17β-Estradiol prevents oxidative stress and decreases blood pressure in ovariectomized rats

ISABEL HERNÁNDEZ, JUAN L. DELGADO, JULIAN DÍAZ, TOMAS QUESADA, M. JOSÉ G. TERUEL, M. CARMEN LLANOS, AND LUIS F. CARBONELL. 17β-Estradiol prevents oxidative stress and decreases blood pressure in ovariectomized rats. Am J Physiol Regulatory Integrative Comp Physiol 279: R1599–R1605, 2000.—In this study, we tested whether estrogen deficiency is associated with oxidative stress and decreased nitric oxide (NO) production, which could be responsible for an increased blood pressure in ovariectomized rats. Hemodynamic studies were performed on conscious, chronically instrumented rats. Chronic estrogen replacement on ovariectomized rats lowered blood pressure ~13 mmHg, from 119 ± 3 mmHg in ovariectomized rats to 106 ± 3 mmHg in ovariectomized-treated rats; it was also accompanied by an increase in cardiac index and vascular conductance, achieving hemodynamic values similar to those shown by sham-operated rats. N^G-nitro-L-arginine methyl ester administration lowered significantly less the vascular conductance (0.14 ± 0.01 vs. 0.22 ± 0.03 and 0.26 ± 0.01 ml/min ±1/mmHg ±1/100 g; P < 0.05) in ovariectomized rats than in the sham-operated and estrogen-treated ovariectomized rats, respectively. Estrogen replacement prevented the lower plasma levels of nitrates/nitrates observed in ovariectomized rats. The lower plasma total antioxidant status and reduced thiol groups and the increase in plasma lipoperoxides presented in ovariectomized animals were reestablished with the estrogen treatment. These results show that estrogen administration decreases blood pressure and increases vascular conductance in ovariectomized rats. This effect may be related to an increase in NO synthesis and/or preventing oxidative stress, then improving endothelial function.

Address for reprint requests and other correspondence: I. H. García, Departamento de Fisiología y Farmacología, Facultad de Medicina, Universidad de Murcia, Campus de Espinardo, 30100 Murcia, Spain (E-mail: isabelhg@um.es).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
supplementation status was determined by dividing all the ovariectomized groups into treated with pellets of estrogen and not treated animals.

METHODS

Experiments were performed on female virgin and ovariectomized Sprague-Dawley rats (250–400 g). At 12 wk of age, animals were anesthetized with Talamonal (mix of 16 mg/kg Fentanyl and 0.83 mg/kg Droperidol), and a small abdominal incision was made with the use of a sterile technique. Ovariectomy was performed in 28 rats, and 13 rats were exposed to sham operation. After convalescence (4 days), 13 ovariectomized rats were given a subcutaneous implant of pellets (Innovative Research of America, Toledo, OH) containing 17β-estradiol (1.5 mg/8 wk). The animals were cared for according to the principles established in the Guide for the Care and Use of Laboratory Animals.

Hemodynamic measurement. While rats were under anesthesia, catheters were placed into the left femoral artery and vein for measurement of mean arterial pressure (MAP) and heart rate (HR) and into the left femoral vein for infusion. A right atrial catheter and a thoracic aortic thermocouple were implanted via the right external jugular vein and right carotid artery, respectively. The catheters were brought out through the skin on the dorsal side of the neck. Finally, the distal ends of these lines were threaded through a lightweight flexible spring connected to a swivel. All surgical procedures were performed under aseptic techniques. Rats were placed in plastic cages with the swivels mounted above, allowing complete freedom of movement and free access to chow and tap water. Three full days were permitted for recovery from surgery. Cardiac output was measured by thermodilution as previously described in our laboratory (10). The thermodilution curve and the pressure signal were processed with a microcomputer system (Cardiomax IIR, Columbus Instruments). Hemodynamic values were the mean of three determinations. Cardiac output was measured by rapid injection of 200 μl 0.9% saline at room temperature (20°C) through the jugular catheter, with the use of a spring-loaded constant-rate, constant-volume syringe (Hamilton, CR 700–200). Cardiac index (CI) was calculated by dividing cardiac output by animal weight (100 g) and total vascular conductance (TVC) by dividing CI by MAP. Baseline hemodynamic parameters and the hemodynamic response to intravenous injections of the NO synthase inhibitor L-nitro-arginine methyl ester (3 mg/kg + 50 μg·kg⁻¹·min⁻¹ during 30 min) were evaluated in the three groups of rats, 8 wk after ovariectomy or sham operation.

