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Pinkham, M. I., & Barrett, C. J. (2015). Estradiol alters the chemosensitive cardiac afferent reflex in female rats by augmenting sympathoinhibition and attenuating sympathoexcitation. *Clinical and Experimental Pharmacology and Physiology*, 42(6), 622-631. doi: [10.1111/1440-1681.12392](https://doi.org/10.1111/1440-1681.12392)

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**Estradiol alters the chemosensitive cardiac afferent reflex
in female rats by augmenting sympathoinhibition and
attenuating sympathoexcitation**

Maximilian I. Pinkham¹ and Carolyn J. Barrett¹

Circulatory Control Laboratory, Department of Physiology and Bioengineering Institute,
University of Auckland, Auckland, New Zealand¹

Running head:

Sex and estradiol alter cardiac afferent reflex

Key words:

Cardiac afferent, myocardial infarction, sex, sympathetic nerve

Address for correspondence:

Dr. Carolyn Barrett

Circulatory Control Laboratory

Department of Physiology

Faculty of Medical and Health Sciences

The University of Auckland

Private Bag 92019

Auckland 1142

New Zealand

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1440-1681.12392

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Accepted Article

Fax: (649) 373 7499

Phone: (649) 373 7599 ext 86909

Email: c.barrett@auckland.ac.nz

Abstract

The chemosensitive cardiac vagal and sympathetic afferent reflexes are implicated in driving pathophysiological changes in sympathetic nerve activity (SNA) in cardiovascular disease states. This study investigated the impact of sex and ovarian hormones on the chemosensitive cardiac afferent reflex. Experiments were performed in anesthetized, sinoaortic baroreceptor denervated male, female and ovariectomized female (OVX) Wistar rats with either intact cardiac innervation or bilateral vagotomy. To investigate the chemosensitive cardiac afferent reflexes renal SNA, heart rate (HR) and arterial pressure (AP) were recorded before and following application of capsaicin onto the epicardial surface of the left ventricle. Compared to males, ovary-intact females displayed similar cardiac afferent reflex mediated changes in renal SNA albeit with a reduced maximum sympathetic reflex driven increase in renal SNA. In females, ovariectomy significantly attenuated the cardiac vagal afferent reflex mediated inhibition of renal SNA (renal SNA decreased $2\pm 17\%$ in OVX vs. $-50\pm 4\%$ in ovary-intact females, $P < 0.05$) and augmented cardiac sympathetic afferent reflex mediated sympathoexcitation (renal SNA increased $91\pm 11\%$ in OVX vs. $62\pm 9\%$ in ovary-intact females, $P < 0.05$) so that overall increases in reflex driven sympathoexcitation were significantly enhanced. Chronic estradiol replacement, but not progesterone replacement, begun at time of ovariectomy restored cardiac afferent reflex responses to be similar as ovary-intact females. Vagal denervation eliminated all group differences. The current findings show ovariectomy in female rats, mimicking menopause in women, results in greater chemosensitive cardiac afferent reflex driven sympathoexcitation and does so, at least partly, via the loss of estradiols actions on the cardiac vagal afferent reflex pathway.

Introduction

The chemosensitive cardiac vagal and sympathetic afferent reflexes are implicated in driving changes in sympathetic nerve activity (SNA) in cardiovascular diseases such as myocardial infarction (MI), heart failure and hypertension [1-6]. In pathophysiological conditions, increased cardiac SNA directly drives arrhythmias, fibrillation and sudden death [7], whilst elevated peripheral SNA, particularly renal SNA, drives an increase in total peripheral resistance, fluid retention and renin release that ultimately results in adverse changes in heart structure and function [8, 9]. Clinically significant differences between men and women exist in both the mortality and morbidity associated with cardiovascular diseases, however the cause of these differences are poorly defined [10-12]. The incidence of cardiovascular disease is age-related with females having a lower rate than males pre-menopause, but higher rate post-menopause, suggesting an effect of changes in sex hormone levels [10, 13, 14]. Experimentally, ovarian hormones are associated with cardioprotection following MI [15, 16].

The release of metabolites within the heart muscle stimulates both cardiac sympathetic and vagal chemosensory afferents which subsequently leads to centrally-mediated changes in SNA and hemodynamics [3, 4]. The cardiac sympathetic afferent fibres travel via the middle cardiac nerve, with cell bodies in the dorsal root ganglion and project via secondary spinal afferents to the nucleus tractus solitarii (NTS). The cardiac vagal afferent fibres travel bilaterally via the vagus, with cell bodies in the nodose ganglion, to the NTS. Activation of cardiac sympathetic or vagal afferents results in increases and decreases in SNA respectively, therefore the autonomic changes that occur depend on the balance of activation between the two parallel, yet opposing, cardiac afferent pathways [2]. In this context it is understood that ovarian hormones, and female sex, do affect the regulation of the sympathetic nervous system.

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Current evidence in animals and humans suggest that estrogen is capable of attenuating reflex mediated increases, and augmenting reflex mediated decreases, in SNA [17, 18]. The general view is that estrogen has an inhibitory influence over sympathetic outflow [19]. This is supported by the observations that, in females, resting muscle SNA increases following menopause, when circulating estrogens significantly decrease [20]. Furthermore, acute administration of estradiol directly lowers resting muscle SNA in women [21]. There is very limited evidence regarding the effects of progesterone on sympathetic control [22].

