NO mediates effects of estrogen on central regulation of blood pressure in restrained, ovariectomized rats

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Estrogen replacement therapy is common in oophorectomized [ovariectomized (OVX)] and postmenopausal women. Although its benefits in conferring cardiovascular responses to psychological stress may have important consequences for long-term cardiovascular health, it is important to study these effects in a systematic manner. Nitric oxide (NO) is a central autonomic neurotransmitter implicated in maintenance of body homeostasis (reviewed in Ref. 17). Central NO is believed to mediate decreases in arterial pressure and sympathetic drive during homeostatic instability. Furthermore, we have shown that NO plays an important role in reducing arterial pressure during recovery from psychological stress (34). Finally, psychological stress activates NO-producing neurons, including those that are important in cardiovascular regulation (3, 14, 18), and NO donors attenuate the increases in cardiovascular responses to shaking stress (9). A positive correlation between circulating estrogen levels and levels of plasma NO has been described in humans (4, 12), but, until the present study, nothing was known about the effects of estrogen replacement on levels of NO production in the brain.
To test the hypothesis that estrogen replacement in OVX rats attenuates cardiovascular responses to psychological stress, we used our stress protocol of two cycles of 1-h restraint/1-h rest (19, 34) to study cardiovascular responses during restraint stress and during recovery from stress in OVX-E and OVX-V rats. To test the hypothesis that NO in the brain mediates the effects of estrogen replacement on cardiovascular responses to stress, we measured the effects of intracerebroventricular injections of the NO synthase (NOS) inhibitor \( N^\omega \)-nitro-L-arginine (\( L^-\text{NNA} \)) on these responses. Finally, we measured NO production in the hypothalami and brain stems of unrestrained and restrained OVX-E and OVX-V rats to verify that NO levels are elevated in restrained OVX-E rats.

**METHODS**

**Animals**

Female Sprague-Dawley rats (200–300 g) were purchased from the Biosciences Animal Center, University of Alberta. Rats were housed at 21°C with a 12:12-h light-dark cycle and had full access to food and water. All experimental procedures were approved by the Health Sciences Lab Animal Services at the University of Alberta.

**Preparation of Rats**

**Ovariectomy and estrogen replacement.** Rats were anesthetized with pentobarbital sodium (45 mg/kg ip, Somnotol; MTC Pharmaceuticals, Hamilton, ON). Rats received injections of buprenorphine hydrochloride (15 \( \mu \)g im Buprenex; Reckitt and Colman Pharmaceutical, Richmond, VA) and penicillin G procaine (30,000 units im Ethacillin; Regar/STB, London, ON, Canada). Ovaries were removed through a midline abdominal incision, and a pellet delivering 17\( \beta \)-estradiol (OVX-E; 21-day release, 0.25 mg/pellet; Innovative Research of America, Sarasota, FL) or vehicle (OVX-V) was implanted subcutaneously between the scapulae. The wounds were closed, and rats were allowed to recover for 7 days.

**Instrumentation.** After ovariectomies (7 days), rats were anesthetized again with pentobarbital sodium anesthesia (see above), descending aortas were cannulated, and the free ends of the catheters were tunneled under the skin and exteriorized between the scapulae as previously described (29, 32, 34). In the same surgical session, intracerebroventricular guide cannulas were implanted in the lateral cerebroventricles of each rat, as described previously (34). Rats were allowed to recover for an additional 4–5 days.

**NO assays.** Groups of noninstrumented OVX-V and OVX-E rats were OVX as described above and were left undisturbed for 11–12 days.

