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The role of estradiol in adrenal insufficiency and its interaction with corticosterone on hydromineral balance

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ABSTRACT

Estradiol (E2) plays an important role in controlling the homeostasis of body fluids. Several studies have reported the involvement of the hypothalamic pituitary adrenal axis (HPA) in the homeostatic control of hydromineral balance and the influence of estrogens on the modulation of this system. Nevertheless, until now, the physiological relevance of HPA axis activity on the hydromineral balance in females has not yet been fully elucidated. Therefore, the objective of the present study was to evaluate the effects of E2 (20 μg/animal) pretreatment on neuroendocrine and hydroelectrolyte changes induced by adrenalectomy (ADX) with or without glucocorticoid hormone replacement (corticosterone, CORT; 10 mg/kg) in ovariectomized rats (OVX). The results show that sodium appetite, natriuresis and the elevated plasma angiotensin II (ANG II) concentration induced by ADX were attenuated by E2 pretreatment. Additionally, a reduction of AT1 mRNA expression in the subfornical organ (SFO) and an increase in plasma atrial natriuretic peptide (ANP) concentrations by E2 pretreatment were observed. E2 pretreatment reversed the reduction in water intake induced by ADX in ADX CORT-replaced rats. Moreover, E2 pretreatment attenuated corticotropin releasing factor (CRF) mRNA expression in the paraventricular nucleus (PVN) induced by ADX. In contrast, E2 pretreatment increased CRF mRNA expression in the PVN in ADX CORT-replaced rats. Taken together, these results suggest that E2 has an important role in the modulation of behavioral and neuroendocrine responses involved in the maintenance of body fluid homeostasis in ADX rats with or without glucocorticoid replacement therapy.

Introduction

Appetite for sodium and thirst are important homeostatic behaviors that contribute to the maintenance of body fluid homeostasis. Adrenal insufficiency induced by bilateral adrenalectomy (ADX) is known to alter water and sodium balance due to the inability of the renal to maintain sodium and water balance (Fitzsimons, 1998) in response to an aldosterone deficit (Krause and Sakai, 2007). Sodium depletion and circulating volume reduction induce an increase in renin activation and, consequently, an increase in the circulating levels of angiotensin II (ANG II). ANG II mediates the dipsogenic and natriorexigenic responses elicited by hypovolemic and hyponatremic stimuli (Fitzsimons, 1998), and these ANG II effects have been postulated to be primarily mediated by its type AT1 receptor (Beresford and Fitzsimons, 1992). Additionally, ANG II exerts important renal effects such as antidiuresis and antinatriuresis, which greatly contribute to the hydroelectrolyte balance (Hollenberg, 1984).

Several studies have investigated the involvement of the hypothalamic–pituitary–adrenal (HPA) network in the control of body fluid homeostasis. Experimental evidence shows that thirst and sodium appetite are stimulated by systemic administration of adrenocorticotropic hormone (ACTH; Li and Whitworth, 1992; Denton et al., 1999), whereas other studies indicate that CRF also participates in the regulation of sodium intake, but the mechanisms influenced by CRF in the control of sodium appetite have not been elucidated (Watts, 1992).

Gonadal hormones, especially estrogen, play important roles in the control of body fluid homeostasis. It is well established in the literature that estradiol (E2) regulates thirst and sodium appetite in females, although the precise nature of their control has not been fully elucidated (Covian and Antunes-Rodrigues, 1963; Danielsen and Buggy, 1980; Jonklaas and Buggy, 1984; Fitzsimons, 1998). Additionally, E2 has an important antinatriuretic effect, which induces an increase in renal sodium tubular reabsorption (Brunette and Leclerc, 2001). The neuroendocrine control of the secretion of vasopressin (AVP), oxytocin (OT) and atrial natriuretic peptide (ANP) is also modulated by estrogen. Several studies have shown that E2 has a stimulatory effect on the secretion of OT, ANP and AVP (Forsling et al., 1982; Jankowski et al., 2001; Mecawi et al., 2011; Vilhena-Franco et al., 2011; Yamaguchi et al., 1979). The secretion of AVP and OT occurs in response to osmotic, volemic and non-osmotic stimuli (for review see Antunes-Rodrigues et al. (2004)). In addition to its function in controlling blood pressure, ANP also plays an important role in the control of hydromineral homeostasis, where

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it exerts opposite effects to ANG II (Antunes-Rodrigues et al., 1985, 1986; Quirion et al., 1988).

HPA axis activity is also influenced by gonadal steroids as illustrated by the elevated plasma levels of glucocorticoids observed in females (Critchlow et al., 1963; Kitay, 1961). In parallel with corticosterone (CORT) hypersecretion, estradiol was shown to increase ACTH secretion and mRNA expression of CRH in the PVN (Silva et al., 2010; Viau and Meaney, 1991). However, other studies have shown that E2 decreases or does not change the plasma levels of ACTH and CORT as well as mRNA expression of CRF in the PVN (Ferreira-Silva et al., 2009; Figueiredo et al., 2002; Gerrits et al., 2005). In this context, the physiological relevance of HPA axis activity related to the control of body fluid homeostasis as well as the mechanisms related to reproductive function in females are not yet fully elucidated. Therefore, this study aims to elucidate the influence of E2 and their interaction with corticosterone on behavioral, endocrine and molecular changes involved in the control of body fluid homeostasis under adrenal insufficiency, using the experimental model of adrenalectomy with or without glucocorticoid replacement in ovariectomized rats.

Materials and methods

Animals

Female Wistar rats (~250 g) obtained from the animal facility located on the Campus of Ribeirao Preto, University of Sao Paulo, Brazil, were maintained under controlled temperature (23 ± 1 °C) and were exposed to a daily 12:12 h light–dark cycle (6:00 A.M.:6:00 P.M.) with free access to tap water and pelleted food. All experimental procedures were performed in the morning between 08:00 and 11:00 A.M. This study was conducted according to the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 85-23, revised 1996), and the experimental protocols were approved by the Ethical Committee for Animal Use of the School of Medicine of Ribeirao Preto, University Sao Paulo (protocol # 034/2010).

Surgeries

All surgeries were performed under anesthesia induced by 2,2,2-tribromoethanol (250 mg/kg, 2.5%, ip, Sigma Aldrich, St Louis, MO, USA), followed by prophylactic doses of veterinary pentabiotic (Fort Dodge, Campinas, SP, Brazil).

Ovariectomy and treatment with estradiol

Rats were subjected to bilateral ovariectomies (OVX) and randomly separated into OVX groups treated with vehicle (corn oil, 0.2 mL per rat, subcutaneous) or OVX rats treated with estradiol cypionate (E2; Pfizer, New York, NY, USA) at a dose of 20 μg/animal, subcutaneous (OVX-E2). The administration of vehicle or E2 began 24 h after surgery and was conducted once a day for 14 days between 07:00 and 10:00 A.M. The last administrations of estradiol or vehicle were performed 24 h before euthanasia. The efficiency of the surgical procedure and treatment with E2 was confirmed by body weight gain during the first 7 days and the uterine index at the end of the experiments (after 14 days). On the seventh day of treatment with E2, the rats had a lower weight gain compared to the OVX rats (21.62 ± 1.58 vs. 33.56 ± 2.14 g, $t_{(47)} = 4.45$, $p < 0.001$, $n = 24/25$). At the end of the experiment, after 14 days of treatment with E2, the treatment increased the uterine index of the animals ($F_{7,93} = 737.89$, $df = 1$, $p < 0.001$); however, the uterine index was not affected by adrenalectomy or replacement with corticosterone.