Clinical and experimental evidence have demonstrated that sex and ovarian hormones can influence the autonomic response to MI, although exactly how is unknown [23-25]. Previously, we have shown that ovarian hormones in female rats attenuate the initial sympathetic excitation in response to acute MI, in a manner that is not wholly explained by a sex difference in the arterial baroreceptor reflex [24]. Based on our previous findings it is possible that sex and ovarian hormones may impact the sympathetic response to MI via the cardiac afferent reflex [2, 24]. Importantly, estrogen receptors have been found in areas associated with the cardiac afferent reflex pathway including sympathetic and vagal afferent fibres and central autonomic regions [26-29]. Therefore in the current study we tested the hypothesis that estrogen in females augments the cardiac vagal afferent reflex mediated sympathoinhibition and attenuates cardiac sympathetic afferent reflex mediated sympathoexcitation. To test our hypothesis, in ovariectomized females we investigated the impact of estradiol and progesterone on the cardiac afferent reflex and compared to responses in males and ovary-intact females.

Results

Baseline variables

Baseline MAP, HR and renal SNA were not significantly different between the 10 groups (Table 1). Despite being the same age, OVX female rats with estradiol replacement (OVX+E) had significantly lower body weights compared to OVX females with no hormone replacement and OVX females with progesterone replacement (OVX+P) (Table 1).

Estradiol mediates cardiac vagal afferent reflex driven inhibition of renal SNA in females

Figure 1 illustrates raw recordings of cardiac afferent reflex mediated changes in arterial pressure and renal SNA in response to capsaicin placed onto the anterior surface of the left ventricle. In male and ovary-intact female rats with intact cardiac sympathetic and vagal innervation, the epicardial application of capsaicin caused a biphasic response with an initial sympathoinhibitory phase and hypotension, followed by a secondary sympathoexcitatory phase and hypertension consistent with previous observations using similar doses of capsaicin and method of application [30]. Bilateral vagotomy abolished initial inhibition of renal SNA and hypotension confirming these responses were driven by cardiac vagal afferents.

The initial cardiac vagal afferent reflex driven decrease in renal SNA in response to epicardial capsaicin application was dose dependent as shown in Figure 2. In animals with intact cardiac innervation, the initial decrease in renal SNA driven by the cardiac afferent reflex response to epicardial capsaicin application was not different between males ($-47\pm 7\%$ in response to $10\mu\text{g}$ capsaicin) and ovary-intact females ($-50\pm 4\%$, $P>0.05$, Figure 2). Ovariectomy in females resulted in a significant attenuation in the initial cardiac afferent reflex driven decrease in renal SNA (post- $10\mu\text{g}$ capsaicin change in renal SNA during the sympathoinhibitory phase was $2\pm 17\%$ in OVX females) when compared to males ($-47\pm 7\%$,

P<0.001) and ovary-intact females (-50±4%, P<0.001, Figure 2). Vagal denervation significantly attenuated the change in renal SNA during the sympathoinhibitory phase post-capsaicin in males and ovary-intact females but did not alter the response in OVX females (post-10µg capsaicin change in renal SNA was 7±4% in vagal denervated OVX vs. 2±17 in intact cardiac innervation OVX, P>0.05, Figure 2).

In animals with intact cardiac innervation, chronic estradiol replacement begun at time of ovariectomy restored the cardiac vagal afferent reflex driven inhibition in renal SNA to be similar to ovary-intact females (post-10µg capsaicin change in renal SNA during the sympathoinhibitory phase was -42±7% in OVX+E females vs. -50±4% in ovary-intact females, P>0.05) and significantly different to responses in OVX females without hormone replacement (vs. 2±17 in OVX females, P<0.001) and OVX females with chronic progesterone replacement (vs. -9±5 in OVX+P females, P<0.001, Figure 2).

Estradiol attenuates cardiac sympathetic afferent reflex driven maximum increases in renal SNA in females

Cardiac sympathetic afferent reflex driven increases in renal SNA in response to epicardial capsaicin application were dose dependent (Figure 3). As shown in Figure 3 the differences between groups only reached significance at the highest dose of capsaicin.

In animals with intact cardiac innervation, ovary-intact females displayed significantly attenuated cardiac afferent reflex driven increases in renal SNA compared to males and OVX females (post-10µg capsaicin change in renal SNA during the sympathoexcitatory phase was 91±11% in OVX females vs. 62±9% in ovary-intact females, P<0.05, Figure 3). Vagal denervation did not alter the sympathoexcitatory response to cardiac afferent reflex activation in males and OVX females (Figure 3). However, vagal denervation did eliminate the differences in sympathoexcitation when comparing ovary-intact females to males (post-10µg capsaicin change in renal SNA during the sympathoexcitatory phase was 90±18% in

vagal denervated ovary-intact females vs. $106 \pm 19\%$ in vagal denervated males, $P > 0.05$) and OVX females (vs. $94 \pm 10\%$ in vagal denervated OVX females, $P > 0.05$, Figure 3).

