Estrogen replacement suppresses stress-induced cardiovascular responses in ovariectomized rats

Keiko Morimoto,1 Youko Kurahashi,1 Kaori Shintani-Ishida,2 Natsuko Kawamura,1 Mariko Miyashita,1 Masami Uji,1 Nobusuke Tan,3 and Ken-ichi Yoshida2

1Department of Environmental Health, Faculty of Life Science and Human Technology, Nara Women’s University, Nara 630-8506; 2Department of Forensic Medicine, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033; and 3Department of Exercise and Health Science, Faculty of Education, Yamaguchi University, Yamaguchi 753-8513, Japan

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There are contradictory results on the effects of estrogen on the hypothalamic-pituitary-adrenal axis responses to various stresses (4, 6, 11). Although there are initiative studies by Clarkson et al. (25, 26, 41) that showed the suppressive effect of estrogen on atherosclerosis in the social subordinate female monkey, there are only two animal studies on the effects of estrogen on acute pressor responses to stress. The first study (14) reported that the pressor response to open-field stress was attenuated by estrogen plus progesterone treatment but not by estrogen alone in ovariectomized spontaneously hypertensive rats with the use of radiotelemetry. The second study (9) reported that estrogen reduced the pressor responses to restraint in ovariectomized and catheterized rats. These contradictory results may stem from differences in experimental conditions; notably, the type and the degree of stress, the type and the dose of estrogen treatment, or the parameter employed and its measurement of stress response. In particular, because of the difficulties involved in the observation of physiological variables in conscious rats without any other stress during experiments, it has not been revealed whether estrogen practically modulates cardiovascular responses induced by a mild psychological stress. In this respect, we have successfully studied cardiovascular responses to mild psychological stress with the use of the radiotelemetry system in freely moving rats with minimum additional stress after adequate recovery surgical procedures (33–36, 38).

Recently, several lines of evidence have supported the contribution of nitric oxide (NO) to the cardiovascular protective effects of estrogen in experimental and clinical studies (32, 39). Acute administration of estrogen rapidly activates endothelial NO synthase (eNOS) through phosphatidylinositol 3-kinase/Akt-mediated phosphorylation (21, 40), whereas shear stress activated nitric oxide synthase; blood pressure; heart rate; radiotelemetry and psychological stress

PSYCHOLOGICAL STRESS evokes hypertension, tachycardia, and arrhythmia (33, 34, 36). Some human studies (29, 44) demonstrated that pressor responses to mental stresses were enhanced in postmenopausal women compared with premenopausal women. Moreover, it has been reported (8, 12, 29, 44) that estrogen treatment attenuates cardiovascular responses to mental stresses in postmenopausal women. However, it is unknown how estrogen attenuates the pressor response to psychological stress.

Morimoto, Keiko, Youko Kurahashi, Kaori Shintani-Ishida, Natsuko Kawamura, Mariko Miyashita, Masami Uji, Nobusuke Tan, and Ken-ichi Yoshida. Estrogen replacement suppresses stress-induced cardiovascular responses in ovariectomized rats. Am J Physiol Heart Circ Physiol 287: H1950–H1956, 2004. First published July 1, 2004; doi:10.1152/ajpheart.00341.2004.—We assessed the hypothesis that chronic estrogen replacement in ovariectomized rats has the beneficial effect of suppressing stress-induced cardiovascular responses through endothelial nitric oxide synthase (eNOS). We employed a radiotelemetry system to measure blood pressure and heart rate (HR). Female Wistar rats aged 11 wk were ovariectomized and implanted with radiotelemetry devices. After 4 wk, the rats were assigned either to a placebo-treated group (Placebo; n = 6) or a group treated with 17β-estradiol (Estrogen; n = 8) subcutaneously implanted with either placebo- or 17β-estradiol (1.5 mg/60-day release) pellets under anesthesia. These rats underwent either of the two types of stress after 4 wk of estrogen or placebo treatment, Cage-switch stress and restraint stress rapidly and continuously elevated the mean arterial pressure (MAP) and HR both in the Placebo and Estrogen groups. However, the MAP and HR responses to cage-switch stress and the MAP but not HR response to restraint stress were attenuated significantly in the Estrogen group compared with the Placebo group. A NOS inhibitor, Nω-nitro-l-arginine methyl ester, given in drinking water, reduced the difference in the pressor response to cage-switch between the Estrogen and Placebo groups. In addition, Western blot analysis showed that eNOS expression in the mesentery was increased in the Estrogen group compared with the Placebo group. Thus for the first time we showed that mesenteric eNOS overexpression could explain at least partly why chronic estrogen treatment suppressed the enhanced cardiovascular responses to psychological stress in the ovariectomized rat.

