Chronic estradiol-17β exposure increases superoxide production in the rostral ventrolateral medulla and causes hypertension: reversal by resveratrol

Madhan Subramanian,1 Priya Balasubramanian,2 Hannah Garver,2 Carrie Northcott,2 Huawei Zhao,2 Joseph R. Haywood,2 Gregory D. Fink,2 Sheba M. J. MohanKumar,2 and P. S. MohanKumar1

1Departments of Pathobiology and Diagnostic Investigation and 2Pharmacology and Toxicology, Colleges of Veterinary, Osteopathic and Human Medicine, Michigan State University, East Lansing, Michigan

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Cardiovascular disease remains one of the leading causes of morbidity and mortality in women. Postmenopausal increases in blood pressure (BP) in women make hypertension more prevalent in women compared with men of the same age (6, 42). Because premenopausal women have lower blood pressure compared with age-matched men, estrogens were thought to play a protective role against hypertension. Estrogens are reported to improve lipid profile (52), decrease vascular resistance (28), and modulate the activity of brain nuclei involved in cardiovascular regulation (18, 47). However, recent reports from the Women’s Health Initiative study, National Institutes of Health have provided evidence that hormone replacement therapy (HRT) using estrogen alone or a combination of estrogen and progestin does not confer cardiovascular protection and may actually increase the risk for coronary heart disease among postmenopausal women (30). These clinical studies have brought to light the importance of clinical and basic research in understanding the role of estrogen in BP regulation (57). Oral estrogen administration, either given alone or in combination with progestins, was found to promote systolic blood pressure in postmenopausal women (34, 46). Although the magnitude of the increase in BP was only between 1 and 2 mmHg, similar increases in systolic BP and pulse pressure are known to be associated with a higher rate of progression of coronary atherosclerosis (35) and development of cardiovascular events (34) in large clinical trials.

In addition to women who are on HRT, younger women who take oral contraceptives are also at increased risk for potentially developing cardiovascular disorders. Oral contraceptives have been used worldwide for more than 30 years. Most women who take oral contraceptives are also at increased risk for potential cardiovascular events (34) in large clinical trials. The cortex, limbic system, hypothalamus, brain stem, and the autonomic nervous system are all known to be involved in the maintenance of BP. In the brain stem, the rostral ventral lateral medulla (RVL M) is a critical area involved in the regulation of sympathetic activity and BP. The RVL M integrates sympathetic outflow and provides excitatory input to preganglionic sympathetic cells in the spinal cord (51). The RVL M maintains basal vasomotor tone, and an increase in RVL M activity is associated with hypertension and heart failure (26). Recently, oxidative stress in the RVL M has been suggested to increase sympathetic nervous system.
activity and BP in several animal models of hypertension (4, 9, 25). Therefore, we hypothesized that E2-induced increase in blood pressure could be mediated by oxidative stress-related changes in RVLM.

To test this idea, young, adult cycling female Sprague-Dawley rats were exposed to low levels of E2 on a chronic basis. We attempted to reduce oxidative stress in these animals by treating them with resveratrol, an antioxidant.

MATERIALS AND METHODS

Experimental animals and treatment. All of the protocols followed in this experiment were approved by the Institutional Animal Care and Use Committee at Michigan State University. Adult (3–4 mo old) female Sprague-Dawley (SD) rats (Harlan Sprague-Dawley, Indianapolis, IN) were housed in temperature (23 ± 2°C)- and light-controlled (14:10-h light-dark cycle) rooms with ad libitum food and water. Estrous cycles were monitored, as described previously (22) for 2 wk, and animals that were cycling regularly were used in the experiments. In experiment 1, animals were divided into two groups (n = 4/group); sham-implanted (control); or implanted with E2 (20 ng/day, 90-day slow-release pellet; Innovative Research America, Sarasota, FL). After 60 days, sham and E2-treated rats were implanted with radiotelemetry transmitters to monitor BP, as described below. After 90 days of exposure to E2, all of the animals were killed at noon on the day of estrus. Most of the E2-treated animals were in persistent estrus after 90 days of treatment. The control animals were killed on the

Fig. 1. Effect of chronic E2 exposure on blood pressure parameters. Line graphs depicting mean arterial pressure (MAP; mmHg) (A), heart rate (HR; beats/min) (B), systolic blood pressure (SBP) (C), and diastolic blood pressure (DBP; mmHg) (D), respectively. Solid circles (●) represent E2 pellet implanted (20 ng/day, 90-day slow-release pellets), and open circles (○) represent control rats. E–H: bar graphs showing the average values of the BP parameters shown in A–D, between E2-implanted and control rats. *Significant difference (P < 0.05) from control rats.
day of estrus after 90 days of sham implantation for comparison to the treatment group. The brains and brain stem were removed, frozen on dry ice, and stored at −70°C. The trunk blood was collected, and serum was separated and stored at −70°C until processed.