In animals with intact cardiac innervation, chronic estradiol replacement begun at time of ovariectomy resulted in attenuated cardiac sympathetic afferent reflex driven increases in renal SNA when compared to OVX females without hormone replacement (post- $10\mu\text{g}$ capsaicin change in renal SNA during the sympathoexcitatory phase was $40 \pm 15\%$ in OVX+E vs. $91 \pm 11\%$ in OVX females, $P < 0.05$) and similar to sympathoexcitatory changes in ovary-intact females (vs. $62 \pm 9\%$ in ovary-intact females, $P > 0.05$, Figure 3).

Time to maximum cardiac afferent reflex sympathoexcitation is affected by sex but not ovarian hormones

In animals with intact cardiac innervation, females displayed a significantly faster time to reach maximum sympathetic excitation following capsaicin application compared to males (time to maximum sympathoexcitation post-capsaicin was 20 ± 2 seconds in ovary-intact females vs. 40 ± 2 seconds in males, $P < 0.001$, Figure 4A) regardless of state of circulating ovarian hormones (time to maximum sympathoexcitation post-capsaicin 17 ± 2 seconds in OVX females vs. 40 ± 2 seconds in males, $P < 0.001$, Figure 4A). Compared to animals with intact cardiac innervation, vagal denervation significantly shortened the time to maximum sympathoexcitation in males (time to maximum sympathoexcitation post-capsaicin was 21 ± 3 seconds in vagal denervated males vs. 40 ± 2 seconds in males with intact cardiac innervation, $P < 0.001$) but did not alter the time to maximum sympathoexcitation in females. Therefore vagal denervation abolished the sex difference in time to sympathoexcitation following cardiac afferent reflex activation. In animals with intact cardiac innervation, the time to reach maximum sympathoinhibition following epicardial capsaicin application was not different between males (5 ± 0.4 seconds), ovary-intact females (6 ± 0.4 seconds, $P > 0.05$) and OVX females (5 ± 0.6 seconds, $P > 0.05$, Figure 4A). In vagal denervated groups, the time to

reach maximum sympathoinhibition following epicardial capsaicin application was not determined as there were not clear and consistent decreases from baseline at any time-point post capsaicin application.

Ovariectomy alters the balance between vagal and sympathetic components of the cardiac afferent reflex to favour greater sympathoexcitation

To determine what effect sex and ovarian hormones may have on total change in renal SNA following cardiac afferent reflex activation the average change in renal SNA was taken during the total 40 second period between application of- and then removal of- capsaicin. In all animals the overall effect of cardiac afferent reflex activation over the 40 second period was to drive an average increase in renal SNA. In ovary-intact females with intact cardiac innervation, the combined effect of a faster time to maximum excitation and reduced maximum increase in renal SNA meant that when compared to males, ovary-intact females displayed similar average changes in renal SNA over the total 40 seconds post-capsaicin (Figure 4B). In ovariectomized females with intact cardiac innervation the loss of cardiac vagal afferent reflex mediated inhibition resulted in significantly greater cardiac afferent reflex driven average increases in renal SNA over the 40 seconds post-capsaicin (renal SNA averaged $91\pm 11\%$ in OVX females) compared to ovary-intact females ($41\pm 6\%$, $P=0.034$) and males ($35\pm 11\%$, $P=0.027$, Figure 4B). In ovariectomized females with intact cardiac innervation chronic estradiol replacement, but not progesterone replacement, attenuated the cardiac afferent reflex driven average increase in renal SNA over the 40 seconds period post-capsaicin application compared to OVX females with no hormone replacement (over 40 seconds post- $10\mu\text{g}$ capsaicin renal SNA averaged $21\pm 9\%$ vs. $91\pm 11\%$ in OVX females, $P=0.002$) and resulted in similar changes in renal SNA when compared to ovary-intact females and males (Figure 4B). Vagal denervation eliminated differences between all groups

in the overall renal SNA change over the 40 seconds period following epicardial capsaicin application as shown in Figure 4B.

Hemodynamic changes following cardiac afferent reflex activation in males and females

Timeline changes in arterial pressure and HR following epicardial capsaicin application were unaffected by sex and state of ovarian hormones. In animals with intact cardiac innervation the time to maximum increase in arterial pressure following epicardial capsaicin application was 33 ± 3 seconds in males, 28 ± 1 seconds in ovary-intact females and 22 ± 2 seconds in OVX females and was not altered by vagal denervation. Time to maximum increase in HR following epicardial capsaicin application was not determined as HR had typically not plateaued by 40 seconds post-capsaicin application, regardless of sex.