Address for reprint requests and other correspondence: K. Morimoto, Dept. of Environmental Health, Faculty of Life Science and Human Technology, Nara Women’s University, Kita-Uoya Nishi-machi, Nara 630-8506, Japan (E-mail: kmorimoto@cc.nara-wu.ac.jp).
cular responses to cage-switch or restraint stress, and 2) whether eNOS modulation in peripheral vessels can explain the cardiovascular effect of estrogen replacement in freely moving rats using the telemetry system.

MATERIALS AND METHODS

Three series of experiments were conducted. Experiment 1 was designed to assess the effect of 4-wk estrogen replacement on mean arterial pressure (MAP) and heart rate (HR) responses to two different types of stress in ovariectomized rats. In experiment 2, the effect of a NOS inhibitor on these responses was examined. In experiment 3, the eNOS expression in the mesentery and aorta was examined by Western blot analysis.

The Nara Women’s University Committees on Animal Experiments approved the experimental protocol.

Animals. A total of 38 albino female rats (Wistar strain) was used in this study. In experiment 1, 11-wk-old rats were subjected to ovariectomy and implanted with radiotelemetry devices under anesthesia (45 mg/kg ip pentobarbitone sodium). After 4 wk, rats were assigned randomly to the placebo-treated (Placebo; n = 6) or estrogen-treated (Estrogen; n = 8) group rats 4 wk after pellets implantation. For details, see Experimental protocols.

In experiment 2, to examine the effect of NOS on stress-induced cardiovascular responses, N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME) was orally given to another set of Placebo (n = 6) and Estrogen (n = 6) group rats 4 wk after pellets implantation. For details, see Experimental protocols.

In experiment 3, another set of Placebo (n = 6) and Estrogen (n = 6) groups was used for Western blot analysis of eNOS expression in the mesentery and aorta 4 wk after subcutaneous implants of pellets.

The rats were housed individually in standard plastic rat cages (40 cm long × 25 cm wide × 25 cm deep). The room was maintained at 26 ± 1°C, a temperature within the thermoneutral zone for rats, with a 12:12-h light-dark cycle, with lights on at 6:00 AM. Tap water and rodent chow were provided ad libitum.

Measurement of physiological parameters. Blood pressure, HR, and physical activity were measured using a radiotelemetry system (DATAQUEST, Data Sciences). While the animals were under anesthesia (45 mg/kg ip pentobarbitone sodium), a battery-operated telemeteric transmitter (model TA11PA-C40; Data Sciences) was implanted intraperitoneally. The transmitter contained a sensor, to which a fluid-filled intra-arterial catheter was attached, along with a radio frequency transmitter. The catheter tip was inserted into the abdominal aorta in a retrograde fashion from a point near the bifurcation of theeliac trunk (in Hz) from the transmitter was monitored by an antenna mounted on a receiver board (model RA1310; Data Sciences) placed under the cage. The data were fed each minute into a peripheral processor (matrix model BCM100; Data Sciences) connected to a COMPAQ computer. A “pulse” of activity was monitored as deviation of the transmitter from the antenna mounted in the board.

eNOS expression. The whole mesentery and aorta from both groups of rats were homogenized, and equal amounts of protein (20 μg) were subjected to Western blot analysis with the use of an antibody to eNOS (Transduction Laboratories; Lexington, KY), as previously described (27, 46, 47). The eNOS level was expressed as relative to the mean level of the Placebo group.

Measurement of plasma estradiol. At the end of experiment 1, the blood was sampled from the cardiac ventricles after euthanasia by a pentobarbitone sodium overdose. The plasma estradiol concentration was measured commercially by radioimmunoassay (SRL; Nara, Japan).

Stress exposure. Two types of stress, cage-switch and restraint stress, were used in the experiments. Cage-switch stress was evoked by transfer of a rat from its home cage to another identical plastic cage containing water at 36°C at a depth of 1 cm.

Restraint stress was evoked by confining a rat in a capsule-shaped, metal-framed rat holder with an appropriate size for each rat. To minimize the confounding physiological effects of the circadian rhythm, the rats were exposed to the stress between 11:00 AM and 2:00 PM.