In experiment 2, animals were divided into four groups (n = 6/group): sham-implanted (control); implanted with E2 slow-release pellets (E-90; 20 ng/day for 90 days) (22); control fed with Chow containing 0.84 g resveratrol/kg of Chow (Res) (3); and E2-implanted rats fed with Chow containing resveratrol (Res+E-90). Resveratrol treatment was started 7 wk after pellet implantation. Animals were implanted with telemeters on the 9th wk, as described below. Food intake, water intake, and body weight were monitored weekly throughout the experimental period. The animals were killed at the end of 90 days, while in the state of estrus.

**BP measurement.** In the 9th wk of the treatment period, radio-telemetry transmitters (model TA11-PA-C40; Data Sciences International, St. Paul, MN) were implanted, as described previously (24). Briefly, the tip of the transmitter catheter was placed in the abdominal aorta through the femoral artery under general anesthesia. The body of the transmitter was placed in a subcutaneous pocket in the abdomen. Ten days later, BP data from the transmitter were collected continuously for 11 days in experiment 1, and 14 days in experiment 2 (24 h/d; 10-s averages collected every 10 min). Data were stored and analyzed using the Dataquest A.R.T. software (Data Sciences International, St. Paul, Minnesota).

**Brain microdissection.** Palkovits’s microdissection procedure (32) was used to isolate the RVLM. Briefly, 300-μm serial sections of brain stem were obtained using a cryostat (Slee Mainz, London, UK). The sections were transferred to microscope slides and placed on a cold stage maintained at −10°C. The RVLM of the brain stem was microdissected from sections obtained 12.00 mm to 12.48 mm posterior to the bregma using a 500-μm-diameter punch, using the rat brain stereotaxic atlas as a reference (41). The punches were stored immediately at −70°C until processed for superoxide measurement.

**Superoxide measurement.** Superoxide (O_2^-) levels from the RVLM region were measured using a lucigenin O_2^- chemiluminescence assay adapted from Rey et al. (43). RVLM punches were placed in HEPES buffer (10 mmol/L) containing 119 NaCl, 20 HEPES, 4.6 KCl, 1.0 MgSO_4, 0.15 Na_2HPO_4, 0.4 KH_2PO_4, 5 NaHCO_3, 1.2 CaCl_2, 5.5 glucose (pH 7.4). Diethyldithiocarbamate (DDC; a superoxide dismutase inhibitor; 10 mmol/l) was added, and the samples were incubated at 37°C for 30 min. Lucigenin (5 μmol/l) was added, and the samples were incubated for 10 min at 37°C. Chemiluminescence measurements were obtained using a model MD 20/20 Luminometer (Turner Designs, Sunnyvale, CA) for 10 readings (30 s/reading). Tiron (10 mmol/l; an O_2^- scavenger; Sigma, St. Louis, MO) was added and incubated for 15 min at 37°C, and 10 additional readings were taken. The relative amount of O_2^- was determined by taking the average of 2nd–9th readings prior to the addition of tiron, and subtracting the average of the 7th–10th readings after the addition of tiron. O_2^- generated was reported as change in chemiluminescence per minute per milligram tissue weight.

**Serum estradiol.** Estradiol levels in serum separated from trunk blood were measured by double antibody radioimmunoassay by Dr. A. F. Parlow, National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases. Samples were assayed in duplicate.

**Statistics.** Daily BP measurements and weekly food intake, water intake, and body weight were analyzed by repeated-measures ANOVA followed by post hoc Fischer’s LSD test. Average BP parameters, serum estradiol and O_2^- data were analyzed by ANOVA, followed by post hoc Fischer’s LSD test. Results were considered significant when P < 0.05. Average food intake, water intake, body weight, and heart-body weight ratio were analyzed by ANOVA followed by post hoc Fisher’s LSD test. Results were considered significant when P < 0.05.