Arterial pressure and HR responses to the epicardial application of capsaicin onto the surface of the left ventricle were dose dependent (data not shown). As shown in Figure 5, cardiac afferent reflex mediated changes in renal SNA, heart rate and arterial pressure were similar. Ovary-intact females displayed similar cardiac vagal afferent reflex driven decreases in both HR and arterial pressure when compared to males (Figure 5). Ovariectomy in females significantly attenuated the cardiac vagal afferent reflex driven decreases in both HR and arterial pressure so that the responses were significantly different to both ovary-intact females and males (Figure 5). Chronic estradiol treatment, but not progesterone treatment, begun at time of ovariectomy augmented the cardiac vagal afferent reflex driven decreases in HR and arterial pressure when compared to OVX females with no hormone replacement so that responses were similar to ovary-intact females (Figure 5). Ovariectomized females without hormone replacement and OVX with progesterone replacement with intact cardiac innervation demonstrated augmented cardiac afferent reflex

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driven average increases in arterial pressure over the 40 seconds period post-capsaicin application when compared to ovary-intact females (Figure 4B). OVX with progesterone replacement group demonstrated augmented cardiac afferent reflex driven average increases in heart rate over the 40 seconds period post-capsaicin application when compared to ovary-intact females (Figure 4B).

Discussion

Previously we have shown that ovarian hormones in female rats attenuate sympathoexcitation in direct response to acute MI independent of the arterial baroreceptor reflex [24]. Given the evidence that the cardiac afferent reflex plays an important role in driving initial changes in SNA post-MI we hypothesized that ovarian hormones in females, specifically estradiol, would attenuate the cardiac sympathetic afferent reflex driven increases in renal SNA and augment the cardiac vagal afferent reflex driven decreases in renal SNA. The major findings of the current study are 1) compared to males, ovary-intact female rats displayed similar overall changes in renal SNA in response to cardiac afferent reflex activation, 2) in females, ovariectomy resulted in greater maximum increases- and attenuated cardiac vagal afferent reflex driven decreases in renal SNA thereby resulting in greater overall reflex driven sympathoexcitation 3) chronic estradiol replacement, but not progesterone replacement, reversed the effects of ovariectomy on the cardiac afferent reflex so that responses became similar to ovary-intact females, 4) following cardiac afferent reflex activation the time to maximum sympathetic excitation was significantly faster in females compared to males, 5) all differences between groups in the cardiac afferent reflex responses were eliminated by vagal denervation. The findings suggest that estradiol in female rats has a significant modulatory effect on the cardiac afferent reflex, possibly by acting on the vagal pathway, with a decrease in circulating levels of estradiol in females resulting in augmented reflex driven increases in renal SNA.

Previous studies have only investigated the cardiac afferent reflex in either males or mixed sex groups; therefore the present study is the first to specifically investigate the cardiac afferent reflex control of renal SNA in females. The cardiac afferent reflex is known to drive changes in renal SNA in cardiovascular disease states. Elevated renal SNA in cardiovascular diseases can drive disease progression by increasing fluid load on the heart and by mediating an increase in circulating angiotensin II, via renin release. Therefore, specific investigations of how female sex and ovarian hormones may alter changes in renal SNA, particularly in relation to disease states, are important. In the current study the cardiac afferent reflex responses observed in males were consistent with previous findings using similar doses of capsaicin [30]. Estradiol receptors are expressed in the heart, dorsal root ganglia, nodose ganglia, spinal afferent pathways and in central brain regions involved in the cardiac afferent reflex pathway [26-29, 31]. Furthermore capsaicin sensitive afferent neurons are immunoreactive for estradiol receptors- α and $-\beta$ [26]. Capsaicin, an exogenous substance taken from chili peppers, activates the vanilloid 1 receptor (VR1), which is a non-specific cation channel causing depolarization of the afferent fibre. C-type, non-myelinated and high threshold afferents appear to make up most of the chemosensitive afferents with sensory endings in the heart wall [32-35]. VR1 receptors are expressed in both ventricles of the heart with the highest level of expression on the epicardial surface [36]. To the best of our knowledge no studies have investigated sex specific distributions of VR1 in the heart tissue. Current evidence indicates that sex steroids, and in particular estradiol, are capable of directly modulating VR1 actions [37]. Furthermore, central microinjection of estradiol into the NTS and rostral ventrolateral medulla causes an acute inhibition in efferent renal SNA and an increase in efferent vagal activity suggesting that central estradiol receptors can alter autonomic regulation [38]. It is possible that estradiol can modulate both the peripheral and central components of the cardiac afferent reflex, although the precise mechanisms are currently not understood.

Ovariectomy in females significantly attenuated the cardiac vagal afferent reflex mediated inhibition of renal SNA and augmented the cardiac sympathetic afferent reflex mediated increase in renal SNA. Therefore, the overall effect was that ovariectomy resulted in greater cardiac afferent reflex driven increases in renal SNA, when compared to the responses in males and ovary-intact females. Differences between ovary-intact and OVX females in the cardiac afferent reflex driven sympathoexcitation were eliminated by vagal denervation and/or estradiol, suggesting that estradiol acting on the cardiac vagal afferent pathway at least partly mediated these differences. Capsaicin directly applied to the heart surface increases the neuronal activity of both chemosensory cardiac vagal and sympathetic afferent fibres [34]. In anesthetized rats and cats it has been shown that cardiac vagal afferent input centrally antagonizes the sympathoexcitatory actions of cardiac sympathetic afferents [30, 39, 40]. It is unknown whether estradiol is capable of modulating the central integration of vagal and sympathetic afferent inputs. Previous studies suggest that ovarian hormones, particularly estradiol, are capable of altering the sensitivity of afferents within the vagus nerve [41-44]. However there is currently no research describing the specific effects of ovarian hormones on chemosensitive cardiac afferents, particularly C-fibres that are understood to be responsible for centrally projecting chemosensitive cardiac afferent activity [32-34]. In the current study the doses of capsaicin were larger than in many previous studies as significant differences were not observed at lower doses. Capsaicin applied at higher doses can potentially activate neurons independent of VR1 and therefore it is possible that α -type afferent fibres may have also contributed to the responses observed. However, the possibility that potentially non-chemosensitive α -type afferent fibres were also recruited does not detract from the current observation that estradiol attenuates renal SNA excitation following activation of afferent sensory neurons arising from the heart.

