In Vivo Evidence for Antioxidant Potential of Estrogen in Microvessels of Female Spontaneously Hypertensive Rats

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Abstract—In studies conducted in vitro, it has been demonstrated that estrogen has an antioxidant potential that may contribute to its protective effects on the cardiovascular system. However, the antioxidant effect of estrogen in vivo has not been demonstrated. To address this issue, in this study the effects of estrogen on oxidative stress were evaluated in microvessels studied in vivo. Oxidative stress was evaluated by using intravital microscopy in mesenteric arterioles from female spontaneously hypertensive rats (SHR) in physiological estrous (OE), ovariectomized (OVX), OVX treated with estradiol (E₂), or estradiol + progesterone (E/P). The mesenteries were superfused with hydroethidine, a reduced and nonfluorescent precursor of ethidium bromide (EB). In the presence of reactive oxygen species, hydroethidine is transformed intracellularly in EB, which binds to DNA and can be detected by its red fluorescence. The percentage of EB-positive nuclei along the arteriolar wall in OVX (28.4 ± 4.3) was significantly increased compared with OE (14.2 ± 3.9; P < 0.05). The OVX overproduction of oxyradicals was attenuated by E₂ (15.7 ± 2.2) and E/P (14.8 ± 0.8). Treatment with the superoxide dismutase mimetic MnTMPyP attenuated by 75% the oxidation of hydroethidine in both OE and OVX. Conversely, mannitol, that decomposes hydroxyl radical, and L-NAME, a nitric oxide synthase inhibitor, had no significant effects on hydroethidine oxidation. No differences on hydrogen peroxide plasma concentration were observed among the groups, suggesting that superoxide anion is the most likely oxyradical involved in the increased oxidative stress observed in OVX. The treatment of mesenteries with diphenyleneiodonium (DPI), an nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase inhibitor, but not with oxypurinol, a xanthine-oxidase inhibitor, produced a significant reduction of oxyradical generation in OVX microvessels and a slight decrease in those from OE. Chronic treatment of female SHR with losartan caused similar decreases in oxyradicals in both OE and OVX, whereas diclofenac and verapamil had no effects. Together these data suggest that estrogen reduces superoxide anion bioavailability in vivo. The antioxidant effect of estrogen, which can contribute to a less pronounced endothelial dysfunction in female SHR, may be dependent on a direct modulatory action of estrogen on NADPH activity.

Key Words: hormones ■ estrogen ■ rats, spontaneously hypertensive ■ endothelium ■ anions ■ microcirculation

Several experimental and clinical studies support the hypothesis that estrogen has protective effects on the cardiovascular system. Epidemiological studies have shown that the incidence of cardiovascular disease is higher in men than in premenopausal women and that it increases in postmenopausal women. Because the onset of menopause is marked by a loss in the endogenous estrogen production, estrogen may play a role in the premenopausal protection. Indeed, clinical trials have shown that postmenopausal women receiving hormonal replacement therapy tend to exhibit a lower incidence of cardiovascular disease than women receiving placebo.

Endothelial dysfunction, and a relative deficiency in nitric oxide (NO), have been associated with several cardiovascular diseases including hypertension. Recent data suggest that direct actions of estrogen on the endothelial cells may contribute to its protective effects. Therefore, an impairment in endothelial function after estrogen withdrawal may be considered a risk factor for cardiovascular disease in postmenopausal women. In fact, it has been reported that estrogen deficiency causes a decrease in endothelium-dependent relaxation in both postmenopausal and ovariectomized women. In a previous study, we demonstrated that ovariectomy aggravates the endothelial dysfunction in spontaneously hypertensive rats (SHR).

The mechanisms whereby estrogen exerts its effects on the endothelium are not completely understood. Several studies have suggested that a modulation in endothelial nitric oxide synthase (eNOS) expression and in NO generation may be responsible for the beneficial effects of estrogen on endothel-
lial function. However, it is currently speculated that in essential hypertension, the endothelial dysfunction is evoked not by a decrease in NO generation, but by a decreased bioavailability of NO. The biological NO activity may be modified by reactive oxygen species (ROS), such as superoxide anion. An increase in superoxide concentration leads to scavenging of NO and to cellular damage associated with endothelial dysfunction. Interestingly, it has been demonstrated in hypertensive rats that the impairment in endothelial function after estrogen withdrawal is not related to a reduction in eNOS expression. The increased oxidative stress in these animals provides a potential explanation for the exacerbation of endothelial dysfunction. In addition, in a previous study performed in microvessels from female SHR, we demonstrated that the impairment in endothelial function after ovariectomy is partially due to an augmented superoxide generation.

