Protective effect of sex on chronic stress- and depressive behavior-induced vascular dysfunction in BALB/cJ mice

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Stanley SC, Brooks SD, Butcher JT, d’Audiffret AC, Frisbee SJ, Frisbee JC. Protective effect of sex on chronic stress- and depressive behavior-induced vascular dysfunction in BALB/cJ mice. J Appl Physiol 117: 959–970, 2014. First published August 14, 2014; doi:10.1152/japplphysiol.00537.2014.—The presence of chronic, unresolved stresses leads to negative health outcomes, including development of clinical depression/depressive disorders, with outcome severity being correlated with depressive symptom severity. One of the major outcomes associated with chronic stress and depression is the development of cardiovascular disease (CVD) and an elevated CVD risk profile. However, in epidemiological research, sex disparities are evident, with premenopausal women suffering from depressive symptoms more acutely than men, but also demonstrating a relative protection from the onset of CVD. Given this, we investigated the differential effect of sex on conduit artery and resistance arteriolar function in male and female mice following 8 wk of an unpredictable chronic mild stress (UCMS) protocol. In males, plasma cortisol and depressive symptom severity (e.g., coat status, anhedonia, delayed grooming) were elevated by UCMS. Endothelium-dependent dilation to methacholine/acetylecholine was impaired in conduit arteries and skeletal muscle arterioles, suggesting a severe loss of nitric oxide bioavailability and increased production of thromboxane A2 vs. prostaglandin I2 associated with elevated reactive oxygen species (ROS) and an increased level of systemic inflammation. Endothelium-independent dilation was intact. In females, depressive symptoms and plasma cortisol increases were more severe than in males, although alterations to vascular reactivity were blunted, including the effects of elevated ROS and inflammation on dilator responses. These results suggest that compared with males, female rats are more susceptible to chronic stress in terms of the severity of depressive behaviors, but that the subsequent development of vasculopathy is blunted owing to an improved ability to tolerate elevated ROS and systemic inflammatory stress. vasoilation; endothelium-derived factors; peripheral vascular disease; nitric oxide; models of chronic stress; clinical depression

DURING THE LAST DECADE, EXTENSIVE evidence from epidemiologic and clinical studies has identified a complex relationship between depressive disorders and cardiovascular disease (CVD) outcomes (34, 41, 44, 45, 58). Depression is a powerful risk factor for development of CVD, and can be a predictor of cardiovascular pathologies, including myocardial infarction, coronary artery disease, and cardiomyopathies, regardless of prior history of overt CVD (41, 44).1

A consistent body of evidence indicates that chronic stress, a major contributor to depressive illnesses, may be a potent pathogenic factor linking depression and CVD, in part due to the stress-induced activation of the sympathetic-adrenal medullary and hypothalamic pituitary adrenal (HPA) axes (1, 2, 4, 7, 9, 47). Dysregulation of these two essential systems has been linked to depression and the development of several CVD risk factors, including sex-specific pathophysiological factors associated with autonomic nervous and immune dysfunction, and alterations to vascular reactivity and endothelial function (20, 22, 49, 56). Compared with age-matched male counterparts, premenopausal women are at lower risk of developing CVD (29, 30), but concurrently are twice as likely to suffer from depressive disorders (28, 35). This trend diminishes at menopause, and risk of CVD and neuropsychological disorders in women rapidly increases (32, 35). Current literature supports an association between estrogen and the progression of depressive symptoms and comorbid diseases (17, 35, 55), but also a relationship between estrogen and general cardiovascular protection and antioxidant defense (6, 27, 37).

We (12, 14, 26, 42) and others (3, 5, 27, 39) have previously reported that the impact of chronic stress and depression on aortic ring reactivity is mediated through altered endothelial dysfunction, characterized by an impaired dilator metabolite bioavailability. A comparable outcome, including poor endothelium-dependent reactivity (14, 25, 26, 36), nitric oxide (NO) bioavailability (42, 48), as well as a proinflammatory (24, 36, 39) and prothrombotic (43) environment, has also been observed in human patients with depression, and represents an independent predictor of risk for the development of CVD (18, 48). Because circulating levels of estrogen also exert a positive effect on processes associated with vascular function (6, 27, 35, 37), the effect of sex on CVD under conditions of chronic stress and depression is an area that warrants investigation.

The unpredictable chronic mild stress (UCMS) procedure is a widely used animal model of induced depressive symptoms and has been well established in behavioral studies as an extremely relevant rodent model of human depression on the basis of its ability to reproduce clinical symptoms of depression, including anhedonia and learned helplessness (25, 46, 59). Studies utilizing the UCMS protocol have reported in-

1 This article is the topic of an Invited Editorial by DiVincenzo et al. (18a).
creased behavioral vulnerability to UCMS in female rodents (23, 32, 45) associated with increased cortisol levels (15, 16), altered estrous cycle (15, 16, 23, 31), and decreased serotonergic and dopaminergic turnover ratios in brain regions related to stress and depression (16, 33). However, compared with male counterparts, UCMS female rodents show greater antioxidant defense and increased NO bioavailability, helping to maintain normal endothelial function and enhanced vascular anti-inflammatory and antioxidant capacities (27, 50).