**RESULTS**

**Chronic exposure to estradiol-17β (E2) causes hypertension.** The daily average profiles and the average mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) during the 10–11th wk of treatment in control and E2-treated rats are shown in Fig. 1. As seen in Fig. 1A, the MAP in control animals remained steady over the entire period of observation. In contrast, E2 treatment increased MAP significantly (P < 0.01; Fig. 1A). The average MAP (means ± SE) measured during the 10–11th wk of observation in control rats was 105.1 ± 0.7 mmHg. In contrast, E2 exposure increased MAP significantly to 119.6 ± 0.8 (P < 0.0001; Fig. 1E). The HR profile of E2-treated rats had a tendency to increase compared with control rats during the entire period of observation (Fig. 1B). The average HR (means ± SE, beats/min; Fig. 1F) during the 10–11th wk of treatment in E2-treated rats (371.7 ± 1.5) was significantly elevated compared with control rats (354.5 ± 1.4; P < 0.0001). Similarly, the SBP and DBP profiles in E2-treated rats were significantly elevated in E2-treated rats compared with control rats (P < 0.0001; Fig. 1, C

![Fig. 2. Effect of chronic E2 exposure on superoxide in the rostral ventrolateral medulla (RVLM). A: schematic coronal brain stem section showing the location of micropunches of RVLM (within circles) taken from Paxinos and Watson rat brain atlas, 6th ed. The section coordinate (−12.24-mm bregma) represents the distance caudal to the bregma. ROB, raphe obscurus nucleus; RS, rubrospinal tract; sp5, spinal trigeminal tract; IRt, intermediate reticular nucleus; Sol, nucleus of the solitary tract. B: bar graph denoting O2− levels measured in RVLM brain stem punches of E2-treated (20 ng/day; 90-day slow-release pellets) and sham-implanted female Sprague-Dawley (SD) rats (n = 4 per group) using a luminometer. *Significant difference (P < 0.05) from control rats.
and D). E2 exposure also increased the average SBP and DBP (means ± SE; 140.0 ± 0.7 and 101.1 ± 0.8 mmHg, respectively) significantly compared with control rats (125.4 ± 0.7 and 88.0 ± 0.7 mmHg, respectively; \( P < 0.0001; \) Fig. 1, G and H).

Chronic exposure to E2 increases \( O_2^- \) levels in RVLM. Chronic E2 exposure significantly elevated \( O_2^- \) levels (means ± SE) in the RVLM of E2-treated rats (0.833 ± 0.1 nmol/min·mg) compared with control rats (0.532 ± 0.04 nmol/min·mg; \( P < 0.01 \)) (Fig. 2).

**Fig. 3.** Effect of chronic E2 exposure and resveratrol on food intake, water intake, body weight, and heart weight. A–C: line graphs showing food intake (g), water intake (ml), and body weight (g) between different groups of female SD rats (\( n = 6 \) per group); open circles (○) shows control, solid circle (●) shows E2 pellet implanted (E-90), open inverted triangle (▽) shows resveratrol (Res) treatment alone, and closed triangle shows resveratrol treatment on E2-implanted rats (Res+E-90). *Significant difference from control (\( P < 0.05 \)). D: bar graphs showing the average values of heart weight (g) and heart-to-body weight ratio in the different treatment groups (\( n = 6 \) per group). E–G: bar graphs showing the average values of food intake, water intake, and body weight shown in A–C, respectively. E: *Significant difference (\( P < 0.05 \)) from control and resveratrol-treated groups. F: *Significant difference (\( P < 0.005 \)) from the rest of the groups; “a” denotes significant difference from control (\( P < 0.05 \)). G: *Significant difference (\( P < 0.05 \)) from control.
Chronic E2 exposure increases food intake, water intake and body weight. The average weekly food intake (g/wk; means ± SE), water intake (ml/wk; means ± SE), and body weight (g; means ± SE) of control, E2, resveratrol, resveratrol+E2 animals is shown in Fig. 3. There was no difference in food intake between control and E2-treated groups at the beginning of the experiment. However, food intake in the E2-treated group appeared to increase on the 2nd wk (141.7 ± 4.7 g/wk), the 5th wk (132.1 ± 5.3 g/wk), and the 7th wk (127.6 ± 3.8 g/wk) compared with control rats (123.8 ± 2.8 g/wk, 117.7 ± 2.3 g/wk, and 117.6 ± 2.5 g/wk, respectively; P < 0.05). The average food intake was significantly higher in E2-treated rats (134.6 ± 1.7 g/wk) compared with control rats (122 ± 2.3 g/wk) and those treated with resveratrol alone (123.1 ± 1.3 g/wk; P < 0.05; Fig. 3E). Feeding chow containing resveratrol did not affect food intake in the control and E2-treated groups.