Adrenalectomy and corticosterone replacement

Seven days after treatment with E2 or vehicle, the OVX and OVX-E2 rats were submitted to bilateral adrenalectomies (ADX) or sham operations and randomly separated into groups treated with vehicle (corn oil in 5% ethanol, 0.2 mL per rat, sc, OVX-SHAM, OVX-E2-SHAM, OVX-ADX, OVX-E2-ADX) or corticosterone (B; Sigma, St Louis, MO, USA) at 10 mg/kg, sc (OVX-SHAM-B, OVX-E2-SHAM-B, OVX-ADX-B, OVX-E2-ADX-B). The administration began immediately after ADX surgery and was conducted once a day for 7 days between 5:00 and 6:00 P.M. The last administrations of corticosterone or vehicle were performed on the day before euthanasia. At the end of each study, the success of the surgical procedure was verified by post-mortem inspection to verify the absence of the adrenal glands. The last administrations of vehicle, estradiol and corticosterone were performed 24 h before euthanasia.

Water and sodium intake

Four days after treatment with E2 or vehicle, the animals were placed in individual metabolic cages to appropriately adapt and were provided with two bottles per animal filled with hypertonic saline solution (1.8% NaCl) or tap water, and they were provided with food ad lib. Fluid intake was evaluated daily for 6 days following ADX. The ratio of water to sodium intake was calculated as: (1.8% NaCl intake/water intake + 1.8% NaCl intake) × 100 (Frankmann et al., 1991).

As body weight gain (Fig. 1C) varied significantly in function of treatments (oil vs. estradiol, $F_{7,93} = 8.22$, $df = 1$, $p < 0.01$; sham vs. ADX, $F_{7,93} = 8.77$, $df = 1$, $p < 0.01$; between the three factors, $F_{7,93} = 4.67$, $df = 1$, $p < 0.05$) the measures were analyzed both as absolute and as body weight-adjusted values. The results for absolute and body weight-adjusted measures did not show differences in the pattern of response. Thus only the adjusted results are presented. Therefore, the values are expressed as mL/100 g body weight. In addition, for better visualization the data were presented in the figures as water and sodium intakes accumulated during six days and are expressed as mL/100 g body weight/6 days. Similarly, this procedure was performed with the ratio of water to sodium intake analysis. Table 1 showed the daily values of the analyzed responses.

Determination of urinary sodium

The urine samples were collected on days 1, 3, and 6 after ADX for urinary sodium concentration determined by flame photometry (Flame Photometer, Micronal Model B-262, Sao Paulo, SP, Brazil). The results are expressed as mEq/100 g body weight. In addition, for better visualization the data were presented in the figure as accumulated during three days and are expressed as mL/100 g body weight/3 days. Table 1 showed the daily values of the analyzed responses.

Determination of plasma sodium concentration and hematocrit

Plasma sodium concentration was analyzed using flame photometry (Micronal B-262, Sao Paulo, SP, Brazil). Plasma volume was indirectly inferred from the hematocrit values that were determined using small aliquots of trunk blood collected in capillary tubes and was expressed as the percentage of cells in the blood. To determine these parameters, the animals were decapitated on the fourteenth day of treatment with estradiol or vehicle.

Blood collection, hormonal extractions and immunoassays

After fourteen days of E2 treatment or seven days of replacement with CORT, the animals were decapitated, and their trunk blood was collected for OT, AVP, ANG II, ANP and CORT analysis. Blood collection was performed in chilled plastic tubes containing heparin (10 μL of heparin per mL of blood) to measure plasma OT, AVP and CORT or ethylenediaminetetraacetic acid (2 mg/mL) and proteolytic enzyme inhibitors (10 μL of 1 mM phenylmethylsulfonyl fluoride, 10 μL of 500 mM pepstatin A and 20 μL of p-hydroxymercuribenzoate per mL of blood) for plasma ANP and ANG II determination. Plasma was obtained after

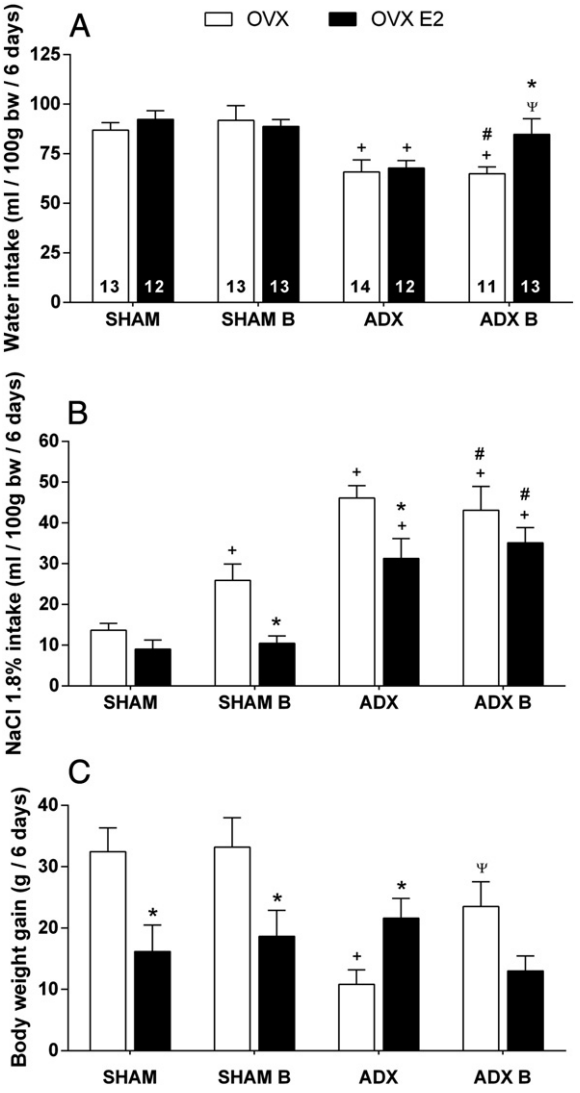

Fig. 1. Effect of pretreatment with estradiol on the water intake accumulated during 6 days (A) and on the sodium intake accumulated during 6 days (B) in OVX rats submitted to adrenalectomy with or without replacement with CORT and CORT treatment in rats with intact adrenal glands. The values are adjusted per 100 g of body weight (bw) and expressed as means ± SEM. Body weight gain 6 days after adrenalectomy (C). +$p < 0.05$ vs. respective OVX SHAM group, #$p < 0.05$ vs. respective OVX SHAM B group, Ψ$p < 0.05$ vs. respective OVX ADX group, *$p < 0.05$ vs. respective OVX oil group. In graphic C sample size (n) is represented inside the columns in graphic A.

centrifugation for 20 min at 3000 rpm at 4 °C and stored at −20 °C until specific extraction and immunoassay procedures were initiated. AVP and OT were extracted from 1 mL of plasma with acetone and petroleum ether, while CORT was extracted from 25 μL of plasma with 1 mL ethanol, and ANP and ANG II were extracted from 1 mL of plasma using Sep-Pak C-18 cartridges (Waters Corporation, Milford, MA, USA).