Recent in vitro studies have indicated that estrogen has an antioxidant activity. The effects of estrogen on ROS have been shown in many studies conducted in cell culture and in isolated blood vessels, but the biological significance of these in vivo or ex vivo studies remains to be elucidated. Although a few clinical studies have observed inhibition of low-density lipoprotein oxidation after estrogen treatment, there are no studies addressing the antioxidant potential of estrogen in physiological conditions. In this study we have evaluated the effects of physiological concentrations of estrogen on oxidative stress in microvessels studied in vivo.

Methods

All of the procedures used were in accordance with guidelines from the University of Sao Paulo, Brazil. We used 16- to 18-week-old female SHR, which were divided into two groups: physiological estrous (OE) and ovariectomized (OVX). Physiological estrus was determined by microscopic evaluation of vaginal smears. Ovariectomy was performed at 12 weeks of age as previously described. Thirty days after ovariectomy, OVX rats were treated during 15 days with subcutaneously implanted 21-day release pellets containing estradiol (0.05 mg) (OVX+E) or estradiol (0.05 mg) and progesterone (50 mg) (OVX+E/P). Arterial blood pressure (BP) was measured in unanesthetized animals by an indirect tail-cuff method, as described by Dringen et al. Hydrogen peroxide measurement was evaluated in the plasma by using the ferric xylenol orange hydroperoxide assay, as described by Dringen et al.

Interventions

To investigate the role of ROS on hydroethidine oxidation in the mesenteric arteriolar wall, we treated the mesenteries with the superoxide dismutase mimetic, MnTMPyP (10 μmol/L, superoxide anion scavenger), and mannitol (10 μmol/L, which decomposes hydroxyl radical), or L-NAME (10 μmol/L, a NOS inhibitor). To evaluate the source of superoxide generation, both OE and OVX SHR were treated with the following drugs: diclofenac (a cyclooxygenase inhibitor, 1 mg/kg/15 days) or losartan (AT1 receptor antagonist, 15 mg/kg/15 days). Because losartan, at this dose, causes a decrease in BP that may contribute to a decrease in superoxide generation, female SHR were treated with verapamil (10 mg/kg/15 days), which does not interfere with pro-oxidative proteins but has an antihypertensive action. In another series of experiments, the mesenteries from OE and OVX were treated with diphenyleneiodonium (DPI 20 μmol/L, an NADPH-oxidase inhibitor) or with oxypurinol (1 mmol/L, a xanthine-oxidase inhibitor). In all treatments, each drug was superfused separately during the 30-minute stabilization period and maintained throughout the experiment.

Hydrogen Peroxide Measurement

Because catalase (which decomposes hydrogen peroxide) seems to be not liposoluble enough to allow mesenteric treatment, hydrogen peroxide formation was evaluated in the plasma by using the ferric xylenol orange hydroperoxide assay, as described by Dringen et al.

Drugs

The following drugs were used: 17β-estradiol and 17β-estradiol + progesterone 21 day-release pellets (Innovative Research of America); hydroethidine (Polysciences); endothium bromide (Gibco); MnTMPyP (Oxis Research); losartan (Merck, Sharp & Dohme); diclofenac potassium (Geigy); verapamil (Knoll); and chloral hydrate, mannitol, DPI, oxypurinol, L-NAME, ferric ammonium sulfate, xylenol orange, sorbitol, sulfuric acid (Sigma).

Statistical Analysis

The results are shown as mean±SEM. Statistical analysis was performed using the nonparametric test Kruskal-Wallis for multiple comparisons. Values were considered statistically significant when P<0.05.

Results

Blood Pressure Measurement

Mean arterial pressure in OVX SHR was higher (175.9±4.6 mm Hg; n=10; P<0.05) than in OE SHR (155.3±2.3 mm Hg; n=10). Treatment with estradiol reduced BP in OVX SHR (148.9±3.1 mm Hg; n=8). The
association of estradiol + progesterone did not change estradiol effects on BP (146.1 ± 5.5 mm Hg; n = 8). Treatment with losartan and verapamil significantly decreased BP in both OE (losartan: 125.8 ± 2.6; n = 6; verapamil: 122.9 ± 1.4; n = 6) and OVX SHR (losartan: 132.9 ± 3.5; n = 6; verapamil: 121.1 ± 1.6; n = 6). Diclofenac treatment had no effects on BP in OE (162.0 ± 3.3; n = 6) or OVX SHR (174.3 ± 4.5; n = 6).