The current study highlights sex specific changes in SNA in response to activation of chemosensory cardiac afferents. It is known that the chemosensitive cardiac afferent reflex is an important proponent of initial changes in autonomic balance in response to MI, with the precise changes depending at least partly on the balance of input from the cardiac vagal and sympathetic afferents [2]. Following MI, a shift in the sympathovagal balance towards sympathetic predominance exposes the patient to a higher likelihood of ventricular fibrillation and sudden death whereas vagal predominance is cardioprotective [7, 45]. Previously we have observed that, compared to males, ovary-intact females demonstrated an attenuated initial sympathoexcitation in response to acute MI [24]. The current findings demonstrate that the cardiac afferent reflex driven change in renal SNA over 40 seconds post-capsaicin is similar between males and ovary-intact females, a period of time similar to the first minute of coronary occlusion in our previous study [24]. Combined with our previous results [24] the current findings suggest that the cardiac afferent reflex may play a minimal role in mediating the differences between males and ovary-intact female rats in the initial renal SNA response to MI. In contrast, the current finding that ovariectomy in female rats resulted in significantly greater cardiac afferent reflex driven increases in renal SNA may help explain our previous findings that ovariectomy augments the sympathoexcitatory response to MI in females, independent of the arterial baroreflex [24]. Whether sex differences in the cardiac afferent reflex do influence sex differences in both or either the initial- and long-term changes in SNA post-MI is unknown.

The current study failed to observe a significant impact of the estrous cycle on the cardiac afferent reflex. However, this is not to say that changing levels of circulating estrogens within ovary-intact females do not alter the cardiac afferent reflex. Ovary-intact females were initially divided into two groups in order to separate females according to estrous phases with high levels of estrogen (proestrous) and low levels of estrogen (metestrous). The estrous cycle in the rat occurs over four days and in proestrous the spikes in estrogens

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happen over a relatively short time, it may be that in the females in proestrous the level of estrogens were not significantly increased at the time of experiment. Previously, it has been demonstrated that the estrous cycle in rat and menstrual cycle in women affects baroreflex control of SNA and we had expected to observe a similar effect on the cardiac afferent reflex [17, 46]. In the present study, the method of hormone replacement, by implanting silastic capsules, has been shown previously to provide levels of circulating hormone consistent with physiological levels in ovary-intact female rats [47, 48].

Limitations

The levels of estradiol and progesterone were not measured which is a major limitation of the current study. However, the significant effect of the estradiol, but not progesterone, replacement treatment on reflex responses and body weight gain supports the assumption that there were significant differences in baseline estradiol levels between ovary-intact and ovariectomized female groups. The current study was performed in anesthetized, open chest animals and therefore resting blood pressures were lower compared to conscious animals. An adequate level of anesthesia was maintained during experiments in order to avoid pain mediated SNA and cardiovascular changes in response to capsaicin that would complicate the cardiac afferent reflex response. Whether the same effects of sex and ovarian hormones on the cardiac afferent reflex exist in conscious animals is unknown. The use of capsaicin as the sole stimulant in the current study is a major limitation, as it is an exogenous substance. We did attempt to use bradykinin, an endogenous substance that has previously been shown to activate the cardiac afferent reflex similarly to capsaicin [36], and found it to have highly inconsistent responses and therefore the results were not presented. For future investigations it is important that endogenous substances be used. Relatively low numbers in each group may have limited the ability to find statistical significance due to the multiple factors and variables being compared thereby decreasing the power of the ANOVA.

Furthermore, some of the variables were not completely independent and so the statistical analysis must be considered with this in mind.

Perspective

The incidence of MI in women, particularly post-menopausal, has been increasing due to the increasing age of populations [13, 49, 50]. To date the majority of treatment strategies for MI have been developed using male subjects. It is important to understand the underlying mechanisms of sex specific responses to MI so that treatment strategies can be improved in women. The autonomic nervous system plays a primary role in driving cardiovascular changes post-MI and, via beta-blockers, is a major treatment target. The current study highlights a pathway that may contribute to sex specific sympathetic responses to MI and therefore outcomes in cardiovascular diseases such as MI [13, 49, 50].

