE− and OvxE+ female mice are presented in Fig. 3. In OvxE−, progressive increases in MAP with ANG II resulted in a linearly related related reflex decrease in HR, with a slope of \( -4.8 ± 1.6 \) beats⋅min\(^{-1}\)⋅mmHg\(^{-1}\) (\( r = -0.90 ± 0.03; \) Table 1). In OvxE+ mice, the reflex inhibition of HR in response to increases in arterial pressure with ANG II was greater than that observed in the OvxE− mice. This is reflected in a significantly greater slope of the baroreflex relation in OvxE+ male mice (\( -7.97 ± 1.06 \) beats⋅min\(^{-1}\)⋅mmHg\(^{-1}\), \( r = -0.92 ± 0.02, P = 0.026; \) Table 1). In intact male mice, progressive increases in MAP with ANG II resulted in a linearly related related reflex decrease in HR, with a slope of \( -5.17 ± 0.95 \) beats⋅min\(^{-1}\)⋅mmHg\(^{-1}\) (\( r = -0.8 ± 0.06; \) Table 1).

Baroreflex Responses to ANG II Compared with PE

It is known that in many species acute ANG II infusion results in blunted baroreflex gain and a reset-
tting of the baroreceptor reflex control of HR to a higher pressure (1, 13, 31, 39). The term “resetting” is generally used to describe a shift in the set point around which the baroreflexes operate. It may not always be accompanied by a change in the gain or slope of the baroreflex curve, which signifies a change in the reflex response to equivalent increases in arterial pressure. In the present study, we analyzed the gain of the baroreflex response to either PE or ANG II. The differential baroreflex response to PE and ANG II and the effect of estrogen have not been investigated in mice. In male mice, reflex inhibition of HR in response to increases in arterial pressure with ANG II was significantly attenuated compared with the PE-induced reflex inhibition of HR (Fig. 4A). A 30-mmHg increase in arterial pressure with PE infusion was accompanied by a 255 ± 40-beats/min decrease in HR (slope: −8.50 ± 0.92 beats·min⁻¹·mmHg⁻¹, Table 1). The same increase in arterial pressure with ANG II infusion produced significantly smaller decreases in HR (148 ± 25 beats/min, P = 0.04), resulting in an attenuated reflex curve (slope: −5.17 ± 0.95 beats·min⁻¹·mmHg⁻¹, P = 0.008; Table 1).

However, in OvxE+ female mice, the reflex inhibition of HR in response to increases in arterial pressure with ANG II was not different from that observed with PE (P = 0.44; Fig. 4B). Similarly, in OvxE− female mice, although the reflex inhibition of HR was attenuated compared with OvxE+, there was no difference in the reflex response to increases in arterial pressure between PE and ANG II (P = 0.41; Fig. 4C).

Baroreflex Responses to SNP

Baroreflex curves relating decreases in MAP to increases in HR during intravenous infusions of SNP in OvxE+, OvxE−, and males are presented in Fig. 5. In OvxE−, progressive decreases in MAP with SNP resulted in a linearly related reflex increase in HR, with a slope of −1.1 ± 0.4 beats·min⁻¹·mmHg⁻¹ (r = −0.76 ± 0.09; Table 1). Estrogen replacement in the ovariectomized females did not alter the baroreflex responses to SNP as indicated by the slope of the baroreflex relation in OvxE+ mice (−1.93 ± 0.47 beats·min⁻¹·mmHg⁻¹, r = −0.73 ± 0.11; Table 1). In males, the slope of the linear relation between decreases in MAP and reflex increases in HR was −2.17 ± 0.58 beats·min⁻¹·mmHg⁻¹ (r = −0.86 ± 0.02; Table 1) and was not significantly different compared with OvxE+ (P = 0.39) or OvxE− (P = 0.11).

**DISCUSSION**

This study is the first to analyze the effects of estrogen and gender on cardiac baroreflex responses to PE, ANG II, and SNP in mice. The first finding was that in female mice, estrogen facilitated the baroreflex inhibition of HR induced by both PE and ANG II.

![Graph](http://ajpregu.physiology.org/Downloaded from 10.220.33.4 on April 25, 2017)
The ability of estrogen to facilitate reflex regulation of HR and sympathetic activity is consistent with observations made in rats (10, 41, 43). In the studies by Saleh et al. (42, 43), improvement of baroreflex sensitivity in ovariectomized female rats was observed following acute intravenous administration of 17β-estradiol. Share and colleagues (41) and El-Mas and Abdel-Rahman (10) observed similar effects but only following chronic subcutaneous administration of 17β-estradiol.

The site of action for the effects of estrogen on facilitation of baroreflex function is not clear. In rats, there is some evidence that estrogen affects baroreceptor afferents and/or central brain stem neurons modulating parasympathetic and sympathetic efferent neurons. Saleh et al. (42) showed that in ovariectomized female rats, estrogen administration into several hindbrain nuclei such as the nucleus of the solitary tract, nucleus ambiguus, parabrachial nucleus, and intrathecal space enhanced PE-induced reflex changes in HR. Abdel-Rahman et al. (32) showed that in ovariectomized rats, frequency-dependent depressor and bradycardic responses elicited by electrical stimulation of aortic depressor nerves were blunted compared with sham-operated rats and estrogen replacement reversed these effects. It is possible that estrogen also acts at central autonomic regulatory nuclei in mice to facilitate PE-induced changes in HR.