Biochemical measurements. In another set of three different groups of rats (sham age-control, ovariocitized, and ovariectomized + estrogen treatment), blood samples were collected (while rats were under anesthesia) from the carotid artery and placed in tubes containing EDTA/K3 as anticoagulant. The samples were centrifuged to 2,500 g during 10 min at 4°C (Beckman Model TJ-6 Centrifuge; Beckman Instruments, Fullerton, CA). The plasma was collected and immediately frozen to −80°C until analysis (within 30 days). Cycles of freezing-unfreezing were avoided. To optimize the stability of the samples and to minimize the processes of lipid peroxidation in vitro, the storage was made in tubes free of trace elements.

Parameters analyzed in the plasma were as follows: the lipoperoxides malonaldehyde and 4-hydroxylkenals as indicators of lipid peroxidation, total antioxidant status (TAS), reduced thiol (−SH) groups, and the stable end products of NO (NOx).

A specific kit (Bioxytech LPO-586 Assay; Oxis International, Portland, OR) was used to measure the plasma levels of malonaldehyde plus (E)-4-hydroxy-2-nonenal by a colorimetric method (5) on the basis of the reaction with 1-methyl-2-phenylindole at 45°C yielding a stable chromophore with maximal absorbance at the 586-nm wavelength. Calibration solutions consisted of 1,1,3,3-tetraethoxypropane and a hydroxynonenal as diethylacetal. Assay range was 0–20 μmol/l. Interassay coefficient of variation was 4.7%. The light wavelength and the low temperature of incubation used for these measurements eliminate interferences and undesirable artifacts (5).

Plasma TAS was measured with the use of a kit supplied by Randox Laboratories (Amtrim, UK). The assay principle is that metmyoglobin reacts with H₂O₂ to form the radical species ferrylmyoglobin. A chromogen ABTS [2,2’-Azinobis(3-ethylbenzthiazoline-sulfonic Acid)] is incubated with the ferrylmyoglobin to produce the radical cation species ABTS⁻. This has a relatively stable blue-green color, which is measured at 600 nm. Antioxidants in the added samples cause suppression of this color production to a degree that is proportional to their concentration (22). The system was standardized with the use of Trolox, a (+)-α-tocopherol analog with enhanced water solubility. The unit of activity is the concentration (mM) of Trolox having the equivalent antioxidant capacity to a 1.0-mM solution of the substance under investigation. Assay range was 0–2.5 mM of Trolox. The interassay coefficient of variation was 5%.

The −SH groups present in plasma samples were determined by the Ellman reaction (4) with DTNB [5,5-Dithiobis(2-nitrobenzoic Acid); Sigma]. −SH groups’ concentration was calculated on the basis of a reduced glutathione (Sigma) standard curve. Assay range was 0–320 μmol/l. Interassay coefficient of variation was 4.9%.

NOx were determined by the Griess method (6); briefly, after protein precipitation of the samples and reduction of nitrate to nitrite with reduced NADPH in the presence of nitrate reductase (Boehringer Mannheim; Germany), nitrite was quantified colorimetrically at 540 nm (Nitric Oxide Colorimetric Assay; Boehringer Mannheim). Interassay coefficient of variation was 4.8%.

Plasma level of 17β-estradiol was measured by microparticle enzyme immunoassay (IMX estradiol assay, Abbot Laboratories, North Chicago, IL).

Statistical analysis. All values are means ± SE. One-way analysis of variance for randomized measures followed by Duncan’s multiple-range test was used to determine the differences in baseline hemodynamic and the effects of N²-nitro-L-arginine methyl ester and also in the biochemical determination among estrogen, sham-operation, and ovariectomized groups. Differences were considered significant at P < 0.05.