The purpose of this study was to determine the differential susceptibility to depressive symptom development following UCMS between male and female mice, and to elucidate sex-specific mechanisms underlying vascular/endothelial dysfunction in both conduit vascular rings and in pressurized resistance arterioles. This study tested the hypothesis that 8 wk of UCMS will result in a more severe development of depressive symptoms in female mice (vs. males), but that vascular function will be preserved to a superior extent than in males. Of special note, this study utilized a protocol wherein the estrous cycle of female mice was not matched. Rather, female mice were randomly exposed to the following stressors on multiple occasions throughout the 24-h period: 1) 10 oz. of water was added to each standard cage for the next 3 h (damp bedding); 2) all bedding was removed and ~0.5 inch of water was added to empty cage for the next 3 h (water temperature was ~30°C; room temperature was ~24°C); 3) each cage was tilted to 45 degrees with or without bedding for 3 h; 4) each mouse was switched into a cage of a neighboring mouse for 4 h (social stress); 5) no bedding lasting for 3 h or, on two occasions each week, overnight; 6) succession of light/dark cycles, lasting 24 h; and 7) exposure to predator smells (e.g., cat fur) and/or sounds (e.g., cat growling) for 8 h. After 8 wk, all mice were subjected to a series of behavioral tests to evaluate the outcomes of the UCMS procedures.

Cost stress test. This evaluation was carried out throughout the duration of the UCMS protocol. The total cumulative score was computed by giving an individual score of 0 (clean) or 1 (dirty) to eight body parts (head, neck, dorsal coat, ventral coat, tail, forelimb, hind-limb, and genital region).

Splash test. This test was used to evaluate acute grooming behavior, defined as cleaning of the fur by licking or scratching. A 10% sucrose solution was sprayed on the dorsal coat of each mouse and grooming activity was recorded for 5 min. The viscosity of the sucrose solution will dirty the coat and induce grooming behavior, with depressive symptoms characterized by an increased latency (idle time between spray and initiation of grooming) and decreased frequency (number of times grooming a particular body part).

MATERIALS AND METHODS

Animals. Male and female BALB/cJ mice (Jackson) were fed standard chow and drinking water ad libitum and were housed in the animal care facility at the West Virginia University Health Sciences Center. All protocols received prior approval of the institutional animal care and use committee. At 9 wk of age, mice from each sex were divided into two groups, control and UCMS (below). After 8 wk duration under either condition, mice were anesthetized with injections of sodium pentobarbital (50 mg/kg ip) and a carotid artery was cannulated for determination of arterial pressure. Venous blood aliquots were collected for biochemical evaluation of plasma biomarkers of treatment outcomes and health status of the mice.

UCMS protocol. All mice were doubly housed, with the control group in a separate quiet room in close proximity to the room used for UCMS treatments. Alternatively, in the UCMS group, mice were randomly exposed to the following stressors on multiple occasions throughout each 24-h period: 1) 10 oz. of water was added to each standard cage for the next 3 h (damp bedding); 2) all bedding was removed and ~0.5 inch of water was added to empty cage for the next 3 h (water temperature was ~30°C; room temperature was ~24°C); 3) each cage was tilted to 45 degrees with or without bedding for 3 h; 4) each mouse was switched into a cage of a neighboring mouse for 3 h (social stress); 5) no bedding lasting for 3 h or, on two occasions each week, overnight; 6) succession of light/dark cycles, lasting 30 min throughout a 24-h period; and 7) exposure to predator smells (e.g., cat fur) and/or sounds (e.g., cat growling) for 8 h.

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In tail suspension test. Mice subjected to the short-term, inescapable stress of tail suspension, will develop an immobile posture, with longer periods of immobility in mice exhibiting a depressed behavior. Mice were completely suspended by the tail on a horizontal bar 35 cm from the base platform using adhesive tape such that contact with the laboratory benchtop was not possible. The latency between the first bout of immobility and the duration of total immobility was recorded for 6 min.

Measurements of vascular reactivity (skeletal muscle resistance arterioles). From each anesthetized mouse, the intramuscular continuation of the gracilis artery was removed and cannulated, as described previously (8). These first-order arterioles were extended to their approximate in situ length and were equilibrated at 80% of mean arterial pressure to approximate in vivo intraluminal pressure. Following equilibration, the reactivity of isolated, pressurized arterioles was assessed in response to increases in concentrations of phenylephrine (10−10 M to 10−7 M) to assess adrenergic constrictor responses and increasing concentrations of acetylcholine (10−10 M to 10−6 M) and sodium nitroprusside (10−10 M to 10−6 M) to assess dilator reactivity. Vascular responses to stimuli were also determined following treatment with Nω-nitro-L-arginine methyl ester (L-NAME; 10−4 M), indomethacin (INDO; 10−5 M), and 1-oxyl-2,2,6,6-tetramethyl-4-hydroxyperipiperide (TEMPOL) (10−4 M) for 45–60 min, to assess the roles of NO, cyclooxygenase (COX), and reactive oxidant stress, respectively, in modulating reactivity.