**Experimental Design**

**Effect of NOS inhibition on mean arterial pressure and heart rate during restraint stress and recovery in OVX-V and OVX-E rats.** Baseline measurements of mean arterial pressure (MAP, mmHg) and heart rate (HR, beats/min) were recorded for 60 min before restraint and during the restraint protocol. MAPs were calculated at minimum intervals of 5 min. Rats received intracerebroventricular injections of \( L^-\text{NNA} \) (88 \( \mu \)g/kg dissolved in 10 \( \mu \)l of saline; Calbiochem, La Jolla, CA) or vehicle alone 15 min in advance of the restraint, as we have described previously (32, 34). \( L^-\text{NNA} \) is an inhibitor of constitutive NOS (neuronal NOS and endothelial NOS) when used at this concentration and inhibits inducible NOS only at much higher concentrations (27). After the injections (15 min), rats were restrained individually in a hemicylindrical, well-ventilated, Plexiglas tube according to a 1-h restraint/1-h rest/1-h restraint/1-h rest (4 h) protocol, as we have described previously (19, 33, 34). Immediately before the second hour of restraint, rats were given a second injection of \( L^-\text{NNA} \) or vehicle. This protocol of \( L^-\text{NNA} \) injections has been shown to ensure continued inhibition of NO production for at least 4 h (32, 34). At the end of the experiment, rats were given an overdose of pentobarbital sodium. Blood samples were collected from the vena cava of rats immediately before perfusion, placed in tubes containing EDTA, and centrifuged at 6,000 rpm for 6 min. The plasma was decanted and stored at –70°C. RIA for estradiol was done according to instructions provided in RIA kits (ICN Biomedical, Costa Mesa, CA). Radioactivity in duplicate samples was quantified using a gamma counter.

**Effect of restraint on NO production in the hypothalamus and brainstem in OVX-V and OVX-E rats.** To determine the effects of restraint (but not rest) on NO production in the brain, noninstrumented OVX-V and OVX-E rats were restrained as above for 1 h. At the end of the experiment, restrained rats and their unrestrained controls were anesthetized and decapitated with a rat guillotine. Hypothalami (0 to –5 mm bregma, 2 mm lateral to midline, and 4 mm below the dura) and brain stems (–14.8 to –10 mm bregma) were microdissected from each brain at 4°C and prepared for nitrate/nitrite (NO\(_x\)) assays, as described previously (32, 34).

**Statistical Analyses**

In each group, data were expressed as means ± SE. MAP and HR data within each group were compared over time using a two-way repeated-measures ANOVA; a Newman-Keuls post hoc test was used when significant differences were found. To further analyze pairs of MAP data, areas under the curve (AUC) were calculated for each animal during each phase of restraint and rest, and the mean AUC was then calculated for each group. Paired t-tests were used to determine whether responses between groups were different. Finally, comparisons of NO levels between individual restrained groups and their unrestrained controls were made using the unpaired Student’s t-test. \( P < 0.05 \) was taken to indicate statistical significance.

**RESULTS**

**Estradiol Levels**

Estradiol levels in OVX-E rats were 62.9 ± 10.4 pg/ml (n = 10); in OVX-V rats, estradiol levels were below the level of detection (n = 9).

**Cardiovascular Responses**

For the purposes of illustration, data for MAP and HR have been divided into the following groups: restrained vehicle-treated OVX-E rats vs. restrained vehicle-treated OVX-V rats (Figs. 1A and 2A); restrained vehicle-treated OVX-E rats vs. restrained \( L^-\text{NNA} \)-treated OVX-E rats (Figs. 1B and 2B); restrained vehicle-treated OVX-V rats vs. restrained \( L^-\text{NNA} \)-treated OVX-V rats (Figs. 1C and 2C); restrained \( L^-\text{NNA} \)-treated OVX-E rats vs. restrained \( L^-\text{NNA} \)-treated OVX-V rats (Figs. 1D and 2D).
treated OVX-E rats vs. restrained L-NNA-treated OVX-V rats (Figs. 1D and 2D). In all groups, $n = 6$. 