RESULTS

Baseline systemic hemodynamic. Mean values for systemic hemodynamic in sham age-control (n = 6), ovariectomized (n = 8), and ovariectomized with estrogen replacement (n = 6) groups are shown in Fig. 1. MAP was 13 mmHg lower in ovariocitized rats with estrogen treatment compared with that of nontreated ovariectomized rats (−13 mmHg); from 119 ± 3 in ovariectomized rats to 106 ± 3 mmHg in ovariectomized-treated rats (P < 0.05). This lower blood pressure was accompanied by a 66% higher (P < 0.05) vascular conductance and a 52% higher (P < 0.05) CI.
compared with that of nontreated ovariectomized rats. In ovariectomized rats, MAP was 119 ± 6 vs. 109 ± 5 mmHg in sham-control rats (P < 0.05). This higher blood pressure was accompanied by a lower level of systemic vascular conductance (0.28 ± 0.02 vs. 0.40 ± 0.04 ml-min⁻¹-mmHg⁻¹/100 g) and lower CI compared with that of the age-control group (33.52 ± 1.54 vs. 42.41 ± 3.10 ml-min⁻¹·100 g⁻¹). As presented in Fig. 1, ovariectomized animals with estrogen treatment show hemodynamic values similar to sham-operated rats. There was no difference in the baseline HR among the three groups of rats. Body weight after 8 wk of sham, ovariectomy, and ovariectomy plus estrogen replacement was 283 ± 13, 391 ± 16, and 280 ± 7 g, respectively.

**Hemodynamic response to NO synthase inhibition.** Administration of N⁵-nitro-L-arginine methyl ester to sham-operated and ovariectomized rats elicited a rise in MAP to an average of 145 ± 6 and 153 ± 11 mmHg, respectively, 30 min after its injection. Estrogen-replacement therapy did not modify the pressor response produced by inhibition of NO synthase in ovariectomized rats (Fig. 2).

The pressor response to N⁵-nitro-L-arginine methyl ester was accompanied by a decrease in HR, CI, and vascular conductance in all groups (Fig. 2). The decrease in HR was more pronounced in the ovariectomized group compared with that of the sham-operated animals. Although the fall in CI (−12.3 ± 1.5 ml·min⁻¹·100 g⁻¹) produced by N⁵-nitro-L-arginine methyl ester in ovariectomized rats was less than that determined in sham-operated rats (−16.6 ± 3.5 ml·min⁻¹·100 g⁻¹), these differences were not statistically significant. The decrease in CI was more pronounced in the ovariectomized estrogen-replaced group compared with that of the ovariectomized rats. Although the increase in MAP produced by N⁵-nitro-L-arginine methyl ester was of similar magnitude in ovariectomized rats, the decrease in vascular conductance (0.14 ± 0.01 ml·min⁻¹·mmHg⁻¹/100 g) was lower than that recorded in sham-operated animals (0.22 ± 0.03 ml·min⁻¹·mmHg⁻¹/100 g, P < 0.05). Furthermore, estrogen-replacement therapy restored the response in vascular conductance produced by inhibition of NO synthase, achieving a higher (0.26 ± 0.1, P < 0.05) decrease in ovariectomized estrogen-replaced rats compared with ovariectomized animals.

**Biochemical measurements.** Compared with the sham-operated (n = 7) group, ovariectomy (n = 7) induced a significant increase in plasma lipoperoxides that was accompanied by a lower TAS and a lower plasma level of −SH groups (Fig. 3). Estrogen-replacement therapy (n = 7) induced a significant increase in plasma TAS and −SH groups that was associated with a decrease in plasma lipoperoxides concentration; these parameters returned to values similar to those of sham-operated animals. Furthermore, the stable end products of NO NOx were significantly lower in ovariectomized animals compared with those of sham-operated rats (Fig. 3). Again, estrogen therapy significantly increased plasma NOx.
increased NOx to a level similar to that observed in sham-operated animals.

Plasma 17β-estradiol concentration was (in pg/ml) 97.4 ± 4.3 in sham-operated, 6.9 ± 0.4 in ovariectomized, and 164.6 ± 4.2 in ovariectomized estrogen-replaced rats.