Methods

Experimental preparation

Experiments were conducted in 54 Wistar rats and were approved by, and carried out following the guidelines of, the Animal Ethics Committee of the University of Auckland. Preparatory surgery was performed in a subset of female rats weighing 120-150 grams and aged between 5-6 weeks old. Briefly, bilateral ovariectomy (OVX) was performed through a dorsal incision under isoflurane anesthesia (2% in oxygen). Chronic hormone replacement, begun at the time of ovariectomy, was achieved as has been previously described [51]. Briefly, silastic capsules (0.93 I.D./3.23 O.D, Dow Corning, VWR International, Buffalo Grove, IL, USA) implanted sub-cutaneously during ovariectomy surgery were used to chronically administer estradiol (1x20mm tube length filled with estradiol (solution; 200 μ g 17 β -estradiol/ml peanut oil (Sigma-Aldrich Inc. USA)), or progesterone (4x30mm tube length

packed with crystalline progesterone (Sigma-Aldrich Inc. USA)). Rats with no hormone replacement did not receive silastic capsule implantation. At the beginning of surgery, all animals were given antibiotics (12.5 mg/kg enrofloxacin, Baytril, Bayer, New Zealand) and analgesia (20 µg/kg buprenorphine, Temgesic, Reckitt Benckiser, New Zealand) and again 24 hours later. As soon as the rats regained consciousness they were returned to their home cages. A heating pad was placed under the cage for 24 hours after the surgery. Prior to the experimental day all rats were housed 2-4 per cage with water and food *ad libitum* in a room of constant temperature (22±1°C) with a 12h:12h light:dark cycle. Experiments were performed 4-6 weeks following ovariectomy.

Female animals with intact ovaries had their stage of estrous cycle determined by examining the cytology of vaginal smears. Animals were divided into 10 groups according to sex, state of ovarian hormones and whether the animal had intact cardiac innervation or had undergone vagotomy thereby leaving only the cardiac sympathetic afferents intact. In some cases (n=15), animals already used in the intact cardiac innervation group subsequently underwent bilateral vagotomy and were also used in the vagal denervated group.

On the day of experiment the animals, aged between 8-14 weeks old, were anesthetized by intraperitoneal injection of urethane (1000-1500 mg/kg IP, Sigma-Aldrich Inc. USA) and α -chloralose (80-120 mg/kg IP, Sigma-Aldrich Inc. USA) to effect over the course of an hour. Sufficient anesthesia was confirmed by testing for the complete removal of the hind-limb-, tail pinch- and blink reflex. Anesthesia was maintained throughout the experiment by intravenous infusion of anesthetic (10% of initial dose every hour) and body temperature was maintained at 37°C by a heating pad and heating lamp.

Surgical Preparation

Once sufficient level of anesthesia was obtained the trachea was cannulated and the rat artificially ventilated (model 680; Harvard apparatus, Holliston, MA) with room air supplemented with oxygen. The femoral artery and vein were cannulated to monitor arterial pressure and for administration of drugs, respectively. To record renal SNA a retroperitoneal incision was used to expose the left kidney. The renal nerve was identified and placed within a pair of coiled stainless steel electrodes. The electrode wires and nerve were then coated in a silicone elastomer (Kwik-sil, World Precision Instruments, Sarasota, FL). Renal SNA was wirelessly recorded via a high gain amplifier (model TR46SP, Telemetry Research Ltd., Auckland, NZ) with the signal transmitted to a receiver (TR162, Telemetry Research Ltd., Auckland, NZ). Renal SNA recordings were validated by listening to the nerve signal and by observing the entrainment of the sympathetic nerve signal to the arterial pulse. Arterial baroreceptors were denervated by cutting the carotid sinus and aortic depressor nerves. The carotid sinus was exposed and denervated bilaterally by cutting all visible nerves between the internal and external carotid arteries and stripping these vessels. The aortic depressor nerve was located at the point where the superior laryngeal nerve meets the vagal nerve and transected bilaterally. Baroreceptor denervation was confirmed by the absence of changes in renal SNA and heart rate (HR) when arterial pressure was increased by intravenous infusion of phenylephrine (PE, 20 μ g/kg). In a subset of rats, vagal denervation was also performed in addition to baroreceptor denervation by cutting the cervical vagal nerves running alongside the carotid artery. The chest was then opened via an incision through the 4th intercostal space and the pericardium removed. A positive end pressure of 3-4 mmHg was used to ensure the lungs remained inflated. Mean arterial pressure (MAP), heart rate (HR) and renal SNA were recorded throughout the course of the experiment in all groups.

Experimental protocol

Cardiac afferent reflex responses to capsaicin

Following preparatory surgery and prior to beginning the experimental protocol a minimum of 30 minutes was allowed to ensure all recorded variables were stable. To test the cardiac afferent reflex in anesthetized, baroreceptor-denervated rats, dose-dependent responses to capsaicin (1, 5 and 10 μ g per 10 μ l of saline (10% ethanol and 1% Tween 80)) were applied, via a ~3x3mm piece of filter paper, onto the epicardial surface of the left ventricle. MAP, HR and renal SNA responses were recorded for 40 seconds post-capsaicin application. Previous studies [39], and our own observations (not shown), have identified that maximum sympathetic excitation following the epicardial application of capsaicin is achieved by 40 seconds. After 40 seconds the filter paper was removed and the heart rinsed in warm saline (~50ml). A recovery period of at least 15 minutes was allowed between applications of the different doses of capsaicin in each rat. Application of vehicle or saline alone onto the epicardial surface of the left ventricle had no effect on hemodynamics and renal SNA. Capsaicin was the sole stimulant used in the current study because our preliminary findings suggested that 1) capsaicin produced consistent reflex responses in the males, therefore confident comparisons could be made with the responses in females and, 2) bradykinin, another stimulant for the cardiac afferent reflex that is typically used in these types of studies [36], produced very inconsistent responses in both males and females (see Supporting Figure 1).