**Restrained vehicle-treated rats.** Resting MAPs in OVX-V and OVX-E rats were not significantly different (Fig. 1A), although there was a tendency for MAPs in OVX-V rats ($\sim$98 mmHg) to be higher than in OVX-E rats ($\sim$90 mmHg). Upon restraint, MAPs in OVX-V rats rose significantly above baseline ($\sim$17 mmHg) and remained elevated for the remainder of the restraint hour (Fig. 1A). During the first hour of rest, MAPs in OVX-V rats returned to baseline values. Similar responses were observed for the second hour of restraint and second hour of rest. In OVX-E rats, restraint stimulated increases in MAPs ($\sim$12 mmHg) with a return to baseline within $\sim$15 min (Fig. 1A); during the first hour of rest, MAPs were similar to baseline values for OVX-E rats. During the second hour of restraint, MAPs in OVX-E rats rose only briefly by $\sim$8 mmHg and returned to baseline values. During the second hour of rest, MAPs in OVX-E rats were not significantly different from baseline values. A comparison of MAPs between OVX-V and OVX-E rats showed that restraint elicited MAPs that were significantly higher in OVX-V rats, both during restraint and during rest periods. In addition, comparisons of AUCs showed that increases in MAP in OVX-V rats were significantly lower in OVX-E rats.
greater than those in OVX-E rats, compared with their respective baseline values, during all restraint and rest periods (Fig. 3A).

HRs in restrained vehicle-treated OVX-V and OVX-E rats were variable, and few significant differences were observed between the two groups of rats, although OVX-V rats tended to show more pronounced HR responses to placement in and removal from the restraint chamber (Fig. 2A).

**Effects of L-NNA.** In restrained L-NNA-treated OVX-E rats, MAP responses to restraint were elevated compared with restrained vehicle-treated OVX-E rats, and many of these differences reached significance as the experiment proceeded (Fig. 1B). Similarly, most MAPs during rest periods remained significantly elevated in L-NNA-treated OVX-E rats. Comparisons of AUC for each hour showed significant differences between the two groups during the two rest periods. Although no differences were found during the restraint periods, a trend toward a significantly greater AUC was found in L-NNA-treated OVX-E rats compared with vehicle-treated OVX-E rats during the second restraint period (Fig. 3B). HRs between the two groups of OVX-E rats were variable, and only a few points were significantly different (Fig. 2C). When data between L-NNA-treated...

**Fig. 2.** Effects of NO inhibition on heart rate (HR) values (means ± SE) during restraint stress and recovery in OVX-E or OVX-V rats. In all groups, n = 6 rats. A: OVX-E and OVX-V rats receiving icv injections of vehicle. B: OVX-E rats receiving icv injections of L-NNA (88 μg/kg) or vehicle. C: OVX-V rats receiving icv injections of L-NNA or vehicle. D: OVX-E and OVX-V rats receiving icv injections of L-NNA. For A–D, the thin arrow is the time of vehicle or L-NNA injection. Thick, black arrows are the times at which rats were placed in the restraint chamber; white arrows are the times at which rats were removed from the restraint chamber and allowed to rest. Lines below values represent points of significant difference (P < 0.05) between the 2 groups at that time.
OVX-E rats and L-NNA-treated OVX-V rats were compared, no significant differences were found between the two groups (Figs. 1D and 3D). HRs showed several points where significant differences were found, with higher HRs found in L-NNA-treated OVX-V rats (Fig. 2D).

**NO Production in the Hypothalamus and Brain Stem**

In response to restraint, no changes in NOx levels were found in hypothalami or brain stems of OVX-V rats, whereas NOx levels were elevated significantly in both regions (~2.4- and 2.3-fold increases in hypothalamus and brain stem, respectively) of OVX-E rats (Fig. 4). In all groups, n = 6.

**DISCUSSION**

We have measured the effects of estrogen replacement on cardiovascular responses to psychological stress in OVX rats using an experimental paradigm that allows us to measure responses both during administration of restraint stress and during recovery from the stress. Our results show that OVX-E rats respond to restraint with smaller increases in MAP than OVX-V rats and that MAPs during recovery are also significantly lower in OVX-E rats. We have also investigated the role of NO in mediating the attenuation of arterial pressure in response to restraint. Although inhibition of brain NO production in OVX-V rats has no effect on their cardiovascular responses to restraint, it augments the responses of OVX-E rats, especially during periods of recovery, so that the responses are indistinguishable from those of OVX-V rats. Finally, we show that NO production is increased in hypothalami and brain stems of restrained OVX-E, but not OVX-V, rats. These results show that estrogen replacement in OVX rats reduces MAP responses to...
restraint stress and that NO plays an important role in mediating these effects.