**DISCUSSION**

This study shows that estrogen plays a significant role in modulating the blood pressure of female rats. Our results showed that treatment with estrogen decreased baseline blood pressure compared with ovariectomized animals. These data agree with accumulating evidence of a link between increased prevalence of hypertension and postmenopausal stages in women (29). Our results are further complemented by recent studies showing that transdermal estrogen exerts beneficial effects, both in lowering elevated blood pressure and in maintaining a uniform blood pressure control over 24 h (22). In addition, Brosnihan et al. (2) showed that both ovariectomized Sprague-Dawley normotensive and transgenic hypertensive rats (mouse Ren-2 gene) treated with estrogen for 4 wk presented significantly lower blood pressure levels than nontreated animals, suggesting a beneficent effect of estrogen on cardiovascular control system. On the other hand, this is one of the first studies that shows an elevation in blood pressure in normotensive rats after ovariectomy. Recently, Milsted et al. (23) have reported an increase in systolic blood pressure in female Wistar-Kyoto rats after 7 wk after ovariectomy. However, other studies have reported no increase in blood pressure after ovariectomy. Nickenig et al. (24) reported no significant differences in blood pressure between ovariectomized or sham-operated Wistar-Kyoto rats. These discrepancies may be explained by the different times after ovariectomy. Nickenig et al. (24) reported that rats were killed 5 wk after ovariectomy, instead 8 wk were allowed after ovariectomy in the present work.

In our study, ovariectomy significantly decreased CI and TVC of female rats, and estrogen replacement prevented this hemodynamic disorder. This result is in agreement with those of Huang et al. (12) reporting that ovariectomy decreased the basal tone of arterioles of female rats, and estrogen replacement restored the myogenic tone of arterioles to a level identical to that of normal female rats. These results suggest that the chronic absence of circulating estrogen may well be responsible for the observed differences in basal TVC in our ovariectomized rats. Our data are further sup-
ported by a recent study in postmenopausal women showing that transdermal estradiol treatment significantly increased cardiac output and stroke volume that were accompanied by a decrease in blood pressure (1).

In the present study, ovariectomy reduced plasma levels of NO metabolites, nitrites and nitrates. Furthermore, as other previous studies in postmenopausal women show (27), 17β-estradiol administration increased the circulating levels of nitrites and nitrates in ovariectomized rats. Our findings are supported by our previous results (4) and of Hayashi et al. (8) where basal release of NO from the aortic rings of ovariectomized rats and rabbits was lower than their respective female control animals. In addition, other studies in vitro showed that endothelial nitric oxide synthase increases in response to 17β-estradiol in human (11), bovine aortic endothelial cells (9), and ovine fetal pulmonary artery endothelial cells (21). Therefore, it was logical to assume that a greater release of NO, due to the presence of estrogen, may be responsible for the difference in basal TVC between ovariectomized and both sham-operated and ovariectomized estrogen-treated rats.

$N^G$-nitro-l-arginine methyl ester administration elicited a similar hemodynamic effect in the ovariectomized group as that previously published by our laboratory (10) in Sprague-Dawley rats. In addition, although there was no difference in the magnitude of the pressor response elicited by $N^G$-nitro-l-arginine methyl ester among the three groups, the decrease in vascular conductance was significantly greater in normal female and ovariectomized estrogen-treated rats than in ovariectomized rats. These data suggest that the significant reduction in basal vascular conductance that was found in ovariectomized rats may reflect a minor dependence of vascular conductance on NO in this group.