Measurements of vascular reactivity (conduit arteries). Following removal of the resistance arteriole in each mouse, the thoracic aorta was removed, rinsed in physiological salt solution, cleared of surrounding tissue, and cut in 2- to 3-mm ring segments. Each ring was mounted in a myobath chamber between a fixed point and a force transducer (World Precision Instruments), and set to 0.5 g tension for 45 min to equilibrate. The organ baths contained physiological salt solution at 37°C, and were aerated with 95% O2 and 5% CO2. Rings were preconditioned by treatment with 10−7 M phenylephrine for 5 min, at which time 10−5 M methacholine was added to the bath to assess endothelial integrity. Any ring that failed to demonstrate both a brisk constrictor response to phenylephrine and viable endothelial function was discarded. Subsequently, rings were treated with increasing concentrations of phenylephrine (10−10 M to 10−4 M) to assess constrictor reactivity. For assessment of dilator reactivity, rings were pretreated with 10−6 M phenylephrine and exposed to increasing concentrations of methacholine (10−10 M to 10−4 M) and sodium nitroprusside (10−10 M to 10−4 M). To assess the roles of NO, COX, and reactive oxidant stress in modulating vascular responses to the agonist treatments, concentration-response curves were also conducted following pretreatment of the rings for 45–60 min with l-NAME (10−4 M), INDO (10−3 M), and TEMPOL (10−4 M), respectively.

Measurement of vasoactive metabolite bioavailability. From mice within each group, the remaining sections of the thoracic and abdominal aorta that were not used for measurements of reactivity were used to assess vascular NO, prostaglandin I2 (PGI2; from levels of 6-keto PGF1α), and thromboxane A2 (TXA2; from levels of 11-dehydro-TXB2) bioavailability using amperometric sensors (World Precision Instruments) and a commercially available kit (Cayman), respectively. Briefly, aortae were isolated, cleaned, sectioned into ~1 mm lengths and placed within a chamber filled with physiological salt solution equilibrated with 21% O2, 5% CO2, balance N2. Within this chamber, an NO sensor (ISO-NOPF 100) was inserted and a baseline current was obtained. Subsequently, methacholine (10−8, 10−6, and 10−4 M) was added to the chamber and the changes in current were determined. To verify that responses represented NO release, these procedures were repeated following addition of L-NAME (10−4 M) to the chamber. In response to challenge with 10−4 M methacholine, an aliquot of physiological salt solution was removed from the chamber and used for determination of PGI2 and TXA2 production.

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Biochemical analyses. In all samples, fasting blood glucose (8 h) was determined using a commercially available glucometer (Freestyle). Chronic oxidant stress was assessed through determination of plasma nitrotyrosine, and plasma cortisol was determined using standard kits (Cayman). Finally, using a multiplexed procedure, plasma insulin and biomarkers of inflammation were assessed using commercially available kits (Millipore).

Data and statistical analyses. Mechanical responses following challenge with methacholine or phenylephrine were fit with the three-parameter logistic equation:

$$y = \min + \left[ \frac{\max - \min}{1 + 10^{\log ED_{50}}} \right]$$

where \(y\) represents the isometric tension; \(\min\) and \(\max\) represent the lower (minimum) and upper (maximum) bounds, respectively, of the change in tone with agonist concentration; \(x\) is the logarithm of the agonist concentration; and \(\log ED_{50}\) represents the logarithm of the agonist concentration \(x\) where the response \(y\) is halfway between the bounds. The use of the three-parameter logistic equation is appropriate for the analysis of sigmoidal concentration-response relationships because it simultaneously provides estimates of the curve maximum (upper bound), minimum (lower bound), and the dose at which the dependent variable reaches 50% of maximum (ED\(_{50}\)). In a dilator response in which the initial condition is set to 100% (the upper bound), the asymptotic minimum (the lower bound from the equation) is reflective of the degree of dilator reactivity for that vessel. If the vessel becomes more reactive, the lower bound will decrease toward reduced levels, whereas if the vessel becomes less reactive, the lower bound will increase toward higher values. This situation is reversed for constrictor responses, in which the vessel starts at the lower bound, and then goes through a range of increased tensions to reach an upper bound/asymptotic maximum. For the presentation of results, we have focused on the changes in the upper or lower bounds, because we did not determine a consistent or significant change to the ED\(_{50}\) values between relevant experiment groups.