All hormone measurements were performed using specific radioimmunoassay techniques as previously described by Elias et al. (1998), Haanwinckel et al. (1995), Gutkowska et al. (1984), Botelho et al. (1994), and Vecsei (1979) for AVP, OT, ANP, ANG II and CORT, respectively. All measurements were performed in duplicate in the same assay. The sensitivity of the assay and the coefficient of variation intra-assay were 0.12 pg/mL, 10.6% for AVP; 0.12 pg/mL, 8.3% for OT; 0.7 pg/mL, 3.7% for ANP; and 0.4 pg/mL, 9.4% for ANG II. The sensitivity of the assay and the coefficient of variation for the intra- and inter-assays were 0.4 μg/dL and 8.0%–10.6% for CORT, respectively.

Microdissection, RNA isolation and semi-quantitative real-time PCR

After fourteen days of treatment with E2 or seven days of replacement with CORT, the animals were decapitated, their brains were collected under RNAse-free conditions, and they were immediately frozen on dry ice and stored at −80 °C to determine CRF mRNA expression in the PVN and AT1 receptor mRNA expression in the subfornical organ (SFO). Tissue samples of the PVN and SFO were obtained by microdissection of coronal sections of 1800 μm in cryostat, according to the coordinates of the atlas (Paxinos and Watson, 1997): −0.7 mm to −2.5 mm, in relation to the bregma. Samples of PVN and SFO were collected through a 1.5 mm diameter sterile needle, transferred to a microtube containing RNAlater reagent (Ambion, Austin, TX, USA), maintained for approximately 12 h at 4 °C and then stored at −70 °C until RNA extraction.

Total RNA was extracted using TRIzol Reagent (Invitrogen®, Carlsbad, CA, USA) and quantified by UV spectrophotometry. The synthesis of complementary DNA (cDNA) was performed using 500 ng of total RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using Applied Biosystems Step One Plus Real-Time PCR System. The reactions were performed in triplicate, and water (instead of cDNA) was used as negative control. The results were analyzed according to the ΔΔCt method. β-Actin was used as the endogenous control (4352340E, Applied Biosystems). The Ct of β-actin was subtracted from the Ct of the gene of interest (CRF, Rn 01462137_m1, AT1, Rn 00578456_m1, Applied Biosystems) to determine the ΔCt of each sample ($\Delta Ct = Ct_{unknown} - Ct_{reference\ gene}$). The ΔCt of the calibrator (control group OVX SHAM) was subtracted from the ΔCt of each sample to determine the ΔΔCt ($\Delta\Delta Ct = \Delta Ct_{unknown} - \Delta Ct_{calibrator}$). This value was used in the equation $2^{-\Delta\Delta Ct}$, and the results represent the relative expression of mRNA expressed in arbitrary units.

Statistical analysis

Results are presented as the mean ± standard error (SEM). Data were analyzed using Statistica (StatSoft, USA) through the factorial analysis of variance (three-way ANOVA) with the hormonal profile (E2 and B) and ADX surgery as independent variables. For the analyze of the dependent variables: daily water and sodium intake, ratio of water to sodium intake and natriuresis; with the hormonal profile (E2 and B), ADX surgery and days as independent variables, it was performed general linear models univariate (GLM) with days as random factor (mixed-model). Post-hoc comparisons of Fisher's least-significant-difference test were performed only when the F value was significant, that is, when the test showed statistically significant effects and interactions. The level of significance was set at 5% ($\alpha = 5\%$).

Results

Effect of pretreatment with estradiol on sodium appetite, thirst and natriuresis in OVX control rats and OVX rats submitted to ADX with or without glucocorticoid replacement

Water intake (Table 1 and Fig. 1A) varied significantly in function of treatments (oil vs. estradiol, $F_{7,93} = 8.54$, $df = 1$, $p < 0.01$; sham vs. ADX, $F_{7,93} = 69.34$, $df = 1$, $p < 0.001$) but it was not influenced by treatment with CORT. Significant interactions were observed between the three treatments ($F_{7,93} = 8.42$, $df = 1$, $p < 0.01$). The ADX induced a reduction in water intake in both OVX animals ($p < 0.01$) and in OVX animals treated with E2 ($p < 0.01$). However, E2 pretreatment increased water intake in the ADX B groups compared to oil treatment ($p < 0.05$).

In regard to salt intake, the results obtained indicate that the intake of hypertonic saline (1.8% NaCl, Fig. 1B and Table 1) increased after ADX ($F_{7,93} = 303.17$, $df = 1$, $p < 0.001$), and replacement with CORT did not reverse this effect. Furthermore, sodium intake also varied in function

Table 1
Daily water intake (mL/100 g body weight), sodium intake (mL/100 g body weight), ratio of water to sodium intake (%) and urinary sodium (mEq/100 g body weight) in OVX rats submitted to adrenalectomy with or without replacement with CORT and CORT treatment in rats with intact adrenal glands.

