Ovarian Hormones Modulate Endothelin-1 Vascular Reactivity and mRNA Expression in DOCA-Salt Hypertensive Rats

Flavia L. David, Maria Helena C. Carvalho, Ana L.N. Cobra, Dorothy Nigro, Zuleica B. Fortes, Nancy A. Rebouças, Rita C.A. Tostes

Abstract—We previously demonstrated a differential activation of the endothelin-1 (ET-1) pathway in male and female deoxycorticosterone (DOCA)-salt hypertensive rats, with the male rats exhibiting marked alterations in vascular and pressor responses to ET-1 and Suc-[Glu,9-Ala11,15]-ET-1(8-21) (IRL-1620), an ETB agonist. Mechanisms underlying these gender differences are unclear, and we hypothesized that the ovarian hormones attenuate vascular ETB responses in female DOCA-salt rats. Female Wistar rats were randomized in 3 groups: sham-operated, ovariectomized (OVX), and OVX plus hormone replacement with estradiol (E) or estradiol/progesterone (EP). Two weeks later, rats were uninephrectomized and further randomized in DOCA-salt (subcutaneous injections of deoxycorticosterone and drinking water containing NaCl/KCl) and control normotensive (subcutaneous injections of vehicle and tap water). Blood pressure was evaluated both by direct and standard tail-cuff methods. Responses to IRL-1620 were evaluated in vivo/in situ in the mesenteric microcirculation. mRNA expression of ET-1 and ETA/B receptors was evaluated in mesenteric arteries by reverse transcription–polymerase chain reaction and expressed relative to GAPDH. OVX-DOCA rats developed a more severe form of hypertension than did DOCA rats. Treatment with E or EP restored blood pressure to levels observed in DOCA rats. In the mesentery, IRL-1620 induced vasodilatation in control rats, a mild vasoconstriction in DOCA rats, and marked vasoconstriction in OVX-DOCA rats. Both E and EP decreased IRL-1620–induced vasoconstriction in the DOCA group. In the normotensive group, OVX did not change blood pressure or IRL-1620–induced vasodilation. Removal of the ovaries increased ET-1 mRNA in arteries from DOCA and control rats, although treatment with E or EP reversed these changes. Vascular ETB receptor mRNA levels were greatly enhanced in OVX-DOCA but not OVX-control rats. Hormone replacement with E or EP restored ETB receptor expression in the DOCA group. A greater blood pressure–lowering effect of bosentan (ETA/ETB blocker) was observed in OVX-DOCA rats. The observation that OVX worsens hypertension as well as the altered ETB receptor–mediated responses and the effects of bosentan in female DOCA rats supports our suggestion that the ovarian hormones modulate ET-1/ETB receptor vascular responses/expression in DOCA-salt hypertension. (Hypertension. 2001;38[part 2]:692-696.)

Key Words: deoxycorticosterone ■ vasoconstriction ■ endothelin ■ estrogen ■ receptors, endothelin

In deoxycorticosterone (DOCA)-salt hypertension and other experimental models of hypertension, male rats develop an earlier and more severe form of hypertension than do female rats.1–3 We have recently suggested that differential activation of endothelin-1 (ET-1) pathways, expressed by a functional upregulation of ETB receptors, may play a role in the higher blood pressure (BP) levels observed in male DOCA-salt hypertensive rats.3–4 We observed that mesenteric arterioles from male but not female DOCA-salt rats display increased ET1– and ETB–mediated responses and also that male DOCA-salt animals exhibit a marked and greater BP-lowering effect in response to bosentan, a mixed ETA/ETB antagonist, compared with that of female DOCA rats.3–4 Mechanisms underlying the attenuated alterations in the vascular ET-1 pathway in female DOCA-salt rats are unclear but may be related to the cardioprotective effects of ovarian hormones. Increasing evidence suggests that ovarian hormones suppress or modulate ET-1 production and its effects: (1) 17β-estradiol attenuates ET-1–induced coronary artery constriction both in vitro5 and in vivo6; (2) plasma ET-1 levels fluctuate during the menstrual cycle7 and are higher in men than in age-matched women8; (3) ET-1 decreases during estrogen replacement therapy, during pregnancy (when estrogen levels are high), and during estrogen administration in transsexual male patients9,10; and (4) in the absence of ovarian hormones, ET-1 mRNA increases, as does the peptide.10,11