Data Collection

The original renal SNA signal was amplified, filtered between 50-2000 Hz, full-wave rectified and integrated using a low pass filter with a 20ms time constant. All data were sampled at 500Hz using an analog-to-digital acquisition card (PCI-6024E National Instruments, Austin, TX). All subsequent data analysis was performed using a data acquisition program

(Universal Acquisition and Analysis, ver. 11). SNA was normalized as a percentage change from resting level. Changes in arterial pressure and HR were taken as absolute values of the change from resting level. The resting level for each variable was taken as a period of 60 seconds directly prior to capsaicin application. The noise level was taken to be the integrated SNA value when SNA was at the lowest level between two distinct bursts of nerve activity.

Statistical analysis

The changes in arterial pressure (mmHg), HR (beats per minute, BPM), and renal SNA (%) were assessed as a change from the average of arterial pressure, HR, and renal SNA over 60 seconds directly prior to capsaicin application (termed baseline). To determine the effects of sex and ovarian hormones on the two adjacent components of the cardiac afferent reflex (the cardiac vagal and sympathetic afferent reflexes) two different time points following capsaicin application were subsequently investigated. The sympathoinhibitory phase of the cardiac afferent reflex was determined as the average over a period of 5 seconds of recording between 2-7 seconds following capsaicin application. The sympathoexcitatory phase of the cardiac afferent reflex was determined as the average over a period of 5 seconds of recording between 35 to 40 seconds following capsaicin application (see results for further explanation). Overall change in renal SNA driven by cardiac afferent reflex activation was analysed as the percentage change from baseline in renal SNA over 40 seconds directly following capsaicin application and prior to the rinsing off of capsaicin. There were no significant differences between ovary-intact females in the metestrous and proestrous phases of the estrous cycle (See Supporting Figure 2). Therefore ovary-intact females are grouped together regardless of estrous phase for subsequent analysis. Furthermore, cardiac afferent reflex responses in animals used in both intact cardiac innervation group and vagal denervated group indicated that following vagal denervation the initial vagal mediated sympathoinhibitory phase was abolished and the sympathoexcitatory response was not significantly altered in these animals (in the 15 animals the average

change in renal SNA during the sympathoexcitatory phase was $76\pm 9\%$ above baseline following bilateral vagal denervation vs. $58\pm 9\%$ above baseline before vagal denervation, $P=0.166$, Supporting Figure 3). Therefore these animals were included in the overall group analysis. Statistical analysis was performed in Sigmaplot 11.0. Three-way ANOVA with Bonferroni post-hoc analysis was used to analyse the cardiac afferent reflex responses between all groups with the three factors being *sex/hormonal status*, *dose of capsaicin* and *state of innervation*. Two-way ANOVA was used to analyse the baseline variables and when analysing the time to maximum decrease in renal SNA, HR and arterial pressure in all groups (because animals with vagal denervation did not display a clear decrease in all variables). P values less than 0.05 were considered significant.

Grant support

This work was supported by the Lottery Health New Zealand and University of Auckland Faculty Research Development Fund.

Disclosure

All authors have contributed sufficiently to the project to be included as authors.

No financial or other conflicts of interest exist.

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Table 1: Baseline body weight, mean arterial pressure (MAP, mm Hg), heart rate (HR, beats per minute (BPM), and renal sympathetic nerve activity (RSNA (μ V)) prior to the application of capsaicin onto the epicardial surface of the left ventricle in males, ovary-intact females (female), ovariectomized females: without hormone replacement (OVX), with estradiol replacement (OVX+E), or with progesterone replacement (OVX+P) with intact cardiac innervation (intact inner.) or vagal denervated and only cardiac sympathetic innervation intact (Vagal den.). * indicates significant difference compared to OVX and OVX+P groups, $P < 0.05$.

Variable	Male		Female		OVX		OVX+E		OVX+P	
	Intact inner.	Vagal den.	Intact inner.	Vagal den.	Intact inner.	Vagal den.	Intact inner.	Vagal den.	Intact inner.	Vagal den.
Number in group	7	5	13	11	6	5	7	7	4	4
Weight (grams)	278 \pm 8	284 \pm 8	274 \pm 6	266 \pm 6	293 \pm 8	289 \pm 9	223 \pm 8*	223 \pm 8*	325 \pm 10	310 \pm 10
MAP, mmHg	87 \pm 5	77 \pm 5	79 \pm 3	73 \pm 4	74 \pm 4	75 \pm 5	77 \pm 5	73 \pm 5	72 \pm 6	75 \pm 6
HR, bpm	417 \pm 17	480 \pm 18	430 \pm 13	466 \pm 13	456 \pm 18	480 \pm 18	406 \pm 18	479 \pm 18	430 \pm 22	492 \pm 22
RSNA, μ V	3.6 \pm 0.6	3.9 \pm 0.7	3.8 \pm 0.5	2.5 \pm 0.4	3.0 \pm 0.8	3.4 \pm 0.7	3.3 \pm 0.7	3.4 \pm 0.7	4.0 \pm 0.8	2.6 \pm 0.9