All data are presented as means $\pm$ SE. Significant differences between groups were determined using ANOVA. In all cases, a Student-Newman-Keuls post hoc test was used when appropriate and \(P < 0.05\) was taken to reflect statistical significance.

RESULTS

Table 1 presents data describing the characteristics of mouse groups in the present study. At the time of final use, plasma insulin and nitrotyrosine were significantly greater in UCMS mice than in controls. No consistent and significant differences were determined between body mass, mean arterial pressure, and blood glucose between conditions or sex. Plasma levels of cortisol were significantly increased with UCMS in both sexes compared with controls, and levels in UCMS females were significantly greater than those in UCMS males.

The effect of the UCMS protocol on depressive behaviors in mice is summarized in Fig. 1. Throughout 8 wk of UCMS, the coat status in mice undergoing the stress protocol was consistently poorer compared with that in control animals (Fig. 1A), although the severity of this degradation in coat status was significantly worse in UCMS females. In response to the sucrose spray, control mice demonstrated both a more rapid (Fig. 1B) and more frequent (Fig. 1C) grooming response compared with that exhibited by mice of either sex following the UCMS protocol. However, the latency of the grooming response was greater in UCMS females compared with that in UCMS males. Figure 1D presents the total period of immobility in response to tail suspension, where control mice demonstrated a significantly shorter period of immobility compared with that in UCMS mice of either sex. Consistent with the previous measurements, UCMS females demonstrated a longer period of immobility compared with UCMS males.

The constrictor responses of aortic rings from control and UCMS-mice in response to increasing concentrations of phenylephrine are summarized in Fig. 2. Compared with responses in aortic rings from control mice, rings from UCMS males exhibited a similar constrictor response to the adrenergic agonist (Fig. 2A). In contrast, the constrictor response of aortic rings from UCMS females was not different from that in controls. Following pretreatment of vessels from all groups with L-NAME, responses of aortic rings from the control and UCMS groups remained similar in response to increasing concentrations of phenylephrine (Fig. 2B). Pretreatment of aortic rings with INDO did not significantly alter responses to phenylephrine from that determined under untreated conditions in any group (data not shown).

A summary of the methacholine-induced dilator responses of aortic rings from control and UCMS mice is presented in Fig. 3. Compared with responses from control mice, the dilation to increasing concentrations of methacholine was significantly reduced in both male and female UCMS mice, although the severity of this blunted response was greater in the aortic rings from UCMS males (Fig. 3A). In aortic rings from control animals, pretreatment with either L-NAME or INDO resulted in a significant inhibition of methacholine-induced dilator reactivity, whereas pretreatment with both nearly abolished all responses to the agonist (Fig. 3, B and C). In aortic rings from UCMS males, pretreatment with L-NAME did not further

Table 1. Baseline characteristics between mouse groups in the present study

<table>
<thead>
<tr>
<th></th>
<th>Males, n = 14</th>
<th>Females, n = 9</th>
<th>UCMS Male, n = 12</th>
<th>UCMS Females, n = 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass, g</td>
<td>29 $\pm$ 2</td>
<td>28 $\pm$ 3</td>
<td>30 $\pm$ 3</td>
<td>29 $\pm$ 4</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>87 $\pm$ 4</td>
<td>91 $\pm$ 4</td>
<td>94 $\pm$ 5</td>
<td>90 $\pm$ 5</td>
</tr>
<tr>
<td>Insulin$_{plasma}$, ng/ml</td>
<td>1.1 $\pm$ 0.3</td>
<td>1.2 $\pm$ 0.4</td>
<td>4.1 $\pm$ 0.7*</td>
<td>4.5 $\pm$ 0.5*</td>
</tr>
<tr>
<td>Glucose$_{plasma}$, mg/dl</td>
<td>82 $\pm$ 7</td>
<td>80 $\pm$ 8</td>
<td>94 $\pm$ 8</td>
<td>101 $\pm$ 10</td>
</tr>
<tr>
<td>Cholesterol$_{plasma}$, mg/dl</td>
<td>71 $\pm$ 7</td>
<td>68 $\pm$ 8</td>
<td>78 $\pm$ 6</td>
<td>77 $\pm$ 10</td>
</tr>
<tr>
<td>Triglycerides$_{plasma}$, mg/dl</td>
<td>94 $\pm$ 6</td>
<td>101 $\pm$ 8</td>
<td>109 $\pm$ 8</td>
<td>116 $\pm$ 12</td>
</tr>
<tr>
<td>Nitrotyrosine$_{plasma}$, ng/ml</td>
<td>12 $\pm$ 3</td>
<td>11 $\pm$ 4</td>
<td>29 $\pm$ 5*</td>
<td>36 $\pm$ 6*</td>
</tr>
<tr>
<td>Cortisol$_{plasma}$, pg/ml</td>
<td>12 $\pm$ 3</td>
<td>14 $\pm$ 4</td>
<td>29 $\pm$ 5*</td>
<td>44 $\pm$ 4*</td>
</tr>
<tr>
<td>TNF-α$_{plasma}$, pg/ml</td>
<td>2.3 $\pm$ 0.3</td>
<td>2.0 $\pm$ 0.2</td>
<td>4.1 $\pm$ 0.4*</td>
<td>6.4 $\pm$ 0.4*†</td>
</tr>
<tr>
<td>MCP-1$_{plasma}$, pg/ml</td>
<td>2.8 $\pm$ 3</td>
<td>3.3 $\pm$ 0.5</td>
<td>10.2 $\pm$ 1.0*</td>
<td>14.8 $\pm$ 1.3*†</td>
</tr>
</tbody>
</table>