**Plasma Hormonal Levels**

Hormonal levels in female SHR in physiological estrous were: estrogen (203.2 ± 13.3 pg/mL; n = 8); progesterone (596.3 ± 9.4 pg/mL; n = 8); LH (9.2 ± 1.5 ng/mL; n = 8); and FSH (25.6 ± 1.3 ng/mL; n = 8). A decrease in estrogen (67.3 ± 11.5 pg/mL; n = 8; P < 0.05) and progesterone (275.2 ± 6.0 pg/mL; n = 8; P < 0.05) levels as well as an increase in LH (31.3 ± 3.7 ng/mL; n = 8; P < 0.05), but not in FSH (38.5 ± 3.0 ng/mL; n = 8) levels were observed after the ovariectomy. Treatment with estradiol increased serum estrogen (184.5 ± 10.8 pg/mL; n = 8), but not progesterone (279.5 ± 5.8 pg/mL; n = 8), to levels observed in OE SHR. Estradiol + progesterone treatment increased the serum concentrations of both estrogen (190.4 ± 17.3 pg/mL; n = 8) and progesterone (615.6 ± 8.2 pg/mL; n = 8) in OVX SHR.

The elevated LH levels in OVX decreased after treatment with estradiol (8.2 ± 2.1 ng/mL; n = 8) and estradiol + progesterone (8.6 ± 1.2 ng/mL; n = 8), demonstrating the effectiveness of the hormonal treatments in OVX SHR.

**Intravital Microscopy**

During continuous superfusion with hydroethidine, no significant changes in microvascular diameter were observed (data not shown). As illustrated in Figure 1, EB fluorescence was markedly enhanced in OVX arterioles compared with OE. Figure 2A represents the time course for the relative number of EB-positive nuclei (percent) along the mesenteric arteriolar wall of OE and OVX. Compared with OE, the number of EB-positive nuclei in arterioles from OVX was significantly increased at 15, 30, 45, and 60 minutes after the onset of hydroethidine superfusion. The OVX overproduction of oxyradicals was corrected by estrogen treatment (Figures 1 and 2B). The association of estradiol with progesterone did not change the responses observed with estradiol (Figures 1 and 2C).

The oxidation of hydroethidine along the mesenteric arterioles was significantly attenuated by the superoxide dismutase mimetic MnTMPyP (Figure 3A), but not by the
hydroxyl radical scavenger mannitol (Figure 3B), suggesting that superoxide anion is the most likely ROS involved in the increased oxidative stress observed in OVX. L-NAME superfusion did not have any significant effect in the number of EB-positive nuclei in both OE and OVX arterioles (Figure 3C), demonstrating that there was no correlation between NO generation and hydroethidine oxidation.

The enhanced number of EB-positive nuclei observed in OVX was significantly attenuated by treatment with the NADPH oxidase inhibitor DPI (Figure 4A). DPI also induced a slight and less significant decrease in hydroethidine oxidation in microvessels from OE SHR (Figure 4A). There were no changes after oxypurinol treatment (Figure 4B). Changes in BP do not seem to be associated with superoxide generation, since losartan (Figure 5A), but not verapamil (Figure 5B) treatment attenuated the hydroethidine oxidation. Although an effect of AT1 receptor blockade in superoxide generation was observed, the decrease in EB-positive nuclei after losartan treatment was similar in OVX and OE arterioles (Figure 5A). Diclofenac treatment did not have any effect either in BP (data not shown) or in hydroethidine oxidation (Figure 5C).

Figure 2. Time course of the EB-positive nuclei along mesenteric arterioles of female SHR in physiological estrous (OE, n=6) and after ovariectomy (OVX, n=6) (A); OVX treated with estradiol (OVX+E, n=6) (B); and OVX treated with estradiol + progesterone (OVX+E/P, n=5) (C). Each point represents the mean±SEM. * P<0.05 compared with OE; # P<0.05 compared with OVX.

Figure 3. Time course of the EB-positive nuclei along nontreated mesenteric arterioles of estrous (OE, n=6) and ovariectomized (OVX, n=6) SHR, and in mesenteric arterioles treated with MnTMPyP (10 μmol/L, n=5) (A); mannitol (10 μmol/L, n=6) (B); or L-NAME (10 μmol/L, n=6) (C). Each point represents the mean±SEM. * P<0.05 compared with OE; # P<0.05 compared with OVX.