**Arterial Pressure Responses to Restraint**

*Baseline MAPs in OVX-E and OVX-V rats.* We found no significant differences in baseline MAPs or HRs of OVX-E and OVX-V rats, in agreement with studies in which no changes in resting MAP were found in OVX rats with 2 days (23) or 5 wk (24) of estrogen replacement compared with vehicle-treated rats. It is interesting to note, however, that baseline MAPs in OVX-E rats in our study tended to be lower than those in OVX-V rats. Although it is difficult to draw strong conclusions at this time, it is tempting to speculate that the presence of estrogen in OVX-E is responsible for this trend. Because the levels of estradiol in our OVX-E rats were found to be equivalent to those in the late diestrus or proestrous phases of the rat estrous cycle (2), our results are physiologically relevant.

*Restricted vehicle-treated OVX-E and OVX-V rats.* Not surprisingly, the psychological stress of restraint elicited increases in MAP responses in both groups of rats, as the brain increases sympathetic output to mobilize the animal. These results are generally similar to those that we obtained for male rats in a previous study (34), although increases in MAP appear to have been larger in restrained males than in restrained OVX-V or restrained OVX-E rats. In restrained vehicle-treated OVX-V rats, MAPs (but not HRs) were significantly greater than in restrained vehicle-treated OVX-E rats during both restraint and rest periods. These results show that estrogen replacement for 11–12 days substantially affects cardiovascular responses to restraint. Because the cardiovascular responses to psychological stress are generated in the brain, our results suggest that estrogen attenuates the central sympathetic response to stress. Previous work in humans has shown that 8 wk of estrogen replacement in perimenopausal women was associated with attenuated arterial pressures, but not HRs, in response to 10 min of mental arithmetic (16). In other studies, it has been shown that, compared with OVX-V rats, OVX-E rats receiving estrogen for 7 days showed decreased struggling behavior and decreased activation of forebrain neurons (c-fos expression) in response to the forced swim test (25). OVX-E rats (7-day treatment) exposed to noise stress showed attenuation of neuronal activation in central autonomic centers, including the paraventricular nucleus of the hypothalamus (PVN), amygdala, and catecholaminergic neurons in the brain stem (5). These results, combined with ours, suggest that chronic estrogen replacement in OVX rats not only attenuates behavioral and neuronal activation responses to stress but also attenuates the arterial pressure responses to stress. It is also important to note that, because of our use of pellets, we assume that the levels of estrogen in the OVX-E rats were relatively constant during treatment. Thus it will also be important to elucidate the effects of estrogen both on resting MAP and on MAP responses to stress in normally cycling female rats.

*Restricted L-NNA-treated OVX-E and OVX-V rats.* To investigate the role of brain NO in estrogen’s effects on brain-derived attenuation of arterial pressure responses to restraint, we blocked the production of constitutive NO in the brain using intracerebroventricular injections of L-NNA in advance of the administration of stress. NO has emerged as an important neurotransmitter utilized by autonomic neurons during periods of homeostatic imbalance (17). For example, psychological stress activates central autonomic NO systems (3, 14, 18), and NO donors attenuate the increases in cardiovascular responses to psychological stress (9). We used the protocol for use of L-NNA that we developed in a previous study (32) to show that blockade of NO production in the brain with L-NNA leads to elevated arterial pressure responses to restraint in OVX-E rats compared with restrained vehicle-treated OVX-E rats. In OVX-V rats, L-NNA had no effect on the arterial pressure responses so that the MAP responses to L-NNA-treated OVX-E and L-NNA-treated OVX-V rats were now indistinguishable from one another. The effects of NO inhibition on MAP in OVX-E rats were most clearly observed during the periods of rest, where analysis of AUC showed significantly higher values in OVX-E rats treated with L-NNA compared with OVX-E rats that received vehicle. Thus NO appears to be especially important in mediating estrogen’s effects to reduce MAP during recovery from psychological stress. Although significant differences in AUC were not found during periods of restraint, a trend toward a