	Oil				Estradiol			
	SHAM	SHAM B	ADX	ADX B	SHAM	SHAM B	ADX	ADX B
Water intake								
Day 1	14.5 ± 1.0	16.3 ± 1.4	10.2 ± 1.0[a]	11.7 ± 1.2[b]	14.2 ± 1.2	13.1 ± 0.7	10.4 ± 0.9[a]	12.6 ± 1.8
Day 2	17.0 ± 1.1	18.3 ± 1.8	12.4 ± 1.2[a]	11.7 ± 1.3[a,b]	18.1 ± 1.1	16.8 ± 0.7	13.0 ± 0.6[a]	15.1 ± 1.6
Day 3	15.7 ± 1.0	17.8 ± 1.7	11.8 ± 1.4[a]	12.2 ± 0.8[a,b]	17.2 ± 1.0	16.5 ± 0.9	11.7 ± 0.9[a]	14.4 ± 1.1
Day 4	13.0 ± 0.8	12.8 ± 1.2	10.1 ± 1.3	10.0 ± 0.5	14.0 ± 0.9	14.5 ± 1.1	11.4 ± 1.0	14.0 ± 1.4[d]
Day 5	15.9 ± 1.0	16.1 ± 1.7	11.2 ± 1.4[a]	11.5 ± 0.9[a,b]	16.3 ± 0.9	15.6 ± 0.9	11.6 ± 1.1[a]	14.7 ± 1.3
Day 6	10.8 ± 1.0	10.3 ± 2.2	10.0 ± 1.2	7.9 ± 1.0	12.5 ± 1.5	12.2 ± 0.7	9.7 ± 0.8	14.0 ± 2.0[c,d]
Sodium intake								
Day 1	1.8 ± 0.5	3.2 ± 0.7	4.3 ± 0.8[a]	4.1 ± 1.0[a]	1.8 ± 0.5	1.7 ± 0.3	3.9 ± 0.4	3.2 ± 0.5
Day 2	2.9 ± 0.5	4.6 ± 0.7	7.3 ± 0.7[a]	7.0 ± 0.9[a,b]	2.3 ± 0.5	2.1 ± 0.4[d]	5.0 ± 0.8[a,d]	5.0 ± 0.8[a,b]
Day 3	1.7 ± 0.3	4.1 ± 1.0[a]	8.1 ± 0.4[a]	6.0 ± 1.1[a,c]	1.1 ± 0.3	1.4 ± 0.3[d]	5.1 ± 1.1[a,d]	5.4 ± 0.9[a,b]
Day 4	1.9 ± 0.4	4.0 ± 0.8[a]	9.2 ± 0.6[a]	7.9 ± 1.0[a,b]	1.2 ± 0.3	1.4 ± 0.3[d]	5.4 ± 0.8[a,d]	7.2 ± 1.0[a,b]
Day 5	2.2 ± 0.5	4.6 ± 1.3[a]	8.6 ± 0.5[a]	8.6 ± 1.5[a,b]	1.3 ± 0.5	1.9 ± 0.4[d]	5.7 ± 1.1[a,d]	7.1 ± 0.9[a,b]
Day 6	3.2 ± 0.7	5.3 ± 0.7[a]	8.6 ± 0.7[a]	9.5 ± 1.4[a,b]	1.4 ± 0.6	1.9 ± 0.5[d]	6.2 ± 1.3[a,d]	7.1 ± 1.0[a,b,d]
Ratio S/TF intake								
Day 1	10.3 ± 2.5	15.9 ± 3.4	29.7 ± 4.6[a]	25.2 ± 4.0[a]	9.6 ± 2.5	11.4 ± 2.2	27.5 ± 2.6[a]	21.5 ± 3.0[a]
Day 2	15.1 ± 3.1	20.5 ± 3.6	36.4 ± 2.2[a]	39.6 ± 5.4[a,b]	10.3 ± 1.9	10.8 ± 2.0	27.3 ± 3.4[a]	23.8 ± 2.6[a,d]
Day 3	10.1 ± 2.0	18.5 ± 3.7	41.7 ± 2.1[a]	32.3 ± 4.8[a,b,c]	5.7 ± 1.7	7.7 ± 1.7[d]	28.0 ± 5.3[a,d]	25.9 ± 3.6[a]
Day 4	12.4 ± 2.6	24.0 ± 4.8[a]	48.2 ± 3.0[a]	43.9 ± 3.6[a,b]	7.4 ± 2.1	9.1 ± 1.5[d]	32.4 ± 4.5[a,d]	33.7 ± 3.9[a]
Day 5	11.9 ± 2.5	21.7 ± 4.5	44.6 ± 2.8[a]	41.6 ± 5.7[a,b]	6.2 ± 1.9	11.1 ± 2.7[d]	32.2 ± 5.5[a,d]	32.5 ± 2.9[a]
Day 6	23.1 ± 4.0	40.0 ± 6.6[a]	48.5 ± 3.7[a]	53.3 ± 5.2[a,b]	9.7 ± 3.7[d]	12.7 ± 3.1[d]	36.2 ± 5.7[a,d]	34.8 ± 3.9[a,d]
Natriuresis								
Day 1	0.64 ± 0.3	0.45 ± 0.1	1.12 ± 0.2	1.02 ± 0.2	0.26 ± 0.09	0.26 ± 0.07	0.59 ± 0.2	0.77 ± 0.2
Day 3	0.70 ± 0.2	1.10 ± 0.3	2.11 ± 0.3[a]	2.05 ± 0.3[a,b]	0.38 ± 0.08	0.39 ± 0.06[d]	1.32 ± 0.3[a,d]	1.38 ± 0.3[a,b,d]
Day 6	1.25 ± 0.4	1.44 ± 0.2	2.70 ± 0.4[a]	2.55 ± 0.5[a,b]	0.39 ± 0.08[d]	0.41 ± 0.08[d]	1.50 ± 0.3[a,d]	1.03 ± 0.2[a,d]

Data are expressed as means ± SEM. S/TF = sodium/total fluid intake. (a) $p<0.05$ vs. respective Oil SHAM group, (b) $p<0.05$ vs. respective Oil SHAM B group, (c) $p<0.05$ vs. respective Oil ADX group, and (d) $p<0.05$ vs. respective Oil group. The sample size is presented in Fig. 1A.

of pretreatment with E2 ($F_{7,93} = 65.31$, df = 1, $p<0.001$), which attenuated ADX-induced sodium intake. Only treatment with CORT in rats with intact adrenal glands was capable of inducing increased sodium intake ($F_{7,93} = 6.27$, df = 1, $p<0.05$), which was also attenuated by E2 pretreatment ($p<0.01$). Significant interactions were observed between the three treatments ($F_{7,93} = 11.21$, df = 1, $p<0.001$).

Adrenalectomy also induced an increase in the ratio of water to sodium intake ($F_{7,93} = 338.73$, df = 1, $p<0.001$, Table 1 and Fig. 2); however, CORT replacement did not reverse this effect. By contrast, pretreatment with E2 attenuated sodium appetite ($F_{7,93} = 82.46$, df = 1, $p<0.001$) in ADX rats with or without CORT replacement and in rats with intact adrenal glands treated with CORT. Post-test analysis showed that treatment with CORT in rats with intact adrenal glands induced an increase in sodium appetite ($p<0.05$). Significant interactions were observed between the three treatments ($F_{7,93} = 4.01$, df = 1, $p<0.05$).

Regarding urinary excretion of sodium (Fig. 3 and Table 1), ADX induced a significant increase in natriuresis in both the OVX group and in the OVX group previously treated with E2 ($F_{7,93} = 65.23$, df = 1, $p<0.001$). These effects were not reversed by replacement with CORT. However, E2 pretreatment reduced natriuresis irrespective to the presence (SHAM) or absence (ADX) of adrenal glands or CORT treatment ($F_{7,93} = 46.13$, df = 1, $p<0.001$). Urinary excretion of sodium was not influenced by CORT replacement in ADX rats or treatment with CORT in SHAM rats.

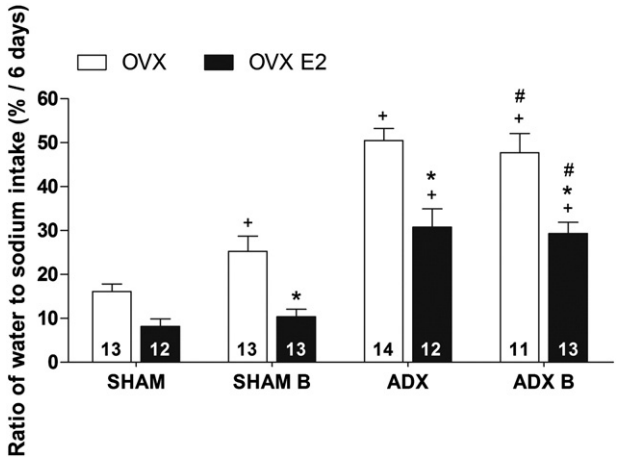

Fig. 2. Effect of pretreatment with estradiol on the ratio of water to sodium intake accumulated during 6 days in OVX rats submitted to adrenalectomy with or without replacement with CORT and CORT treatment in rats with intact adrenal glands. The values are expressed as means ± SEM. +p < 0.05 vs. respective OVX SHAM group, #p < 0.05 vs. respective OVX SHAM B group, *p < 0.05 vs. respective OVX oil group. The sample size (n) is represented inside the columns.