The average weekly water intake in control and experimental animals is given in Fig. 3B. There was a tendency for increased water intake in E2-treated rats compared with control rats in the 1st wk, and this trend continued throughout the period of observation, though the change was not statistically significant. However, the average water intake (Fig. 3F) was significantly higher in the E2-treated group (316.5 ± 5.6 ml/wk) compared with control rats (288.2 ± 5.5 ml/wk), sham-implanted resveratrol-treated group (279.6 ± 2.9 ml/wk) and the resveratrol and E2-treated group (257.5 ± 5.8 ml/wk; P < 0.005). The E2-treated group fed chow containing resveratrol consumed less water (257.5 ± 5.8 ml/wk) compared with the control group (288.2 ± 5.5 ml/wk; P < 0.05).

Changes in body weight among the different groups are shown in Fig. 3C. There was no difference in body weight between the treatment groups at the beginning of the experiment. Although there was a tendency for body weight to increase with time, the average weekly body weight in the E2 group was not statistically different from that of control rats. Feeding control and E2 rats with chow containing resveratrol for 2 wk did not produce any change in body weight. The average body weight over the entire period of observation (Fig. 3G) was significantly higher in the E2 group (297.4 ± 2.7 g) compared with control rats (287.3 ± 1.6 g; P < 0.005). Feeding resveratrol did not alter the average body weight in control and E2-treated rats. Body weight in E2-treated rats fed chow containing resveratrol (299.6 ± 0.4 g) was significantly higher compared with control (P < 0.05).

There were no significant changes in heart weight or the ratio of heart weight to body weight in any of the treated groups compared with the control group (Fig. 3D).

Serum E2. Serum E2 levels (means ± SE) at the end of 90 days of E2 exposure and resveratrol treatment are shown in Fig. 4. Exposure to E2 resulted in significant increases in serum E2 of animals that are treated with E2 alone (68.2 ± 4.07 pg/ml) or treated with E2 and resveratrol (59.4 ± 1.5 pg/ml) compared with control rats (46.7 ± 2.9 pg/ml) or rats treated with resveratrol alone (47.4 ± 4.4 pg/ml, P < 0.0005). There was no difference in E2 levels between the E2-treated groups (with or without resveratrol).

Resveratrol reverses chronic E2-induced hypertension. The daily average profiles and the average MAP, SBP, DBP, and HR during the entire period of observation in control, E2, resveratrol and resveratrol + E2-treated rats are shown in Fig. 5. Similar to what was observed in Fig. 1, exposure to E2 for 90 days significantly elevated MAP, HR, SBP, and DBP compared with control rats. Feeding chow containing resveratrol to sham-implanted control rats did not alter MAP, HR, SBP, and DBP. In contrast, feeding chow containing resveratrol to E-90 rats completely reversed E2-induced increase in MAP, HR, SBP, and DBP in E2-implanted rats (P < 0.01).

Resveratrol attenuates oxidative stress in RVLM. Changes in superoxide levels (means ± SE, nmol/min·mg) in the RVLM of control, E2, resveratrol and resveratrol+E2 groups are shown in Fig. 6. As seen in Fig. 2, superoxide levels increased significantly in E-90 rats (0.608 ± 0.052 nmol/min·mg) compared with control rats (0.454 ± 0.007 nmol/min·mg; P < 0.005). When sham-implanted control rats were fed chow containing resveratrol alone, it did not alter superoxide levels (0.431 ± 0.007 nmol/min·mg) compared with control rats (0.454 ± 0.007 nmol/min·mg). In contrast, feeding E2-treated rats with chow containing resveratrol completely reversed E2-induced increase in superoxide levels in the RVLM (0.431 ± 0.007 vs. 0.608 ± 0.052 nmol/min·mg; P < 0.0005).