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![Graph A](image1.png)  ![Graph B](image2.png)

**Fig. 4.** Nitrate/nitrite levels (µg/ml) in hypothalamus and brain stem of restrained OVX-V (A) and OVX-E (B) rats compared with their respective unrestrained controls. For all groups, n = 6 rats. Brackets denote concentration. *P < 0.05.
greater value in L-NNA-treated rats was found during the second hour of restraint, suggesting that NO may also mediate at least some of estrogen’s effects to reduce MAP responses during restraint.

**Restraint-induced Changes in Brain NO Production**

To further demonstrate that estrogen exerts an important effect on NO production in the brains of stressed rats, we show that restraint stimulates significant increases in NO production within the hypothalamus and brain stem of OVX-E rats, but not OVX-V rats, compared with their respective, unrestrained controls. These results provide evidence that increased production of NO in the brains of OVX rats in response to restraint is related to the presence of estrogen. Although levels of circulating estrogen have been shown to positively affect levels of plasma NO in humans (4, 12), our results are the first to describe a similar relationship in brains of OVX rats exposed to psychological stress.

The hypothalamus contains several NO neuronal cell groups, but one of the largest and most conspicuous groups is the PVN, an important autonomic integrating center (17). NO is known to decrease sympathetic output when applied to the PVN (17), and estrogen receptor-β immunoreactivity and mRNA have been described in neurons of the PVN (30). Thus the increases in hypothalamic NO production that we found in restrained OVX-E rats may occur, at least in part, within neurons of the PVN. Alternatively, other areas in the hypothalamus may also be contributing, since the preoptic area, ventral lateral nucleus, supraoptic nucleus, and arcuate nucleus contain NO-producing neurons, albeit in smaller numbers (18, 22).

Increased NO production was also observed in the brain stems of restrained OVX-E rats. The brain stem contains several cardiovascular/autonomic centers (e.g., nucleus of the tractus solitarius, rostral and caudal ventrolateral medulla, raphe nuclei) that are interconnected with each other and with the PVN. Furthermore, the ventrolateral medulla and raphe nuclei contain large numbers of NO-producing neurons that are recruited by psychological stress (18), and activity of neurons in these areas is affected by NO (17). Finally, estrogen receptor gene expression has been demonstrated in neurons of the nucleus of the tractus solitarius and caudal ventrolateral medulla (31). Therefore, these findings provide the anatomic basis for mechanisms by which estrogen may affect brain stem NO production during restraint in OVX rats.

L-NNA is an inhibitor of neuronal NOS and endothelial NOS, so that either or both of these NOS isoforms may be contributing to the increases in NO production in restrained OVX-E rats. As discussed above, neuronal NOS in hypothalamic and/or brain stem neurons may participate in producing these increases. Endothelial NOS may also be involved, since increased levels of endothelial NOS have been found in cerebral blood vessels of OVX-E rats (21), and endothelial NOS mRNA and protein are increased in the median emi-

**Perspectives**

We show that arterial pressure responses to psychological stress in OVX female rats are attenuated significantly in rats receiving exogenous estrogen. Because psychological stress significantly increases the risk of myocardial ischemia (8) and is associated with increased risk of other pathological cardiac events, especially in patients with cardiovascular disease (13), the effect of estrogen may thus prove to be protective during periods of stress. By showing that the effects of estrogen on cardiovascular responses to stress in OVX-E rats are mediated by brain NO, especially during periods of recovery, our results illustrate that estrogen's effects on NO and arterial pressure extend to the brain, and they contribute to the understanding of the mechanisms by which estrogen in the brain affects autonomic function.

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**DISCLOSURES**

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**REFERENCES**