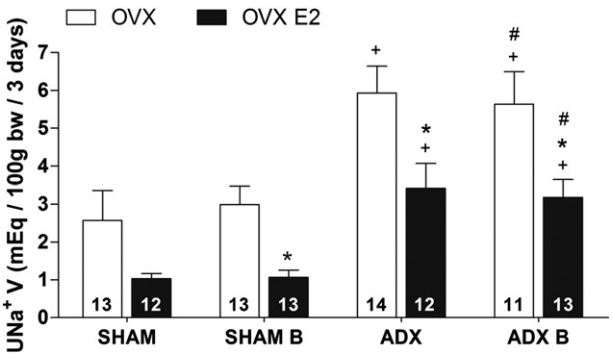

Fig. 3. Effect of pretreatment with estradiol on the sodium urinary excretion accumulated during 3 days in OVX rats submitted to adrenalectomy with or without replacement with CORT and CORT treatment in rats with intact adrenal glands. The values are adjusted per 100 g of body weight (bw) and expressed as means ± SEM. +p < 0.05 vs. respective OVX SHAM group, #p < 0.05 vs. respective OVX SHAM B group, Ψp < 0.05 vs. respective OVX ADX group, *p < 0.05 vs. respective OVX oil group. The sample size (n) is represented inside the columns.

Effect of pretreatment with estradiol on plasma sodium concentration and hematocrit values in OVX control rats and OVX rats submitted to ADX with or without glucocorticoid replacement

No significant differences in plasma sodium concentration were observed in any experimental conditions (Fig. 4A). Hematocrit varied significantly in function of pretreatment with E2 ($F_{7,93} = 13.51$, df = 1, $p < 0.001$) but it was not influenced by ADX or CORT replacement and treatment with CORT in SHAM rats. The post-test analysis showed that treatment with E2 reduced hematocrit in rats submitted to ADX ($p < 0.05$, Fig. 4B).

Effect of pretreatment with estradiol on the plasma levels of AVP, OT, ANP and ANG II in OVX control rats and OVX rats submitted to ADX with or without replacement of glucocorticoids

ADX induced a significant increase in plasma levels of ANG II ($F_{7,81} = 160.51$, df = 1, $p < 0.001$). Significant interactions were observed between the three treatments ($F_{7,81} = 16.45$, df = 1, $p < 0.001$). Analysis performed using the post-test indicated that pretreatment with E2 attenuated ADX-induced increase in plasma ANG II ($p < 0.001$). Replacement with CORT in ADX animals reduced the plasma levels of ANG II rats ($p < 0.001$). However, OVX rats submitted to ADX, pretreated with E2 and replaced with CORT (OVX E2 ADX B) had higher circulating levels of ANG II, demonstrating a reversal of the effect induced by both E2 and by CORT when these hormones were administered separately ($p < 0.01$, Fig. 5A).

The plasma concentration of ANP varied significantly in function of the treatments (oil vs. estradiol, $F_{7,77} = 5.39$, df = 1, $p < 0.05$; sham vs. ADX, $F_{7,77} = 7.83$, df = 1, $p < 0.01$; oil vs. CORT, $F_{7,77} = 12.09$, df = 1, $p < 0.001$). As shown in Fig. 5B, ADX rats previously treated with E2 had higher plasma concentrations of ANP ($p < 0.01$); however, ADX rats replaced with CORT and pretreated with E2 had lower levels of ANP compared to ADX rats treated only with E2 ($p < 0.001$).

Although OT secretion did not change in most of the experimental conditions, the pretreatment with E2 in ADX B rats induced a reduction in the plasma OT concentration (interaction between E2 vs. CORT, $F_{(7,90)} = 6.26$, df = 1, $p < 0.05$). On the other hand, no significant differences were observed in the plasma AVP concentrations in any experimental condition studied (Fig. 5D).

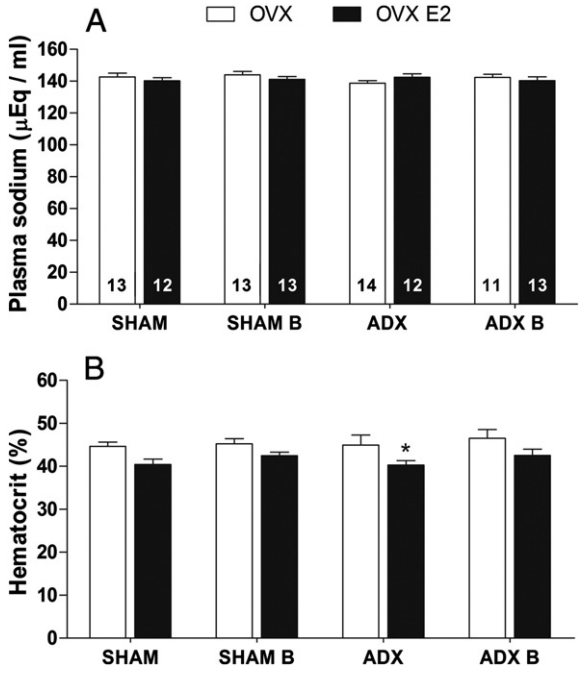

Fig. 4. Effect of pretreatment with estradiol on the plasma sodium concentration (A) and on the hematocrit (B) in OVX rats submitted to adrenalectomy with or without replacement with CORT and CORT treatment in rats with intact adrenal glands. The values are expressed as means ± SEM. *$p < 0.05$ vs. respective OVX oil group. The sample size (n) is represented inside the columns in graphic A.

Effect of pretreatment with estradiol on AT1 mRNA expression in the SFO in OVX control rats and OVX rats submitted to ADX with or without replacement of glucocorticoids

Pretreatment with E2 reduced the relative expression of AT1 mRNA in the SFO of OVX rats with intact adrenal glands and in OVX ADX rats ($F_{7,56} = 15.28$, df = 1, $p < 0.001$), as shown in Fig. 6. By contrast, ADX and replacement or treatment with CORT did not affect the relative expression of AT1 mRNA in the SFO.

Effect of pretreatment with estradiol on CRF mRNA expression in the PVN and plasma corticosterone levels in control OVX rats and OVX rats submitted to ADX with or without replacement of glucocorticoids

The relative expression of CRF mRNA in the PVN (Fig. 7A) varied significantly in function of the treatments (sham vs. ADX, $F_{7,55} = 54.33$, df = 1, $p < 0.001$; oil vs. CORT, $F_{7,55} = 31.51$, df = 1, $p < 0.001$). Moreover, significant interactions were observed among the three treatments ($F_{7,55} = 6.04$, df = 1, $p < 0.05$). The results showed that ADX in OVX rats induced an increase in CRF mRNA expression ($p < 0.001$) that was significantly attenuated by E2 pretreatment ($p < 0.05$). In addition, replacement with CORT decreased both CRF mRNA expression in ADX ($p < 0.001$) and in E2-treated rats ($p < 0.05$). However, OVX E2 ADX B rats showed a higher level of CRF mRNA expression compared to OVX ADX B rats ($p < 0.01$).

Regarding plasma CORT levels (Fig. 7B), they varied significantly in relation to the treatments (oil vs. estradiol, $F_{7,79} = 75.66$, df = 1, $p < 0.001$; sham vs. ADX, $F_{7,79} = 154.43$, df = 1, $p < 0.001$), but they were not influenced by treatment or CORT replacement. Significant interactions between the three factors were also observed ($F_{7,79} = 6.45$, df = 1, $p < 0.01$). Pretreatment with E2 induced an increase in plasma levels of CORT in OVX rats with intact adrenal glands ($p < 0.001$). ADX induced a reduction in CORT plasma concentration levels in both OVX and OVX E2 rats ($p < 0.01$); however, replacement or treatment with CORT at the dose used in our experimental model did not cause CORT plasma levels to significantly differ in OVX rats with intact adrenal glands (OVX SHAM). Therefore, these results provide evidence that validates ADX surgery and CORT therapy.