Received March 28, 2001; first decision April 26, 2001; revision accepted June 27, 2001.
From the Departments of Pharmacology (F.L.D., M.H.C.C., A.L.N.C., D.N., Z.B.F., R.C.A.T.) and Physiology (N.A.R.), Institute of Biomedical Science, University of Sao Paulo, Sao Paulo, SP, Brazil.
Correspondence to Rita C.A. Tostes, University of Sao Paulo, Institute of Biomedical Science, Pharmacology Dept, Av. Lineu Prestes, 1524 Sao Paulo, SP 05508-900 Brazil. E-mail rtostes@usp.br
© 2001 American Heart Association, Inc.
Hypertension is available at http://www.hypertensionaha.org

692
Therefore, the aim of the present study was to investigate whether the ovarian hormones modulate ET-1 vascular reactivity and expression in female DOCA-salt rats. We evaluated (1) responses to the ET₃ agonist Suc-[Glu,Ala₁¹,₁₅]-ET-1(8-21) (IRL-1620) in vivo/in situ in the mesenteric microcirculation, (2) the vascular mRNA expression of ET-1 and ET receptors, and (3) the BP-lowering effect of bosentan in female normotensive and DOCA-salt rats that were sham-operated, were ovarioectomized, or received hormone replacement therapy following the ovarioectomy.

Methods

Animals

Female Wistar rats from the Institute of Biomedical Science’s animal facility were used in this study. All animals were fed standard laboratory rat chow, had ad libitum access to both food and water, and were housed individually in a room with a constant temperature (24°C) and a 12-hour/12-hour light/dark cycle. Experimental protocols followed standards and policies of the University of Sao Paulo’s Animal Care and Use Committee.

Surgical Procedures

At 6 weeks of age, rats underwent ovarioectomy under chloral hydrate anesthesia (300 mg/kg SC). Briefly, under aseptic conditions, a small incision was performed in the lower abdomen, and both ovaries were removed (OVX rats) or touched with the surgical instruments (sham). On the same day, some of these rats received pellets containing 17β-estradiol (E; 0.1 mg/pellet; 60-day release; Innovative Research of America) or E plus progesterone (EP; 0.1 mg and 100 mg/pellet, respectively; 60-day release). Two weeks after surgery, these animals were randomized into 6 groups: (1) normotensive (control); (2) OVX normotensive (OVX-control); (3) OVX-DOCA-salt rats (DOCA); (4) OVX-DOCA-salt rats that received E or EP pellets (E-DOCA or EP-DOCA); (5) OVX DOCA-salt (DOCA); and (6) OVX-DOCA-salt rats that received E or EP pellets (E-DOCA or EP-DOCA). DOCA-salt treatment, measurements of BP, and vascular reactivity, as well as assessment of hormone levels and effectiveness of ovarioectomy and hormone replacement therapy, were performed as we previously reported.²,¹²

Vascular mRNA Expression of ET-1 and ET₃/ET₆ Receptors

Total cellular RNA was isolated from aorta/mesenteric arteries using TRIzol reagent (GIBCO BRL, Life Technologies). After DNA digestion, total RNA (20 ng) was used for reverse transcription (RT) in the presence of a RNAase inhibitor, 200 U Moloney murine leukemia virus reverse transcriptase, and 1 μg oligo(dT)₁₂ to 18 primer at 37°C for 60 minutes. The cDNA products were isolated by phenol-chloroform extraction, precipitated by ethanol, and resuspended in 120 μL TE (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.5). PCR primers were as follows: ET-1, antisense primer CTCGCTCTATGGAATGCTGG and sense primer GCTCTCCTCTTGAT, which should amplify a 471-bp fragment; ET₃, antisense primer CTGGTGGTCTGCGCCCTTGTGA and sense primer GAAGTCGTCCGTGGGCATCA (216-bp fragment); ET₆, antisense primer CACGATGAGAAGAATGAG and sense primer TTACAGACAGCCAAAGACT (565 bp); and GAPDH, antisense primer CACACCCCTGTGCTGTA and sense primer TATGGATCAAAAGGTG (219 bp). GAPDH was used as an internal control for the amplification. The following conditions were selected for polymerase chain reaction (PCR) in a volume of 50 μL: RT products from 20 ng of RNA, 2.5 U Taq polymerase, 28 cycles of amplification for ET-1, 25 cycles for ET₃/ET₆ receptors, and 20 cycles for GAPDH. After an initial denaturing cycle at 94°C for 5 minutes, the subsequent cycles were as follows: denaturation, 30 seconds at 94°C; annealing, 30 seconds at 55°C (ET-1, ET₃); and 60°C (ET₆, GAPDH); and extension, 45 seconds at 72°C. PCR products (10 μL per lane) were electrophoresed by 1% agarose gel containing ethidium bromide 0.5 μg/mL. The band intensities were measured using a software (Kodak Digital Science, Eastman Kodak Co), and the signals were expressed relatively to the intensity of the GAPDH amplicon in each coamplified sample.