**DISCUSSION**

The results from the present study provide evidence that chronic exposure to low levels of E2 for 3 mo increases superoxide levels in RVLM and results in hypertension. Treatment with an antioxidant, resveratrol, reversed E2-induced increases in superoxide levels in the RVLM and reversed the increase in BP, indicating that chronic exposure to low levels of E2 is capable of causing hypertension, possibly by increasing superoxide generation in the RVLM.

Estrogen was originally believed to prevent hypertension based on the observation that postmenopausal women have a higher incidence of hypertension compared with age-matched men (17, 31, 40), and, therefore, HRT containing estrogenic preparations were believed to reduce BP in these women (48). Several possible mechanisms by which estrogens could decrease BP have been suggested. There is evidence that estrogen increases ACh-induced, nitric oxide-mediated relaxation of aorta in male spontaneously hypertensive rats (23) through upregulation of endothelial nitric oxide synthase (eNOS) (20). Also, estrogen is believed to act on some brain stem autonomic centers to decrease sympathetic nervous activity (54, 55), thereby causing reduced vasomotor tone and lowered BP.

Contrary to the belief that estrogens can lower BP, there is a large body of evidence, indicating that repeated exposure to...
low levels of estrogens, as in the case of oral contraceptives, can cause an increase in BP. Numerous human clinical studies have associated chronic use of contraceptive pills with increase in BP (12, 29, 56). Similar results have been seen in animal studies, where exposure to a combination of 1\u03bcg of ethinyl estradiol and 10\u03bcg of norgestrel or ethinylestradiol alone for 10 wk resulted in concomitant increases in systolic and mean arterial BP (7, 36, 38). In another study, female SD rats injected with 0.2\u03bcg of ethinyl estradiol exhibited significant increases in systolic BP of 17 mmHg in 6 wk and 32 mmHg in 12 wk (7). Different mechanisms have been proposed for oral contraceptive-induced hypertension. These include impaired renal handling of water resulting in volume-dependent hypertension (37) and hyperactivity of the renin-angiotensin system.
chose to focus specifically on the RVLM because it is known to be involved in the maintenance of basal vasomotor tone via regulation of sympathetic activity (45). Moreover, brain stem neurons in the RVLM contain estrogen receptor mRNA (1, 27, 49), indicating that this is a potential site of E₂ action. Also, stimulation of the RVLM is known to activate sympathetic preganglionic neurons in the intermediolateral cell column of the spinal cord and increase sympathetic activity resulting in hypertension (14). An E₂-induced increase in O₂⁻ levels in the RVLM observed in the present study may act through similar mechanisms. Other studies have shown an association between O₂⁻ in the RVLM and hypertension in multiple animal models (9, 19, 25, 39). Moreover, reversal of hypertension by increasing the expression of superoxide dismutase using gene transfer and adenoviral vectors or decreasing O₂⁻ in the RVLM substantiates the role of oxidative stress in the pathogenesis of hypertension (10, 11, 13, 15). Because our results clearly indicated that E₂ treatment increases O₂⁻ production in the RVLM, we wanted to explore the possibility of reversing this effect using the antioxidant, resveratrol.

Resveratrol is a red wine polyphenol, which is known to possess antioxidant and O₂⁻-scavenging properties (53) and exerts strong antioxidant effects in the brain (33). In the current study, treatment with resveratrol reduced MAP, HR, SBP, and DBP induced by E₂ treatment with complete quenching of O₂⁻ in the RVLM. Resveratrol has been shown to reduce SBP in obese Zucker rats by increasing eNOS expression in the aorta (44). Resveratrol treatment also prevented hypertension caused by a high-fat diet in female rats (2). In the present study, treatment of E₂-treated rats with resveratrol reversed E₂-induced increase in O₂⁻ in the RVLM and also decreased BP, raising the possibility that E₂’s effects on BP could be mediated through increased production of O₂⁻.

In conclusion, our results support the hypothesis that chronic exposure to low levels of E₂ causes hypertension in young SD female rats. This hypertensive effect of E₂ may be related to increased O₂⁻ production in the RVLM. Although, this study examines the effect of E₂ on O₂⁻ generation in the RVLM, the involvement of other brain sites and peripheral tissues, such as the vasculature and kidney, should also be considered. Collectively, this study provides valuable insights into the mechanisms by which chronic low-dose exposure to E₂ causes hypertension and draws attention to potential cardiovascular risks faced by women on long-term oral contraceptives.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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