Discussion

In the present study, we observed that adrenalectomy induced a reduction in water intake, an increase in sodium intake and an increased ratio of water to sodium intake in OVX rats. In addition, ADX leads to an increase in renal sodium excretion and in body sodium depletion, due to aldosterone deficiency (Fitzsimons, 1998; Krause and Sakai, 2007). This body sodium deficit and consequent reduction in extracellular fluid volume are detected by specialized receptors that convey this information to specific areas of the central nervous system (CNS) such as the circumventricular organs (CVO), which are responsible for an integrated response and induce increased sodium appetite with subsequent restoration of circulating volume (Antunes-Rodrigues et al., 2004; Fitzsimons, 1998). In fact, OVX ADX rats did not show a change in their circulating volume seven days after ADX with free access to tap water and hypertonic saline, which was indicated by the absence of changes in hematocrit values in these animals. Thus, in response to seven days of ADX with free access to water and hypertonic saline, an increase in sodium and reduced water intake was observed, which maintains adequate plasma osmolality (data not shown) and restores their circulating volume. Because the spontaneous consumption of water is associated with feeding, and ADX rats present a hypophagic response, possibly

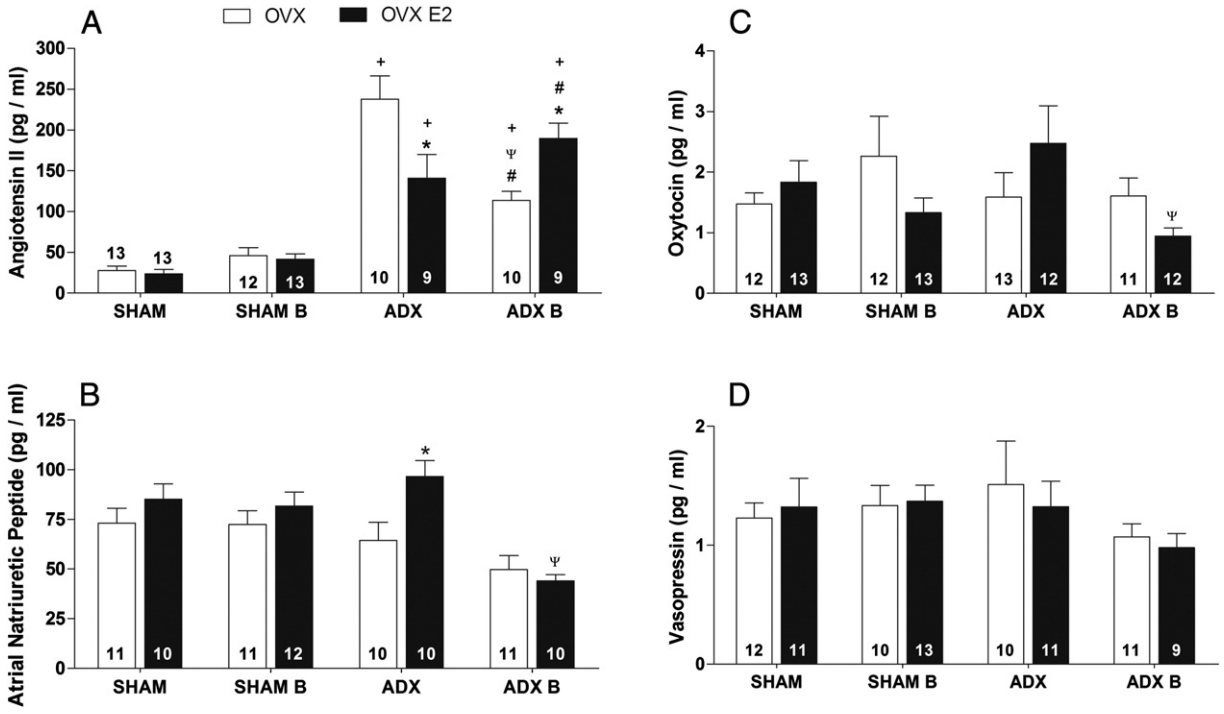

Fig. 5. Effect of pretreatment with estradiol on the plasma levels of angiotensin II (A), atrial natriuretic peptide (B), oxytocin (C) and vasopressin (D) in OVX rats submitted to adrenalectomy with or without replacement with CORT and CORT treatment in rats with intact adrenal glands. The values are expressed as means ± SEM. +$p < 0.05$ vs. respective OVX SHAM group, #$p < 0.05$ vs. respective OVX SHAM B group, Ψ$p < 0.05$ vs. respective OVX ADX group, *$p < 0.05$ vs. respective OVX oil group. The sample size (n) is represented inside the columns.

the reduction of water intake under this condition may be related to the reduction of food intake (Kraly, 2004; Uchoa et al., 2010).

ADX in OVX rats also induced an increase in plasma levels of ANG II, which significantly contributes to the stimulation of sodium appetite in these animals. Sodium appetite in sodium-depleted ADX rats may be mediated by peripheral ANG II via activation of AT1 receptors present in the CVOs, primarily in the SFO and in the organum vasculosum of the lamina terminalis (OVLT; Morris et al., 2002; Weisinger et al., 2000).

Conversely, pretreatment with E2 significantly attenuated sodium appetite, the elevated plasma levels of ANG II and the mRNA expression of the AT1 receptor in the SFO in response to ADX. Thus, E2 may attenuate sodium appetite in response to ADX by reducing circulating levels of ANG II and reduction of central ANG II actions via down-regulation of AT1 receptors in the SFO. These results corroborate data from the literature that demonstrates that E2 attenuates sodium appetite and thirst in female rats during proestrus and OVX rats treated with E2 submitted to dipsogenic and natriorexigenic challenges as well as in response to ANG II central administration (Covian and Antunes-Rodrigues, 1963; Danielsen and Buggy, 1980; Jonklaas and Buggy, 1984; Mecawi et al., 2007, 2008; Stricker et al., 1991). Additionally, recent studies have shown that treatment with E2 in OVX rats reduced the interaction between ANG II and AT1 receptors in the SFO (Kisley et al., 1999) and reduced neuronal activity in the OVLT and SFO during intracarotid administration of ANG II (Tanaka et al., 2001).

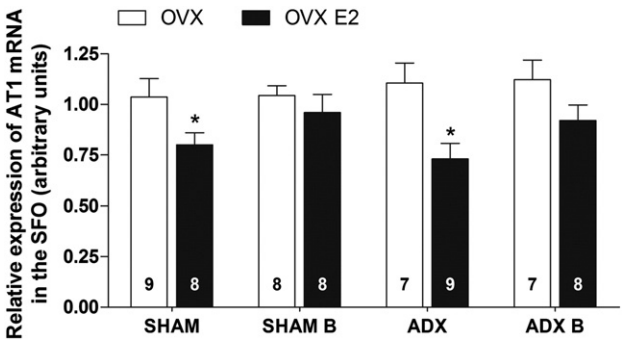

Fig. 6. Effect of pretreatment with estradiol on the relative expression of AT1 receptor mRNA in the SFO in OVX rats submitted to adrenalectomy with or without replacement with CORT and CORT treatment in rats with intact adrenal glands. The values are expressed as means ± SEM. *$p < 0.05$ vs. respective OVX oil group. The sample size (n) is represented inside the columns.