Data Analysis and Statistical Evaluation

Values are expressed as mean±SEM. Statistical evaluation of the data was performed by 2-way ANOVA followed by pair-wise multiple comparison procedures (Tukey Test) (SigmaStat, version 2.0, Jandel Scientific Software). P<0.05 was considered statistically significant.

Results

Ovariectomy accelerated the development and severity of hypertension in DOCA-salt rats (P<0.05). Hormone replacement, both with E or EP, restored BP levels in OVX-DOCA rats to values seen in DOCA rats (P<0.05). No differences were observed between the control, OVX-control and E- or EP-control groups. Systolic BP values are shown in the Table. Ovariectomized rats, both control and DOCA-salt rats, had significantly higher body weights than that of sham-operated rats, and the weight gain was reduced in the groups.
that received hormone treatment ($P<0.05$). To control for the efficacy of ovariectomy and hormone replacement, both the uterine weight and hormone plasma levels were evaluated. As can be observed in the Table, uterine body weight and plasma levels of E and EP were significantly reduced in the OVX groups and were restored with the hormone replacement therapy ($P<0.05$). Because responses in E- and EP-treated rats were similar at all protocols, only responses in the E-groups will be shown.

IRL-1620 (10$^{-9}$ to 10$^{-7}$ mol/L) induced vasodilation in control (initial diameter, 19.3±1.5 μm; after 10$^{-9}$ mol/L IRL-1620, 20.7±1.6 μm) and OVX-control (initial diameter, 18.9±1.3 μm; after 10$^{-9}$ mol/L IRL-1620, 20.1±1.8 μm) rats. Treatment with E or EP did not modify IRL-1620–induced responses (initial diameter, 20.6±0.8 μm; after 10$^{-9}$ mol/L IRL-1620, 21.9±1.2 μm) in the normotensive group. A mild vasoconstrictor response occurred in DOCA rats (initial diameter, 18.9±2.1 μm; after 10$^{-9}$ mol/L IRL-1620, 18.2±1.6 μm), but a greater and marked vasoconstriction was observed in the OVX-DOCA rats on IRL-1620 stimulation (initial diameter, 18.8±1.8 μm; after 10$^{-9}$ mol/L IRL-1620, 17.2±1.5 μm; $P<0.05$) (Figure 1). Treatment with E or EP significantly attenuated IRL-1620–induced vasoconstriction in the hypertensive rats (initial diameter, 20.5±0.9 μm; after 10$^{-9}$ mol/L IRL-1620, 19.9±1.1 μm; $P<0.05$). Figure 2 demonstrates results obtained from RT-PCR showing mRNA expression of ET-1 and ET$_B$ and ET$_A$ receptor genes in mesenteric arteries from sham, OVX, and E-treated control and DOCA-salt rats. Similar results were observed in aortas (not shown). DOCA-salt treatment slightly but significantly increased ET-1 expression in female rats ($P<0.05$). The OVX increased ET-1 mRNA expression in control and DOCA rats ($P<0.05$), whereas treatment with E or EP reversed these changes ($P<0.05$). A trend to increased ET$_B$ receptor mRNA expression was observed in the OVX-control group, but only reached statistical significance in the DOCA-OVX group ($P<0.05$). Treatment with E or EP normalized ET$_B$ receptor mRNA expression ($P<0.05$). No alterations were observed in ET$_A$ receptor mRNA expression. To further investigate alterations in the ET-1 pathway, we assessed effects of the mixed ET$_B$/ET$_A$ antagonist bosentan on BP (Figure 3). Mean arterial BP was higher in DOCA-salt rats compared with control rats ($P<0.05$). Bosentan (10 mg/kg IV) slightly but significantly decreased BP in the control groups. The antagonist lowered BP in DOCA-salt hypertensive rats, but the decrease on BP was significantly greater in the OVX-DOCA group. In the E-DOCA group, the effects of bosentan were similar to those observed in the DOCA group.