Experimental protocols. On the day before experiment 1, each rat was gently picked up, and its transmitter was switched on magnetically. On the experiment day, the basal blood pressure, HR, and activity in freely moving rats were monitored for 30 min before stress exposure, although the parameters were also monitored for 24 h before stress exposure. Half of the rats in each group were assigned random exposure to either cage-switch or the restraint stress. The parameters were monitored continuously during the 60-min period of stress exposure. Seven days later, each rat was exposed to the other stress.

In experiment 2, half of the rats in each group were assigned randomly to be given either plain tap water or L-NAME-supplemented water (250 μg/ml in water). Twenty-four hours later, each group of rats was exposed to cage-switch stress for 60 min. The experiment was repeated 7 days later with each rat being given the other type of water. After 24 h, the rats were exposed to cage-switch stress for 60 min. In the present study, we separately performed these two series of experiments 1 and 2 using different groups of rats to avoid the plasma estradiol variations depending on the replacement duration.

In experiment 3, the whole mesenteries and aortas were harvested after pentobarbitone sodium overdose and stored at −40°C until Western blot analysis for eNOS.

Statistical analysis. The results are expressed as means ± SE in the text and figures. The cardiovascular parameters were analyzed by the two-way repeated-measures ANOVA, whereas the other parameters were analyzed by Student’s unpaired t-test. For details, see the figures.

RESULTS

Four weeks after the replacement, the body weight was significantly lower in the Estrogen group than the Placebo group (285.1 ± 3.5 vs. 308.8 ± 3.7 g; P < 0.05). Evaluation of plasma estradiol concentration. The estradiol concentrations were significantly higher in the Estrogen group than in the Placebo group (60.0 ± 10.0 vs. 6.6 ± 1.2 pg/ml; P < 0.01) at the end of experiment 1.

Evaluations of MAP, HR, and activity. Table 1 shows the resting levels of MAP and HR for 30 min before exposure to each stress and those for 12 h during dark and light periods. The resting MAP was slightly, but not significantly, higher in the Estrogen group than in the Placebo group. The basal HR levels were similar in both the Estrogen and Placebo groups.

<table>
<thead>
<tr>
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<th>Mean Arterial Pressure, mmHg</th>
<th>Heart Rate, beats/min</th>
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<tr>
<td></td>
<td>Placebo group</td>
<td>Estrogen group</td>
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<tr>
<td>Before CS</td>
<td>88.7 ± 4.0</td>
<td>97.7 ± 2.2</td>
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<tr>
<td>Before RT</td>
<td>89.9 ± 1.2</td>
<td>97.5 ± 2.5</td>
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<tr>
<td>Dark period</td>
<td>92.6 ± 4.6</td>
<td>100.0 ± 2.7</td>
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<tr>
<td>Light period</td>
<td>90.8 ± 4.5</td>
<td>96.9 ± 2.3</td>
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Values are means ± SE; n = 6 placebo-treated rats and n = 8 (17β-estradiol)-treated rats. CS, cage switch; RT, restraint. The values were obtained every minute from the rats in each group for 30 min just before CS or RT stress in placebo- and estrogen-treated groups. Dark and light periods refer to the values obtained every minute in each group for 12 h during the light-dark cycle. There were no significant differences between the values for the two groups.
Figure 1 shows significant and sustained elevation of MAP \((P < 0.0001 \text{ in } A)\) and HR \((P < 0.0001 \text{ in } B)\) in the Estrogen and Placebo groups during cage-switch stress. The elevations of the MAP and HR were significantly suppressed in the Estrogen group compared with the Placebo group. Two-way repeated-measures ANOVA confirmed the replacement \((P < 0.01 \text{ in Fig. 1B})\) and time \((P < 0.0001 \text{ in Fig. 1, A and B})\) effects and interaction between replacement and time \((P < 0.01 \text{ in Fig. 1A and } P < 0.05 \text{ in Fig. 1B})\). The systolic pressure, MAP, and diastolic pressure underwent almost parallel changes (data not shown). However, as shown in Fig. 1C, there was no significant difference in locomotor activity between the Estrogen and Placebo groups either before or during cage-switch stress.

Figure 2, A and B, shows changes in MAP and HR before and during restraint stress. In both groups, these two parameters increased immediately after the onset of restraint stress. The elevations of MAP in the Estrogen group were significantly attenuated during the restraint stress compared with those in the Placebo group. Compared with cage switch (Fig. 1), restraint stress induced more sustained elevation in MAP in the Placebo group, whereas the difference between the Placebo and Estrogen groups was kept larger during the time of observation. On the other hand, there were no significant differences between the HR responses for the two groups. Two-way repeated-measures ANOVA also showed replacement \((P < 0.05 \text{ in Fig. 2B})\) and time \((P < 0.0001 \text{ in Fig. 2, A and B})\) effects and interaction between replacement and time \((P < 0.05 \text{ in Fig. 2A})\).