Furthermore, the results showed that OVX E2 ADX rats had higher circulating levels of ANP when compared with OVX ADX rats. This response may contribute to the attenuation of sodium appetite because ANP has been postulated to have an inhibitory role on sodium appetite. Antunes-Rodrigues et al. (1986) demonstrated that the injection of ANP in the anterior ventral third ventricle region (AV3V) dramatically reduces hypertonic saline intake in sodium-depleted rats. Experimental evidence indicated an abundance of ANP receptors in the SFO, and this peptide may modulate the excitatory effects of ANG II in SFO neurons, antagonizing its effects, especially related to ingestive behavior (Ferguson and Bains, 1996). OVX E2 ADX rats showed a greater circulating volume compared to OVX ADX rats as indicated by a reduction in hematocrit values, which may contribute in part to increased levels of circulating ANP observed in these rats. In addition, studies showed that chronic treatment with E2 in OVX rats increases plasma concentration of ANP (Xu et al., 2008), and this effect is modulated by type α E2 receptors (ER-α) present in cardiac atrium (Jankowski et al., 2001).

Pretreatment with E2 attenuated natriuresis induced by adrenal insufficiency despite attenuated plasma levels of ANG II, increased levels of ANP and reduced hematocrit values. However, Winaver et al. (1995) reported that rats with congestive heart failure had reduced urinary sodium excretion although they had elevated ANP plasma levels, demonstrating a hyporesponsiveness of the kidney to ANP. Blocking angiotensin converting enzyme (ACE) induced a significant increase in urinary sodium excretion in these animals, showing an ANG II functional antagonism to ANP-induced natriuresis and diuresis. Other investigators have postulated that ANG II may prevent ANP natriuretic action due to a decrease of cellular ANP signaling. ANG II increases phosphodiesterase activity, which leads to GMPc (ANP second messenger) degradation (Smith and Lincoln, 1987; Wilkins and Needleman, 1992; Winaver et al.,

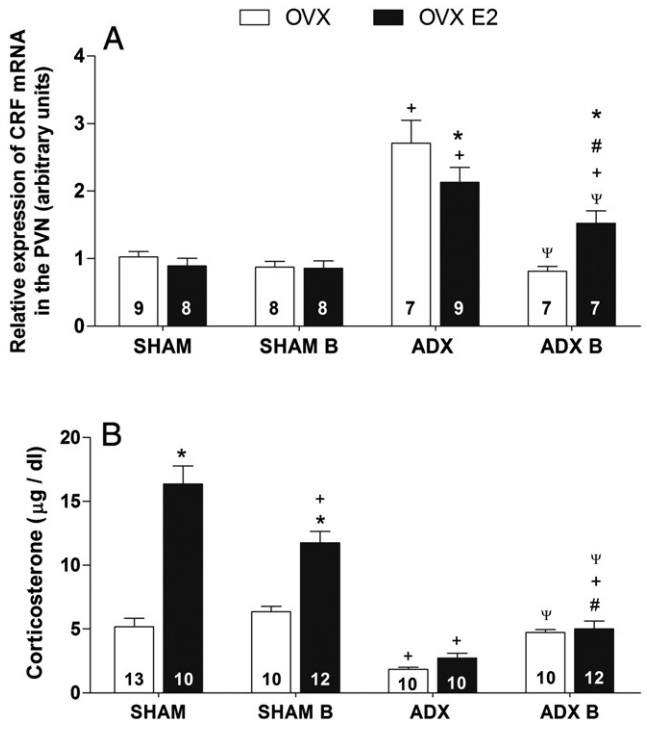

Fig. 7. Effect of pretreatment with estradiol on the relative expression of CRF mRNA in the PVN (A) and on the plasma levels of corticosterone (B) in OVX rats submitted to adrenalectomy with or without replacement with CORT and CORT treatment in rats with intact adrenal glands. The values are expressed as means ± SEM. +p < 0.05 vs. respective OVX SHAM group, #p < 0.05 vs. respective OVX SHAM B group, Ψp < 0.05 vs. respective OVX ADX group, *p < 0.05 vs. respective OVX oil group. The sample size (n) is represented inside the columns.

1995). In this context, higher plasma ANG II levels in OVX E2 ADX rats may prevent the natriuretic action of ANP (despite the high plasma levels of this peptide) and contribute in part to the antinatriuresis observed in these animals. Moreover, some studies have demonstrated that the E2 antinatriuretic effect may be due to its direct effect on renal tubules by increasing sodium reabsorption (Brunette and Leclerc, 2001), as well due to increase of AT1 receptor expression in the mesangial cells of the renal glomerulus, contributing to the reduction of the coefficient of glomerular filtration rate (Baiardi et al., 2005).

CORT replacement in OVX ADX rats did not reverse the sodium appetite induced by ADX. This response may be due to the activity of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) in the kidneys, which can convert the administered CORT in its inactive metabolite, 11-dehydrocorticosterone (Edwards et al., 1998). In fact, replacement with CORT in OVX ADX rats also did not reverse increased ADX-induced natriuresis. In addition, McEwen et al. (1986) demonstrated that the replacement with aldosterone in ADX animals results in reversion of the sodium appetite ADX-induced. Also, the pharmacological inhibition of 11β-HSD attenuated natriuresis ADX-induced in ADX replacement with CORT rats (Bailey et al., 2001). Furthermore, it is known that CORT interacts with MR receptors in the CNS such as the central amygdala, which induces sodium appetite in rats (Zhang et al., 1993). In this context, it was observed that treatment with CORT in rats with intact adrenal glands was shown to induce an increase in sodium appetite, without affecting natriuresis or plasma ANG II levels, suggesting being due to CORT central action. Thus, this central effect of CORT may also contribute to the observed elevated sodium appetite in OVX ADX B rats. In addition, CORT acts in synergy with ANG II in the CNS (Epstein, 1992).

CORT replacement in OVX ADX rats attenuates the ADX-induced high increase in plasma concentration of ANG II, demonstrating a direct peripheral effect of CORT on the renin angiotensin system (RAS) activity. Whereas AT1 mRNA expression in the SFO was not altered by adrenalectomy or CORT replacement in OVX rats treated with oil. AT1 receptors in the SFO have been postulated to mediate the HPA axis response stimulated by circulating ANG II (Jezova et al., 1998; Krause et al., 2008); therefore, we suggest that the decrease in ANG II plasma levels induced by CORT replacement in OVX ADX rats may be associated with a reduction of CRF mRNA expression in the PVN. Furthermore, angiotensinergic activation at SFO has been implicated in the central regulation of blood pressure (Hendel and Collister, 2004; Smith and Ferguson, 2010). Thus, the regulatory effect of CORT replacement in OVX ADX rats on peripheral ANG II is of physiological importance in the regulation of blood pressure. However, more studies are needed to better elucidate the mechanism by which CORT attenuates plasma levels of ANG II and its physiological relevance.