Figure A, B, C.
Hydrogen Peroxide Measurement
Plasma hydrogen peroxide concentration was not increased in OVX SHR in comparison with that in OE SHR (80.6±14.6 versus 74.2±15.2 nmol/L, respectively). In addition, no differences in plasma hydrogen peroxide were detected after estradiol or estradiol + progesterone treatments (67.6±11.3 versus 71.6±10.5 nmol/L, respectively).

Discussion
This study provides the first in vivo evidence for an antioxidant effect of estrogen in resistance vessels, in conditions similar to the physiological ones. The methodology used is based on the oxidation of hydroethidine, a cell permeable nonfluorescent probe, to the fluorescent ethidium bromide (EB) by ROS. Intracellularly and in the presence of ROS, hydroethidine is rapidly converted to EB, which binds to DNA and can be detected through emission of a red fluorescent light. The extent of nuclei labeled with EB along the arteriolar wall quantitatively estimates the vascular oxidative stress.

Under these circumstances, we can suggest that oxidative stress is significantly enhanced after ovariectomy. To confirm that estrogen accounted for the differences in oxidative stress, OVX SHR received pellets containing estradiol or estradiol + progesterone. After the hormonal treatment, the concentrations of estrogen and progesterone were comparable with that seen in OE SHR, demonstrating that the oxidative stress in microvessels from female SHR is influenced by physiological levels of estrogen. Our data also imply that progesterone does...
not interfere with estrogen’s antioxidant effects. In fact, many in vitro studies have shown that various estrogens, but not progesterone, are important scavengers of oxiradicals generated both in an aqueous and lipophilic environment. Furthermore, even though progesterone itself has been reported to have pro-oxidative features, many studies have demonstrated that when administrated concomitantly with estrogen, progesterone does not modify the antioxidant potential of estrogen.

Hydroethidine has been used as a tool to detect spontaneous oxidative changes in the microcirculation under in vivo conditions. Although hydroethidine oxidation to EB is caused more rapidly by superoxide than by hydrogen peroxide or hydroxyl radical, we cannot exclude a role for the last two reactive species on the increased oxidative stress in OVX SHR. Our data showing that superoxide is the most likely ROS involved in hydroethidine oxidation are in agreement with other studies that used hydroethidine to detect oxidative stress. In addition, these results allow us to suggest that estrogen withdrawal leads to increased generation of superoxide anion. Corroborating our in vivo data, many in vitro studies have demonstrated that estrogen can decrease superoxide concentrations in both cell culture and in isolated vessels. Recently, we have demonstrated that long-term depletion of estrogen aggravates the endothelial dysfunction in isolated microvessels from SHR by enhancing superoxide production.

In contrast, because of their phenolic molecular structure, several estrogens have been described to act preferentially as hydroxyl scavengers. In the present study, although mannitol had no significant effects on hydroethidine oxidation in OE there was a small decrease in the oxidative stress in OVX rats and we cannot rule out an additional effect of estrogen on hydroxyl radical concentration. Phenolic estrogens, such as 17β-estradiol, estrone, or estriol, are able to act as hydroxyl scavengers by virtue of the hydrogen-donating capacity of their phenolic groups. Differences in the molecular structures among the several types of estrogens may explain this contrast. Recent data have shown that catecholestrogens, the estrogen-derived metabolites with an ortho-diphenol structure (eg, 2-hydroxyestradiol, a major metabolite of 17β-estradiol), are more potent antioxidants and reach a greater variety of reactive species than their monophenolic precursor (eg, 17β-estradiol) in a number of different biological models. Because this study was carried out in vivo, whether the phenolic 17β-estradiol or its catecholic metabolite is responsible for the decreased superoxide concentration remains an open question.

Superoxide is known to react rapidly with nitric oxide (NO) in solution, with a constant rate of $6.7 \times 10^9 \text{M}^{-1}\text{s}^{-1}$, which is about three times greater than that of dismutation catalyzed by SOD ($2.0 \times 10^9 \text{M}^{-1}\text{s}^{-1}$), leading to the reciprocal inactivation of both free radicals. Therefore, a decrease in vascular superoxide concentration may be influenced by an enhancement in NO concentration. Because a modulatory role of estrogen on NO generation has been demonstrated, we decided to investigate the role of NO on hydroethidine oxidation. In agreement with previous studies, our data show that endogenous NO does not appear to regulate superoxide concentration, perhaps because the production of superoxide largely exceeds that of NO in the animal model studied herein.