Figure 3 shows that l-NAME administration for 24 h similarly increased the resting MAP, but not HR, in the Estrogen and Placebo groups. As shown Fig. 4, after l-NAME pretreatment, the difference in the pressor response to cage-switch stress between the Placebo and Estrogen groups became to be very small.

Expression of eNOS. Figure 5 shows the eNOS protein expression in the mesentery and aorta in the Estrogen and Placebo groups. In the mesentery (Fig. 5C), the eNOS level was significantly higher in the Estrogen group than in the Placebo group \((P < 0.01)\). However, in the aorta (Fig. 5D), the eNOS level was marginally higher in the Estrogen group than in the Placebo group.

**DISCUSSION**

The present study demonstrated that chronic estrogen replacement clearly attenuates the cardiovascular responses to mild psychological stress evoked by cage switch or restraint in...
ovariectomized freely moving rats by use of the radiotelemetry system. Chronic estrogen replacement also upregulates eNOS peripherally.

In earlier studies, Clarkson et al. (25, 26, 41) reported that socially subordinate female monkeys showed ovarian dysfunction and exacerbated coronary artery atherosclerosis, which was inhibited by exogenous estrogen. They pointed out the effect of estrogen on atherosclerosis, whereas we did that on stress-induced pressor response. There are only two reports except ours on the beneficial effect of estrogen replacement on acute cardiovascular responses to psychological stress in experimental animals. However, one study reported that estrogen plus progesterone treatment, but not estrogen alone, attenuated the enhanced cardiovascular responses to open-field stress in ovariectomized spontaneously hypertensive rats by use of the radiotelemetry system (14). The other recent study (9) demonstrated that pressor response to restraint stress was blunted by estrogen replacement in the ovariectomized and catheterized rats, whereas the production of NO metabolites was enhanced in the hypothalamus and brain stem in response to restraint stress in the estrogen-replaced rat. It remains to be elucidated which of the central or peripheral NOS upregulation predominantly mediates the effect of estrogen on the stress responses and how it does.

The present study took advantage of telemetric measurements that enabled us to investigate cardiovascular responses to mild psychological stress induced by cage switch in freely moving rats compared with catheter measurements. In addition, the telemetry system minimizes the stress inherent to the experimental procedure itself. In daily life, anyone experiences mild psychological stress evoked by such a mild change in environment as cage-switch, but not by restraint, in the rat. In addition, the plasma estradiol levels achieved by 4-wk estrogen

Fig. 3. Resting mean arterial pressure (A) and heart rate (B) before (Water) and after N^\text{G}-nitro-L-arginine methyl ester (L-NAME) administration for 24 h in drinking water in rats in placebo-treated (n=6) and estrogen-treated (n=6) groups. Data are means ± SE of values obtained every minute from the rats in each group for 30 min just before cage-switch stress. There were significant differences on the resting mean arterial pressures between before and after L-NAME administration in each group. **P < 0.01 in the placebo-treated group; ***P < 0.001 in the estrogen-treated group.

Fig. 4. The course of mean arterial pressure and heart rate in placebo-treated (n=6) and estrogen-treated (n=6) groups before and during cage-switch stress for 60 min (start at time 0) before (A and B) and after administration (C and D) of L-NAME in drinking water (250 µg/ml). Data are means ± SE of values obtained every minute from the rats in each group over 5-min periods. Two-way repeated-measures ANOVA revealed significant difference between the groups for either mean arterial pressure (*P < 0.05 in A) or heart rate (*P < 0.05 in B) response. However, after administration of L-NAME, these responses (C and D, respectively) were similar in both groups.
replacement in our study were comparable to the physiological levels in proestrous normal rats (5). We think that these experimental settings are keys to positive estrogen’s effects on the cardiovascular response to mild psychological stress in our study.

Resting blood pressure is marginally higher in the Estrogen group than in the Placebo group. Many previous studies (3, 17, 19, 24, 42, 49) showed that contraceptives or estrogen treatment induce hypertension in humans and animals. However, the mechanisms on the pressor effect of estrogen are still inconsistent with each other. Notably, in the only other study using the telemetry system, Takezawa et al. (45) showed that implantation of 17β-estradiol produced a gradual increase in resting blood pressure in ovariectomized rats, consistent with our result. In any case, we think it unlikely that the higher resting MAP attenuated the stress response in the Estrogen group (“ceiling effect”), because there was no correlation between the resting MAP and the stress-induced MAP change in the Estrogen and Placebo groups (correlation coefficient = 0.26).