**Discussion**

Ovarian hormones are believed to possess cardiovascular protective effects, and they seem to play a role in the gender-related differences in the development of hypertension in experimental models.$^{1-2}$ Because ET-1 plays a role in the pathogenesis of DOCA-salt hypertension,$^{13-17}$ it is possible that the attenuated development of hypertension in female DOCA-salt rats may be related to a modulation exerted by the gonadal hormones on ET-1 effects on the cardiovascular system.$^{5-11}$ We have recently reported that in DOCA-salt hypertension, male rats display altered vascular and pressor responses to ET-1 and to the ET$_B$ agonist IRL-1620 and that these alterations are attenuated in female rats.$^{3,4}$ We specu-
lated that the differential and gender-related alterations in ET-1-mediated effects in DOCA-salt hypertension may be important in the higher blood pressure levels observed in male DOCA-salt hypertensive rats. On the basis of these results and considering that ovarian hormones modulate ET-1 responses/production, in the present study we tested the hypothesis that the ovarian hormones influence the attenuated changes in ET-1 actions observed in female DOCA-salt rats.

The major observation in this study is that removal of the ovaries and, consequently, a significant reduction in estradiol and progesterone levels worsens the changes in ET-1-induced effects observed in female DOCA-salt rats: it enhances IRL-1620-stimulated vasoconstriction, it further increases ET-1 and ETB receptor mRNA expression, and it exacerbates the BP-lowering effect of bosentan. Moreover, the implant of extended release pellets containing E or EP, which restored plasma levels of the hormones and uterine weight, reversed these changes. These data further support an important role for estradiol and progesterone in these processes. Changes in ETB vascular reactivity and expression have been observed in vessels from DOCA-salt hypertensive rats by us and others, and both functional and molecular up- or down-regulation of ETA and ETB receptors have been described in arterial hypertension. Additionally, gender differences in human saphenous vein ET-1 receptor density, as well as in the ratio of ETA to ETB receptors, favoring vasodilator effects in women, have also been reported. Mechanisms whereby estradiol and progesterone influence the ET-1 system have not been fully evaluated. Here, there is evidence that estradiol influences vascular responses to ET-1 via receptor-dependent and -independent processes. It could also be speculated that estradiol differentially influences expression of endothelial ETB receptors that induce vasodilation and influences expression of vascular smooth muscle cell ETB receptors that are vasoconstrictory.

We found increased mRNA expression of ETB receptors in DOCA-salt rats, which was associated with increased ETB-mediated vasoconstriction. Although we were unable to differentiate the cellular source of ETB receptors, the fact that IRL-1620 induced vasoconstriction suggests that ETB expression may be primarily on vascular smooth muscle cells. The fact that hormone replacement normalize these responses suggests an estrogenic/progesteronic-sensitive process. However, these aspects still await clarification. There is also the possibility that the changes in vascular responsiveness to ETB stimulation and ET-1/ETB receptor expression are secondary to differences in blood pressure rather than because of lack of the ovarian hormones. This is supported by studies that show that chronic increases in blood flow upregulate ET-1 receptors, including ETB receptors in arterial smooth muscle. However, increased BP itself does not change ET-1 responses/production, because spontaneously hypertensive rats, which exhibit high BP, do not display alterations in the ET-1 pathway. In conclusion, the present study demonstrates that the lack of ovarian hormones increases vascular mRNA expression of ET-1 and ETB receptor subtype as well as vasoconstrictor responses to IRL-1620 and the BP-lowering effects of bosentan, whereas hormone replacement with E or EP restores these responses to a pattern similar to that observed in female DOCA-salt rats.

Acknowledgments

These studies were supported by grants from FAPESP and CNPq. We are most grateful to Dr Martine Clozel (Actelion Ltd, Allschwil, Switzerland) for the donation of bosentan and to M.A. Oliveira for excellent technical expertise.

References

and ET-1 in porcine aortic endothelial cells. *Am J Physiol*. 1997;273:


Ovarian Hormones Modulate Endothelin-1 Vascular Reactivity and mRNA Expression in DOCA-Salt Hypertensive Rats
Flavia L. David, Maria Helena C. Carvalho, Ana L.N. Cobra, Dorothy Nigro, Zuleica B. Fortes, Nancy A. Rebouças and Rita C.A. Tostes

Hypertension. 2001;38:692-696
doi: 10.1161/01.HYP.38.3.692

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/38/3/692