Then, we speculate that the physiological importance of our findings is that the greater release of NO in the vasculature of normal and ovariectomized rats with estrogen replacement tends to increase peripheral vascular conductance by eliciting a greater suppression of the constrictor mechanisms compared with what occurs in ovariectomized rats. That mechanism may be responsible for the lower blood pressure present in animals with estrogen in the current study. Indeed, previous studies showed that estrogen augments the contribution of NO to regulate blood pressure in transgenic hypertensive rats expressing the mouse $Ren-2$ gene (2) and that estrogen attenuates the development of hypertension in spontaneously hypertensive rats (35). Even though changes in blood pressure in response to $N^G$-nitro-l-arginine methyl ester infusion were not different among the three groups of rats, a higher HR response was seen in the ovariectomized rats. This contradictory blood pressure and HR response to $N^G$-nitro-l-arginine methyl ester is difficult to explain. However, a possible explanation may be a difference in baroreflex sensitivity among ovariectomized rats and the other two groups that have estrogen. Recently, it has been reported that lower tolerance to lower body negative pressure in females was associated with reduced HR response to carotid baroreceptor stimulation compared with men (3).
In the present study, we also show that ovariectomy induces alterations in the redox state, characterized by decreased plasma TAS and −SH groups and an increase in plasma lipoperoxides. These plasma data may reflect an unbalance in the redox state toward oxidative process that may be associated with a decrease in NO activity. On the other hand, several studies report that bioactivity of NO is closely related to S-nitrosothiols compounds that appear to be more potent smooth muscle relaxants than NO itself (13, 30). Various vascular related disorders, including coronary artery disease, stroke, hypertension, and shock, are thought to be mediated at least in part through changes in the general redox state and thiol content of vascular tissue. Indeed, there is evidence for an oxidative stress in young essential hypertensive patients and spontaneously hypertensive rats (25, 32). Furthermore, hypertension has been associated with an endothelial dysfunction (19, 20). In addition, N-acetyl-L-cysteine increases the antihypertensive response to captopril and enalaprilat in SHR by a NO-dependent mechanism (28), suggesting that an equilibrium exists between NO and S-nitrosothiol in a biological system that may be influenced by redox state. Furthermore, our results showing an alteration of redox state and decrease in NOx levels are further supported by previous studies from our laboratory. These studies showed that N-acetyl-L-cysteine, a thiol-containing compound, reverted vascular endothelium dysfunction present in aortic rings from ovariectomized rats (4). These results suggest that an alteration in endogenous free radical production under a condition of oxidative stress in ovariectomized rats may play an important role in modulating spontaneous and agonist-stimulated NO production and thus in altering vascular function. In addition, in the present study, estrogen-replacement therapy induced a significant increase in plasma TAS and −SH groups that was associated with a decrease in plasma lipoperoxides concentration; these parameters returned to values similar to those obtained from sham-operated animals. Furthermore, ovariectomized rats treated with estrogen presented higher plasma levels of NOx and a bigger response in vascular conductance to NO inhibition. To explain these results, a possibility is represented by the finding that estrogen can preserve endothelial function by acting as antioxidant. Several studies have shown that 17β-estradiol reduces lipid peroxidation in plasma and platelet membranes from postmenopausal women (23, 34). Furthermore, Kim et al. (17) reported that estrogen protects against endothelial and myocardial dysfunction resulting from brief ischemia/reperfusion. This effect was associated with increased serum NOx concentration and reduced superoxide anion generation in arterial segments after hypoxia/reperfusion. These results suggest that impairment in endothelium-dependent vasodilation in absence of estrogen could be at least partly related to the production of oxygen-derived free radicals, which inactivate NO. It is also suggested that estrogen protects endothelial dysfunction through its antioxidant effect. However, estrogen has been shown to increase endothelial NO synthase protein and mRNA (21). Therefore, after ovariectomy, NO synthase may be reduced, which is in agreement with the plasma NOx levels reported in the present manuscript. Therefore, the effects of estrogen on blood pressure may be mediated both by altering endothelial NO synthase expression and its antioxidant effects.

In summary, our data indicate that estrogen deprivation induced an alteration in redox state that may be responsible for the decreased release of NO and therefore the decrease in systemic vascular conductance that is reflected by a higher blood pressure. Furthermore, estrogen treatment to ovariectomized rats decreases blood pressure, and this effect may be mediated both by altering endothelial NO synthase expression and its antioxidant effect.

Perspectives

The results of the present study and others support the hypothesis that the availability of −SH groups may be an important modulating factor in the expression of the endothelium-derived NO effect, hence of vascular tone. Thus it may be speculated that situations where oxidant stress may decrease the capability of the antioxidant mechanisms could cause an impairment of action of NO on vascular network beds. Our results show an association of oxidative stress and deficiency of estrogens that is prevented by replacement therapy. The pathophysiological significance of this interaction among estrogens, redox state, and NO could be of interest in preventing the major prevalence in vascular abnormalities conducing hypertension and atherosclerosis present in postmenopause.

This investigation was supported by Dirección General de Investigación Científica y Técnica Grant from el Ministerio de Educación y Ciencia, PB94–1150 and FISS-98/0606.

REFERENCES

8. Hayashi T, Fukuto JM, Ignarro LJ, and Chaudhuri G. Basal release of nitric oxide from aortic rings is greater in female