Figure captions

Figure 1: Example traces of arterial pressure (mm Hg), original renal sympathetic nerve activity (RSNA, μV) and integrated RSNA (μV) showing 10 seconds before and 40 seconds following epicardial application of capsaicin (time of application indicated by arrow) onto the left ventricle in a male, ovariectomized female (OVX), OVX with estradiol replacement (OVX+E) and OVX with progesterone replacement (OVX+P) with either intact cardiac innervation (top panels) or vagal denervated and therefore only cardiac sympathetic innervation (bottom panels).

Figure 2: Dose response curves showing the renal sympathetic nerve activity (SNA) response as a percentage (%) change from baseline during the initial sympathoinhibitory phase of the biphasic cardiac afferent reflex response (as illustrated in figure 1) following epicardial application of capsaicin at three different dose concentrations (1, 5 and $10\mu\text{g}$ per $10\mu\text{l}$ saline) in male, female and ovariectomized female (OVX) groups (top row) and OVX with estradiol replacement (OVX+E) and OVX with progesterone replacement (OVX+P, bottom row) in animals with intact cardiac innervation (figures on left) and animals with only cardiac sympathetic afferents intact and bilateral vagal denervation (figures on right). Data are presented as mean \pm SEM. * indicates significant difference between OVX and ovary-intact females, $P < 0.05$, # indicates significant difference between OVX and males, $P < 0.05$, + indicates significant difference between OVX+E and OVX+P, $P < 0.05$.

Figure 3: Dose response curves showing the renal sympathetic nerve activity (SNA) response as a percentage (%) change from baseline during the secondary sympathoexcitatory phase of the biphasic cardiac afferent reflex response (as illustrated in figure 1) following epicardial application of capsaicin at three different dose concentrations (1, 5 and $10\mu\text{g}$ per $10\mu\text{l}$ saline) in male, female and ovariectomized female (OVX) groups (top row) and OVX with estradiol replacement (OVX+E) and OVX with progesterone replacement (OVX+P, bottom row) in animals with intact cardiac innervation (figures on left) and animals with only cardiac sympathetic afferents intact and bilateral vagal denervation (figures on right). Data are presented as mean \pm SEM. * indicates significant difference between OVX and ovary-intact females, $P < 0.05$, # indicates significant difference between OVX and males, $P < 0.05$, + indicates significant difference between OVX+E and OVX+P, $P < 0.05$.

Figure 4: Top graphs (A) show time in seconds (s) to maximum inhibition- (left graph) or maximum excitation- (right graph) of renal sympathetic nerve activity (SNA) following the administration of capsaicin onto the surface of the left ventricle in males, ovary-intact females and ovariectomized females without hormone replacement (OVX) with either intact cardiac innervation or vagal denervated (den.). # indicates significant difference when compared to male group with intact cardiac innervation, $P < 0.05$. Filled circles indicate individual data points and group average data shown as mean \pm SEM.

Bottom graphs (B) show the average percentage (%) change in renal SNA, and absolute change in heart rate (HR, beats per minute (BPM)) and arterial pressure (AP) from baseline over 40 seconds directly following the application of $10\mu\text{g}$ capsaicin onto the surface of the left ventricle in males, females, OVX, OVX with estradiol replacement (OVX+E) and OVX with progesterone replacement (OVX+P) with either intact cardiac innervation (black bars) or vagal den. (White bars). * indicates significant difference when compared to OVX group with intact cardiac innervation, $P < 0.05$. # indicates significant difference when compared to OVX+P group with intact cardiac innervation. Data are shown as mean \pm SEM.

Figure 5: Changes in renal sympathetic nerve activity (SNA, A and B, as a percentage (%) change), heart rate (beats per minute (bpm), C and D) and arterial pressure (mm Hg, E and F) from baseline during initial sympathoinhibitory phase (A, C and E) or secondary sympathoexcitatory phase (B, D and F) of biphasic cardiac afferent reflex response (illustrated in figure 1) following application of $10\mu\text{g}$ capsaicin onto epicardial surface of left ventricle in males, females, ovariectomized females: without hormone replacement (OVX), with estradiol replacement (OVX+E), and with progesterone replacement (OVX+P). Black bars indicate animals with intact cardiac innervation and white bars indicate animals with vagal denervation and only cardiac sympathetic innervation. Data are presented as mean \pm SEM. # indicates significant difference with male group with same cardiac innervation, $P < 0.05$. Δ indicates significant difference with female group with same cardiac innervation, $P < 0.05$. + indicates significant difference with OVX+E group with same cardiac innervation, $P < 0.05$. * indicates significant effect of vagal denervation when compared to respective group with total cardiac innervation, $P < 0.05$.