We demonstrated that NOS inhibition by l-NAME reduced the difference in pressor response to cage switch between the Placebo and Estrogen groups, suggesting the contribution of NOS to the depressor effect of estrogen (Fig. 4). However, we must take the indirect or nonspecific effect of l-NAME into consideration because l-NAME appears to reduce stress-induced pressor response in the Placebo group rather than enhance it in the Estrogen group (Fig. 4). In addition to the transcriptional upregulation of eNOS (32, 48), estrogen also rapidly activates eNOS through phosphorylation of eNOS at Ser1177 by phosphatidylinositol 3-kinase-Akt, thereby dilating vessels (7, 28, 31, 32, 40). On the other hand, endothelial cells in normal blood vessels secrete NO tonically and increase NO production dynamically in response to increased shear stress (10, 15, 16) through these pathways.

We also showed that estrogen replacement enhances eNOS expression in the mesentery. It is tempting to speculate that chronic estrogen replacement causes overexpression of eNOS in the resistant arteries of the mesentery. Thus it is plausible that the pressor response to the stress induces stretch and shear stress in the vascular wall, followed by eNOS activation and NO production, which attenuates the stress-induced vasoconstriction to a greater extent in the Estrogen than Placebo group.

In further support of our hypothesis of the effect of estrogen on the peripheral vascular wall, it was reported (23) that flow-induced arteriolar dilation in the muscle was reduced in the ovariectomized spontaneously hypertensive rat, and estrogen preserved the dilation in a NOS inhibitor-dependent manner. This finding suggests that estrogen preserves the NO-mediated dilation in response to flow/shear stress. A future study to examine whether estrogen increases the phosphorylation of eNOS in the mesentery during stress may reveal this point.

It is also reminded that the eNOS overexpression in the estrogen-treated rats is not contradictory to the higher resting MAP because the eNOS overexpression can be dissociated from its activation. It might be supported by the fact that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (mevastatin) upregulated eNOS in the mouse aorta, although the resting arterial blood pressure slightly increased (1). It is likely that eNOS expression increases but may not be activated more at the basal condition in the Estrogen group.

The estrogen replacement significantly attenuated tachycardia induced by cagemswitch but not restraint stress in a l-NAME-dependent manner. It is unlikely that the effect of estrogen on the pressor response is secondary to its effect on tachycardiac response because estrogen inhibited the pressor response to a greater extent than the HR response (Figs. 1 and 2). In addition, because it is well known that NO promotes tachycardia (37), it is unlikely that the NO overproduction by estrogen replacement suppresses pressor response to stress through attenuation of tachycardia. On the other hand, the difference in pressor responses to the restraint stress between the Placebo and Estrogen groups was kept as long as the stress continued (Fig. 2). This finding suggests that estrogen does not solely affect pressor response through modulation of the initial sympathetic surge in response to stress. However, the possibility of estrogen action on either the central or sympathetic nervous system awaits further investigation.

**Fig. 5.** Representative Western blots indicating endothelial nitric oxide synthase (eNOS) expression in mesentery (A) and aorta (B) and relative eNOS expression of mesentery (C) and aorta (D) in the placebo-treated (P; n = 6) and estrogen-treated (E; n = 6) groups. Student’s unpaired t-test revealed a significant difference (**) between the relative eNOS expression of mesentery for the groups.
Prenopausal women are protected against coronary artery disease and hypertension compared with men or postmenopausal women (2, 20, 30, 43). In addition, psychological stress evokes cardiac events (50) and stroke (22). The attenuation in stress-induced pressor response by estrogen shown in this study is not so large, but the influence on women’s health may be crucial when they are frequently exposed to such a mild stress as cage switch evokes. Our findings provide a clue to a better understanding of the effects of estrogen on the prevention of cardiovascular diseases as well as events and sudden death induced by psychological stresses.

In conclusion, the present study indicates that chronic estrogen replacement attenuates cardiovascular responses to mild psychological stresses at least partly through eNOS overexpression and activation in the periphery. However, further study is required to address the question of whether the effect of estrogen is mediated solely through peripheral overexpression of eNOS or also through central or sympathetic nervous system modulation.

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