All mice were aged 17–18 wk. *\(P < 0.05\) vs. control; †\(P < 0.05\) vs. UCMS-males.
affect the reduced dilator responses to methacholine, although treatment with INDO reduced dilator responses from the untreated condition (Fig. 3D). In conduit artery segments from UCMS female rats, treatment with L-NAME reduced methacholine-induced dilation, whereas responses following treatment with INDO were less striking (Fig. 3E). In all cases, combined treatment with both L-NAME and INDO nearly abolished the reactivity of aortic rings to increasing concentrations of methacholine.

Fig. 4 summarizes the responses of aortic rings from control and UCMS mice to increasing concentrations of sodium nitroprusside. In all cases, application of the NO donor resulted in a significant dilator response that was not different between control and UCMS mice of either sex.

Fig. 1. Depressive symptoms following 8 wk of the unpredictable chronic mild stress (UCMS) protocol in male and female BALB/cJ mice. Data are presented for coat status (A), latency (B), and frequency (C) of facial grooming following a 10% sucrose solution spray, and the total period of immobility during the tail suspension test (D) for control and UCMS mice. Control males n = 14; control females n = 9; UCMS males n = 12; UCMS females n = 12. *P < 0.05 vs. control in the sex; †P < 0.05 vs. UCMS male.

Fig. 2. Constrictor responses of aortic rings to increasing concentrations of phenylephrine from mice under control conditions and after 8 wk of UCMS. Data are presented under untreated conditions (A) and in response to nitric oxide synthase inhibition with Nω-nitro-L-arginine methyl ester (L-NAME) (B). Control males n = 12; control females n = 8; UCMS males n = 10; UCMS females n = 10.
The production of dilator metabolites in response to methacholine challenge is presented in Fig. 5. Vascular production of NO following application of methacholine was significantly reduced in conduit arteries of UCMS mice of either sex, although the severity of this reduction was greater in UCMS males (Fig. 5A). A similar pattern was determined for the vascular production of PGI2, with the methacholine-induced production of this metabolite, estimated from 6-keto-PGF1α, being reduced in UCMS mice of both sexes (male > female) compared with responses in arteries from control mice (Fig. 5B). Vascular production of TxA2 (Fig. 5C) was low and similar in male and female mice under control conditions. In response to the UCMS protocol, the vascular production of TxA2 was significantly elevated in both sexes, but was significantly increased in UCMS males compared with UCMS females.

The impact of pretreatment of aortic rings with the antioxidant TEMPOL on vascular production of NO following methacholine challenge is presented in Fig. 6. Although bioavailability of NO...
was attenuated in vessels from both male and female mice following UCMS treatment (males > females), pretreatment of vessels with TEMPOL significantly increased the bioavailability of NO in vessels from UCMS males. Although a trend for this was evident in UCMS females, this effect was not as striking, likely owing to the more modest reductions in NO bioavailability as a result of the UCMS protocol itself in these mice. Treatment of vessels with L-NAME abolished methacholine-induced NO bioavailability in all conditions.

The effects of pretreatment of aortic rings from UCMS mice with TEMPOL on dilator responses are summarized in Fig. 7. In aortic rings from both UCMS male (Fig. 7A) and UCMS female (Fig. 7B) mice, pretreatment with TEMPOL significantly increased dilator responses to methacholine from the untreated responses; subsequent treatment of vessels with L-NAME or INDO significantly reduced dilation from this improved level of reactivity.

The constrictor responses of isolated skeletal muscle (gracilis) arterioles in response to increasing concentrations of phenylephrine are summarized in Fig. 8. In arterioles from male rats, imposition of the UCMS protocol resulted in a significant increase in the vasoconstrictor responses to phenylephrine compared with responses in vessels from control animals (Fig. 8A). Pretreatment of vessels with L-NAME abolished all differences in phenylephrine-induced constriction between sexes and treatment condition (Fig. 8B).