The exact mechanisms through which estrogen has antioxidant effects are not completely elucidated. As previously mentioned, the antioxidant effects of estrogens may be related to its phenolic or catecholic structure implying a direct, nonreceptor-dependent mechanism. However, the scavenger property of estrogen was observed at low micromolar concentrations. Because the physiological plasma concentration of estrogens is in the nanomolar range, a direct scavenger effect might not be the major mechanism involved in the antioxidant effects of estrogens. It has been hypothesized that estrogen modulates the generation of free radicals by interacting with its nuclear receptor and by decreasing oxidative proteins and/or increasing antioxidant enzymes expression. Many studies have established that the antioxidant effect of 17β-estradiol (an estrogen with high affinity for the estrogen receptor) is specific, it is not observed with 17α-estradiol (low affinity for the estrogen receptor despite its phenolic group), and it is reduced in the presence of estrogen-receptor antagonists. In fact, changes in estradiol levels have been associated with altered levels of glutathione peroxidase, catalase, and superoxide dismutase. Recent studies have shown a modulatory effect of estrogen on superoxide generation, via a modulation in AT-1 receptor gene expression. On the other hand, there are no studies evaluating the influence of estrogen on other sources of free radicals, such as NADH/NADPH oxidases and xanthine oxidases in endothelial and vascular smooth muscle cells.

Further studies are necessary to identify the exact mechanisms by which estrogen exerts its antioxidant effects. As a primary effort, we evaluated the effect of estrogen withdrawal on the activity of pro-oxidative enzymes. There are a variety of intracellular sources of free radicals that include, but are not limited to, NADPH oxidase, xanthine oxidase, and cyclooxygenase. Considering that information, hydroethidine oxidation was studied in the presence of NADPH oxidase, xanthine oxidase and cyclooxygenase inhibitors (DPI, oxypurinol, and diclofenac, respectively). The finding that DPI treatment, but not oxypurinol or diclofenac, substantially attenuated the oxidation of hydroethidine in OVX microvessels and caused a slight decrease in vessels from OE, suggests an increased activity of NADPH oxidase during estrogen deficiency.

Several observations suggest that the NADPH oxidase system accounts for the majority of superoxide generation in the vessel wall. An overactivity of this enzyme, associated with an overexpression of p22 phox (a component of NADPH-oxidase), has been linked to the increased superoxide generation in some pathological conditions, such as hypertension. In addition, it has been demonstrated that some steroid hormones, such as glucocorticoids, can decrease superoxide levels in aortic smooth muscle cells by modulating p22 phox gene expression. Further studies are necessary to elucidate whether estrogen can also decrease superoxide generation by modulating p22 phox expression.

The effects of estrogen deprivation on AT1 receptor-induced superoxide generation was evaluated as well. In agreement with previous studies, our data show that Ang II, by interacting with AT1 receptors, can stimulate oxidative stress in SHR. However, it is important to state that a similar degree of decrease in EB-positive nuclei after losartan treat-
ment was found in both OVX and OE arterioles. For this reason, we conclude that even though the AT1 receptor may play a role in superoxide generation in female SHR, this pro-oxidative pathway cannot account for the differences in oxidative stress between OVX and OE. These findings seem to be contradictory to reports showing that estrogen deficiency leads to increased free radical production associated with an enhanced expression of vascular AT1 receptors. How- ever, these differences may be related to the vascular bed, because most of previous data were derived from aorta and not from arterioles. In fact, numerous studies have demonstrated disparity in AT1 receptor number, in protein, and mRNA levels among different vessels from different sizes. It has been stated that an increase in intravascular pressure elicits release of superoxide anion. Considering that estrogen and losartan treatments reduced BP in OVX rats, it is plausible that a decrease in intravascular pressure may be responsible for the decreased oxidative stress observed in these animals. However, because verapamil treatment decreased BP, but did not affect hydroethidine oxidation, we can rule out that changes in BP (at least at the levels observed herein) are mediating the changes in superoxide generation.

In summary, our study shows that estrogen at physiological concentrations can act as an antioxidant in vivo. Although the precise mechanisms by which estrogen exerts its antioxidant effect remains to be elucidated, we have shown that NADPH oxidase inhibition attenuated superoxide production in the absence of estrogen suggests that estrogen has a modulatory role on NADPH oxidase activity.

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