PE-induced reflex bradycardic responses in male mice were similar to OvxE+ females and significantly facilitated compared with OvxE− females. In healthy normotensive humans, men in general have been shown to have higher cardiac baroreflex sensitivity to PE than age-matched women, with baroreflex sensitivity being further depressed in older women (26, 35). Estrogen replacement has been found to improve baroreflex function in women toward that seen in men (19). Intact male rats have also been shown to have greater PE-induced baroreflex responses compared with intact females, and this difference was significant when the rats were ovariectomized (10). In this study, estrogen replacement in the ovariectomized rats restored the cardiac baroreflex responses to a level similar to that observed in male rats. Altogether, data from the above studies and from the present study suggest that estrogen replacement normalizes the difference in PE-induced cardiac baroreflex response.

The second finding of the present study is that there is a difference between males and females with regard to ANG II-mediated attenuation of reflex bradycardic responses in conscious mice. In dogs, rabbits, and rats, systemic administration of ANG II has been demonstrated to increase blood pressure without causing the large reflex bradycardia that normally accompanies such increases in blood pressure suggesting that ANG II resets the baroreflex control of HR to a higher pressure (29, 39). It is widely accepted that this effect of ANG II is due to central actions of this peptide on circumventricular organs such as area postrema (30, 34). In the present study, ANG II-mediated attenuation of reflex bradycardic responses was observed only in male mice. Reflex bradycardic responses to ANG II compared with PE were not significantly different in females regardless of the status of estrogen replacement. These data may suggest that ANG II-induced effects on baroreflex control of HR are gender-dependent. It is not clear if gender differences are observed in other species as most of the earlier studies in other species were performed in male animals or in induced ovulators such as female rabbits (3, 17, 30, 31, 38, 39). Reflex bradycardic responses to ANG II have been examined in pregnant and nonpregnant ewes but definitive conclusions about gender differences cannot be made as the estrogen status of the females in these studies is unknown and due to lack of systematic comparisons to males (20, 27). Reflex increases in pulse interval to ANG II-mediated increases in blood pressure were blunted compared with PE in both the pregnant and nonpregnant ewes. However, in contrast to the present study, this blunting was in general observed at pressure increases greater than 30 mmHg (20). In a second study, ANG II-mediated increases in blood pressure in nonpregnant ewes when counteracted with SNP infusion resulted in tachycardia. However, in this study, the responses in the nonpregnant females were grouped together with castrated males (27).

There is some evidence in humans that suggests gender-related differences in ANG II-mediated effects on cardiac baroreflexes (14). In a recent study comparing premenopausal women and age-matched men, ANG II infusion produced similar increases in blood pressures. However, in men, reflex decreases in HR were blunted relative to those observed in women. These findings are similar to that observed in the present study in mice and suggest that mice may prove to be a reasonable model for understanding gender differences in baroreflex and cardiovascular function in humans. However, it should be noted that the gender comparisons in the present study were made between intact males and ovariectomized females with or without estrogen replacement. Other gonadal hormones such as progesterone and testosterone are also known to affect baroreflex function and cardiovascular ho-
meostasis (6, 8, 11). The observations made with regard to gender differences in this study are limited to the contribution of estrogen alone. The 4-day-long estrous cycle in intact female rodents is accompanied by significant variations in estrogen and progesterone levels (15, 47, 51). A complete analysis of gender differences would require evaluation of baroreflex responses in intact females with known serum levels of endogenous ovarian hormones and in males with known levels of testosterone.

The molecular and cellular mechanisms underlying gender differences in ANG II modulation of baroreflex are unknown. However, it has been shown that in ovariectomized rats estrogen replacement decreases AT1 receptor expression and binding affinity at several central sites including the subfornical organ, a circumventricular organ (24, 25, 40, 44). In mice, it is interesting to hypothesize that ANG II receptor (AT1) expression at central nuclei mediating the effects of ANG II on baroreflex function could be greater in males compared with Ovx+E− females. Additional biochemical and molecular studies are planned to test this hypothesis.

Increases in AT1 receptor expression associated with decreases in estrogen levels may also account for the significantly smaller reflex bradycardic responses to ANG II in the OvxE− females. However, unlike the males, reflex bradycardic responses to PE are also significantly blunted in the OvxE−. It is possible that in female mice, estrogen modulates reflex bradycardic responses to PE and ANG II via different mechanisms. It is well documented that the one site of central action of ANG II is the area postrema that has neuronal projections to the nucleus of the solitary tract and the dorsal vagal complex and consequently is able to modulate activity in baroreceptor afferents in the hindbrain (22, 33). ANG II has been shown to activate the area postrema neurons, and lesioning of this region abolishes ANG II-mediated attenuation of reflex bradycardic responses in male rats and rabbits (2, 13, 30). In rats, spontaneous area postrema neuronal activity is inhibited by intravenous infusion of 17β-oestradiol probably by potentiating voltage-gated potassium currents (28). This effect on area postrema neuronal activity was rapid in onset, suggesting a nongenomic mechanism. It is possible estrogen replacement in the OvxE+ mice may alter the membrane properties of the area postrema neurons and attenuate ANG II-mediated increases in area postrema neuronal activity.

Activation of sympathetic outflow and reduction in parasympathetic outflow at least to the heart in response to decreases in arterial pressure with SNP did not appear to be influenced by gender or the levels of circulating estrogen in the mice. A definitive conclusion with regard to differences in reflex regulation of sympathetic tone in these mice cannot be ruled out in the absence of direct measurement of sympathetic nerve activity.

Perspectives

This is the first study to report cardiac baroreflex function and the role of estrogen in a conscious mouse model. Similar to observations made in other species, it is evident that estrogen has significant effects on cardiovascular regulation in the mouse. With the availability of several estrogen receptor knockout models, studies in the mouse species allow for further elucidation of mechanisms underlying the regulatory effects of estrogen on cardiovascular function. Delineation of the receptor subtype involved in mediating the beneficial cardiovascular effects could be useful in designing effective therapies to treat and/or prevent cardiovascular disease in women.

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