Figure 9 presents the dilator responses of gracilis muscle arterioles from control and UCMS-treated mice in response to increasing concentrations of acetylcholine. In males, UCMS treatment blunted dilator responses to acetylcholine from levels in controls; pretreatment of vessels from UCMS males with L-NAME had no effect on reactivity, whereas pretreatment with INDO further blunted this impaired response (Fig. 9A). However, treatment of arterioles from UCMS males with TEMPOL was very effective at restoring dilator reactivity to acetylcholine (Fig. 9B). The dilator response in UCMS females in response to acetylcholine was reduced compared with that from controls, and pretreatment with either L-NAME or INDO caused a further reduction in reactivity (Fig. 9C). Similar to that in UCMS males, treatment of vessels from UCMS females with TEMPOL restored dilator responses to increasing concentrations of acetylcholine (Fig. 9D).

The correlation between UCMS-induced changes in systemic inflammation and vascular dysfunction is summarized in Fig. 10 for tumor necrosis factor-α (TNF-α) (Fig. 10A) and monocyte chemoattractant protein-1 (MCP-1) (Fig. 10B). The degree of vascular dysfunction, estimated from the lower bound of the methacholine concentration-response relationship for aortic rings (determined from the logistic equation described above), demonstrated a positive relationship with the degree of systemic inflammation as a result of the UCMS protocol. However, when split into sexes, a more severe...

![Graph](image-url)
Building on our previous report that vasodilator reactivity is blunted in aortic rings of UCMS male mice (14, 26), our new findings suggest that the impaired dilator responses in UCMS mice of both sexes are due to endothelial dysfunction, because dilations to exogenous NO were similar across all groups, as were constrictor responses to phenylephrine, suggesting that an 8-wk UCMS protocol is not sufficient to significantly alter vascular wall mechanics or vascular smooth muscle function. Vasodilator responses were further blunted in aortic rings and cardiovascular disease risk and outcomes, validated preclinical models offer a valuable translational tool for investigating the underlying mechanisms of disease and offer an efficacious method for studying novel interventional therapies and treatment options. This study utilized the UCMS protocol, a validated model of chronic stress-induced depressive symptoms in rodents, to interrogate sex-specific mechanisms of vascular dysfunction in UCMS mice.

The results of this study provide evidence that male and female mice experience graded outcomes related to the severity of depressive symptom development and an almost paradoxically gradation of the impairment to vascular reactivity after 8 wk of the UCMS protocol. Increased behavioral susceptibility to stress conditions was observed in female mice vs. male counterparts, as evidenced by worsened coat grooming score, increased immobility in the tail suspension test (behavioral despair), and elevated plasma levels of corticosterone. Estrogen levels are known to amplify the magnitude of the stress response at the HPA axis (4, 23, 57, 60, 61), offering a potential explanation for the increased corticosterone and behavioral symptom severity observed in UCMS female mice. UCMS-induced impairments to vascular reactivity and function were observed for both sexes, demonstrated by blunting of dilator responses to methacholine/aceetylcholine in isolated aortic rings and in pressurized resistance arterioles, respectively. However, these physiological impairments were also sex-specific, in that female UCMS mice developed greater elevations to biomarkers of oxidative stress and inflammation, yet demonstrated a more modest impairment to vascular reactivity, despite more severe deterioration in the behavioral measures.

With an escalating body of clinical and epidemiological evidence demonstrating that sex differences influence the link between chronic stress, the onset of depressive symptoms and relationship was evident in males, in which similar levels of either TNF-\(\alpha\) (Fig. 10A) or MCP-1 (Fig. 10B) was associated with a greater degree of vascular dysfunction (i.e., a higher lower bound) in males than in females. This was evident under both control and UCMS conditions. Interestingly, within either sex, imposition of the UCMS protocol did not appear to change the inherent relationship between markers of inflammation and vascular dysfunction. Rather, the relationship between the parameters remained similar, but was shifted to a higher severity of dysfunction.

DISCUSSION

With an escalating body of clinical and epidemiological evidence demonstrating that sex differences influence the link between chronic stress, the onset of depressive symptoms and dysfunction of the vascular system, we sought to determine if the relationship between chronic stress and vascular dysfunction is sex-dependent. We performed vasodilator and vasoconstrictor reactivity experiments, with the results being analyzed using statistical comparisons. The findings suggest that female mice experience a more severe impairment to vascular reactivity compared to male mice, with a greater increase in depression symptom measures, as well as a more pronounced increase in plasma corticosterone levels. These results support the notion that sex differences exist in the response to chronic stress and may contribute to the differential risk of cardiovascular disease between males and females.

**Fig. 6.** Vascular production of nitric oxide (NO) following methacholine challenge in male and female mice following 8 wk of UCMS. Data are presented for vessels under control (untreated) conditions, following pretreatment with TEMPOL, following treatment with TEMPOL and following treatment of vessels from UCMS animals with \(\text{L-NAME}\). Control males \(n=9\); control females \(n=8\); UCMS males \(n=9\); UCMS females \(n=8\). *\(P<0.05\) vs. responses in control vessels from mice of that sex; †\(P<0.05\) vs. responses in untreated vessels of UCMS mice of that sex; ‡\(P<0.05\) vs. responses in TEMPOL-treated mice of that sex.

**Fig. 7.** Dilator responses of aortic rings to increasing concentrations of methacholine from UCMS male (A) and UCMS female (B) mice. A: UCMS males under untreated conditions, following pretreatment with TEMPOL, following treatment with TEMPOL/INDO, and following treatment with TEMPOL/L-NAME. B: UCMS females under untreated conditions, following pretreatment with TEMPOL, following treatment with TEMPOL/INDO, and following treatment with TEMPOL/L-NAME. Control males \(n=8–11\); control females \(n=7–9\); UCMS males \(n=8–9\); UCMS females \(n=7–9\). *\(P<0.05\) vs. responses in untreated vessels; †\(P<0.05\) vs. responses in vessels that were pretreated with TEMPOL.
gracilis arterioles of UCMS females following pretreatment with L-NAME; however, this treatment had minimal effects on vascular reactivity in UCMS males, likely owing to the preexisting severe attenuation of vascular NO bioavailability determined in UCMS males. Instead, the blunted dilator responses in vessels from UCMS males were further impaired as a result of COX inhibition with INDO, suggesting that a dependence on dilator influence of arachidonic acid metabolites (likely PGI2)

Fig. 8. Constrictor responses of gracilis arterioles to increasing concentrations of phenylephrine from mice under control conditions and after 8 wk of UCMS. Data are presented under control conditions (A) and in response to nitric oxide synthase (NOS) inhibition with L-NAME (B). Control males n = 10; control females n = 8; UCMS males n = 10; UCMS females n = 10. *P < 0.05 vs. responses in arterioles from control mice.

Fig. 9. Dilator responses of gracilis arterioles to increasing concentrations of acetylcholine from mice under control conditions and after 8 wk of UCMS. A: male mice under untreated conditions and following pretreatment with L-NAME or INDO. B: male mice after pretreatment with TEMPOL alone or with L-NAME. C: female mice under untreated conditions and following pretreatment with L-NAME or INDO. D: female mice after pretreatment with TEMPOL alone or with L-NAME. Control males n = 8–10; control females n = 7–8; UCMS males n = 9–10; UCMS females n = 8–10. *P < 0.05 vs. responses in vessels from control mice of that sex; †P < 0.05 vs. responses in vessels from UCMS mice of that sex.

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Concentration (pg/ml)

stricting metabolites (e.g., thromboxane, TxA2) that can com-
tabolism by COX in favor of increased production of vasocon-
by chronic stress can alter the balance between constricting and
Indeed, proinflammatory, oxidative stress conditions induced
late across all of the observed mechanical responses, suggest-
converged at very similar levels following inhibition of NO
the degree to which endothelial function was impaired as a result
striction in UCMS males, they largely appear to be a reflection of
functions (among others) of the endothelium, the impact of
comes in terms of perfusion control and the antithrombotic
TxA2 production will have on negative cardiovascular out-
symptoms, and the impact of chronic elevations in vascular
has been demonstrated with chronic stress and depressive
Given the integral role of inflammation and oxidant stress that
vascular) as a result of the UCMS protocol, the fact that these
an elevation in stimulus-induced vascular TxA2 generation
pete against the effect of stimulus-induced vasodilation by NO,
that an elevation in stimulus-induced vascular TxA2 generation
can contribute to the integrated vasoreactivity in this model.
Given the integral role of inflammation and oxidant stress that
has been demonstrated with chronic stress and depressive
symptoms, and the impact of chronic elevations in vascular
TxA2 production will have on negative cardiovascular out-
comes in terms of perfusion control and the antithrombotic
functions (among others) of the endothelium, the impact of
chronic inhibition of these pathways on both the behavioral and
vascular outcomes in UCMS-induced depressive symptoms
clearly represents important areas for future interrogation.
Whereas the results of this study found only very limited
evidence of an increased vascular reactivity to adrenergic con-
striction in UCMS males, they largely appear to be a reflection of
the degree to which endothelial function was impaired as a result
of the UCMS protocol. However, the vasoconstrictor response of
all animal groups to increasing concentrations of phenylephrine
converged at very similar levels following inhibition of NO
synthase (NOS) activity via pretreatment with l-NAME, suggest-
ing that the role endogenous NO bioavailability plays in moder-
ating adrenergic constriction under normal conditions remains
largely intact in females despite UCMS, but is diminished in
males following UCMS protocols.
The relationship between systemic inflammation induced by
UCMS and the severity of vascular dysfunction observed in
males and females were investigated to determine sex differ-
ces in the effects of chronic inflammation on vascular reac-
tivity. Circulating plasma levels of proinflammatory markers
MCP-1 and TNF-α were associated with vascular dysfunction
for all UCMS animals, but a greater degree of vascular dys-
function was observed in UCMS males than in UCMS females,
despite the observation that the magnitude of the inflammatory
markers was somewhat higher in female mice. When taken
together with the higher plasma levels of ROS in female
UCMS mice vs. males as well, these data suggest that males
experience a substantially greater endothelial susceptibility to
UCMS-induced dysfunction, raising the possibility of a pro-
tective mechanism for female sex hormones that effectively
reduces impairment of endothelium-dependent vasodilation
during exposure to chronic stress.
There is extensive evidence for the vasoprotective actions of
estrogen against oxidative and inflammatory stressors. Estro-
gen increases NOS activity and promotes NO bioavailability
from the vascular endothelium (15, 27, 60); it is also linked to
increased responsiveness to β2-adrenergic-mediated vasodila-
tion (19, 21, 38), partially through an NO-related mechanism
(19, 51). In addition to supportive effects on NO, estrogen may
also influence production of PGI2 via upregulation of the COX
pathway, specifically COX-1 and prostacyclin synthase, in
some vascular beds (40, 51, 53, 60). Although less is known
about androgens, it has been reported that testosterone supple-
mentation impairs vascular reactivity and antioxidant capacity
(10) in females by mitigating vascular sensitivity to NO (11,
52). Females may therefore have a more robust and protected
endothelium-dependent vasodilator capacity than males due to
higher levels of circulating estrogen and significantly less
testosterone, thus mitigating the inflammatory and oxidative
effects of chronic stress on vascular reactivity (13, 54, 60).
That being stated, the purpose of the study was not to test the
effects of cyclic variation in estrogen levels on vascular func-
tion; rather, it was to determine the potential effect of a true
difference in sex. As such, the female mice used in this study
were randomized within their estrous cycle. Given the robust-
ness of the differences in outcomes (both behavioral and
vascular) as a result of the UCMS protocol, the fact that these

Fig. 10. Correlations between vascular reactivity and individual markers of chronic inflammation in control male (blue), control female (red), UCMS male (green), and UCMS female (black) mice. Data are presented as the lower bound of the methacholine concentration-response curve for an individual aortic ring vs. plasma concentrations of tumor necrosis factor-α (TNF-α) (A) or monocyte chemoattractant protein-1 (MCP-1) (B). In this figure, the lower bound from methacholine concentration-response curve is reflective of the degree of dilator reactivity for that vessel. If the vessel is more reactive to methacholine, the lower bound is decreased, whereas if the vessel becomes less reactive, the lower bound is increased. Also presented are lines of best fit through the data for each group, with the resulting slope (β) and \( r^2 \) values presented in the legend.

A

B

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mice were not studied at the same points in their estrous cycle has significant implications. Most critically, these results suggest that the protective effect associated with the female sex may not be critically dependent on the specific time within the estrous cycle or on specific hormonal profiles at that time, but that it may be much more stable and afford a degree of protection that is relatively insensitive to the day-to-day fluctuations in hormonal profiles. This is especially compelling because it suggests that the potentially beneficial and protective mechanisms that are associated with the female sex remain in place throughout the estrous cycle and may not fluctuate along the same temporal pattern as the hormonal levels. The molecular basis for this temporal pattern, as well as the alternate hypothesis that the lower levels of androgen hormones is actually the basis for the protection in this model of chronic stress and depressive symptoms and poor cardiovascular outcomes, will require future targeted investigation.

**Summary and conclusions.** In response to imposition of chronic unresolvable stresses onto male and female mice, the results of the present study indicate that behavioral impairments, elevations in plasma cortisol, and plasma markers of oxidant stress and inflammation demonstrated by female mice are significantly greater than those demonstrated by male mice. However, despite these elevated responses, the vasculopathy in both the conduit and resistance vasculature of female mice is less severe than that in males. Much of this blunted impairment appears to be a function of a superior maintenance of endothelial function, primarily via more normal levels of NO bioavailability and a more normal balance between the production of PGI2 and TXA2 to help maintain dilator reactivity. Because female rats were randomized with respect to their time in estrous cycle, these data appear to reflect a longer, more temporally stable, protective effect of being female rather than one that reflects the more labile temporal changes in hormonal levels. In addition, the correlational analyses between inflammatory biomarkers and vascular dysfunction suggest that fundamental differences exist between male and female sex with regard to these associations. However, imposition of the UCMS protocol does not appear to cause the differences in the sex-based protections to diminish. Rather, UCMS appears to right-shift these associations along the same sex-specific lines to a higher degree of vascular dysfunction. Future research into the specific nature of the temporal nature of these protective effects and how they can be exploited therapeutically to minimize the devastating effects of depressive symptoms on cardiovascular health outcomes represents a key area for future investigation.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