AVP plasma levels were not affected by the treatments performed in this study, demonstrating the absence of their involvement in the homeostatic responses observed. Also, no changes in plasma sodium were observed, indicating that the animals were able to restore their plasma sodium concentration despite the large sodium urinary loss, which was expected because hypertonic saline solution was offered to these animals. It is well established that AVP is secreted in response to hyperosmolality and hypovolemia, being more sensitive to stimuli that alter plasma osmolality (Haanwinckel et al., 1995). Thus, maintenance of plasma sodium and plasma osmolality correlates with the absence of changes in AVP secretion under these experimental conditions. The OT secretion also was not affected by most treatments, very probably by same explication as for AVP secretion since these hormones presented the equal secretion stimulus (Godino et al., 2007; Haanwinckel et al., 1995). Nevertheless the pretreatment with E2 attenuated plasma OT level in ADX B rats, demonstrating a specific effect of E2 associated with CORT in the OT secretion.

Pretreatment with E2 also attenuated the elevated natriuresis response observed in ADX B rats. This effect is most likely due to elevated plasma concentration of ANG II and low plasma ANP and OT in addition to the other antinatriuretic effects of E2 discussed above. Additionally, OVX E2 ADX B rats showed a significant increase in water intake, reversing the effect induced by ADX. Treatment with E2 or replacement with CORT alone is not able to prevent the reduction of water intake induced by ADX. Thus, this study demonstrates for first time that it is only when the two treatments (E2 and CORT) are combined that the reversion effect induced by ADX on water intake occurs. Increased plasma levels of ANG II and decreased ANP may contribute to increased water intake observed in these animals, specifically considering the dipsogenic effect of ANG II and the antidipsogenic effect of ANP mentioned above.

Recently, it has been postulated that dipsogenic and natriorexigenic responses induced by ANG II and mediated by the AT1 receptor are ultimately due to the activation of distinct intracellular signaling pathways. Daniels et al. (2005, 2007, 2009) showed that water intake involves the activation of protein kinase C (PKC); nevertheless, the activation of MAP kinase plays a role in sodium intake induced by ANG II. Additionally, a growing number of studies have shown selective binding and activation of PKC conventional isoforms by steroid hormones. Aldosterone, E2 and ANG II (via AT1 receptor) activate the same conventional protein kinase C isoform (PKCα; Alzamora and Harvey, 2008; Sumners et al., 2002). In this context, it is reasonable to postulate that in response to ADX and in the presence of CORT and E2, possible crosstalk between E2 and the CORT cell signaling pathway may occur, which induces higher activation of PKC in CNS structures involved with the dipsogenic response of ANG II, such as the SFO. Similarly, potential crosstalk between these steroid hormones may induce the increase of plasma ANG II and the reduction of plasma ANP and OT levels in response to ADX.

Pretreatment with E2 inhibited the stimulatory effect on sodium appetite induced by treatment with CORT in rats with intact adrenal glands and attenuated urinary sodium excretion. These data demonstrate that E2 modulates homeostatic responses induced by the administration of CORT in rats with intact adrenals. E2 may inhibit

CORT-induced sodium appetite through the modulation of MR receptor expression or signaling in CNS structures involved in this behavior. Some studies have shown that E2 significantly reduces mRNA expression of MR in CNS structures such as the hippocampus, the hypothalamic preoptic area and the adenohypophysis (Burgess and Handa, 1993). Furthermore, no significant change in AT1 mRNA expression in the SFO of the OVX E2 SHAM B rats was observed when compared with the OVX SHAM B group, supporting the hypothesis that E2 may inhibit sodium appetite through potential CNS modulation of MR.

We also observed that pretreatment with E2 induced a significant increase in plasma CORT levels in OVX E2 SHAM and OVX E2 SHAM B rats. E2 also increased the weight of the adrenal glands (data not shown). These results corroborate data reporting the role of E2 on increased plasma CORT levels and an increase of the weight of adrenal glands in both OVX rats and proestrus female rats (Ochedalski et al., 2007; Viau and Meaney, 1991). However, this E2-induced increase in CORT plasma levels was not accompanied by an increase in CRF mRNA expression in the PVN. Therefore, the increase in plasma CORT may be due to a direct effect of E2 on the adrenal gland by stimulating the synthesis of glucocorticoids without altering the basal activity of the HPA axis. In fact, in vitro studies demonstrated that E2 increases the basal synthesis of CORT by adrenocortical cells (Nowak et al., 1995) and in vivo studies showed that E2 enhances adrenal sensitivity to ACTH in response to stress (Figueiredo et al., 2007).

Additionally, OVX E2 ADX B rats have higher CRF mRNA levels in the PVN compared with OVX ADX B rats. These data demonstrate that E2 alters the CORT negative feedback signal in neurons of PVN in ADX rats. Zhang et al. (2009) have shown that E2 reduces ligand-induced glucocorticoid receptor (GR) phosphorylation and inhibits glucocorticoid induction of the mitogen-activated protein kinase phosphatase-1 (MKP-1) and serum/glucocorticoid-regulated kinase genes. In accordance with this study, it is reasonable to suggest that E2 may alter the CORT negative feedback signal in neurons of PVN through changes in glucocorticoid binding and GR signaling pathway.

Pretreatment with E2 attenuated the increase of CRF mRNA expression in the PVN in response to adrenal insufficiency, indicating a reduction of responsiveness of neuron producers of CRF in the PVN activity modulated by E2. This result is in accordance with reports in the literature showing that in response to various stressful stimuli, E2 reduces the responsiveness of the HPA axis activity, decreasing the secretion of ACTH and CRF mRNA expression in the PVN (Figueiredo et al., 2007; Gerrits et al., 2005; Ochedalski et al., 2007; Redei et al., 1994). The reduction in AT1 mRNA expression in the SFO induced by E2 in response to ADX may contribute in part to the attenuation of CRF mRNA expression in the PVN because the AT1 receptor has been postulated to mediate the response of HPA axis stimulated by circulating ANG II via SFO as discussed above.

The effect of E2 in attenuating the responsiveness of neuron producers of CRF in the PVN activity after ADX has important relevance for females, which protects against the physiological changes known to be induced by increased HPA axis activity. The E2 may attenuate CRF mRNA expression in the PVN through OT parvocell stimulation (Dellovade et al., 1999) since these has been recently suggested to modulate the expression of CRF in a negative manner (Bülbül et al., 2011). In this context, Ochedalski et al. (2007) showed that icv administration of OT reduced CRF mRNA expression in the PVN in OVX rats treated with E2 on basal and stress-induced restraint conditions. This effect of E2 probably is due to ER-β because its activation in the PVN induced a reduction of stress-responsive corticosterone and ACTH secretion in gonadectomized rats (Lund et al., 2006). Moreover many fewer CRH neurons of the PVN express ER-β whereas a large number of ER-β immunoreactive cells in PVN are OT positive (Handa et al., 2011) supporting the hypothesis that E2 may attenuate CRF mRNA expression in the PVN through modulation of OT parvocells.

In conclusion, this study indicates that E2 plays an important role in modulating behavioral and neuroendocrine responses involved with the maintenance of hydroelectrolytic homeostasis in response to ADX. Similarly, E2 decreases the responsiveness of neuron producers of CRF in the PVN activity under adrenal insufficiency condition. The results also show that E2 modulates the sodium appetite effect induced by treatment with CORT in rats with intact adrenal glands. However, E2 in the presence of CORT reverses the reduction in water intake induced by ADX, changes hormonal responses induced by ADX and alters the negative feedback exerted by CORT in neurons of PVN in ADX conditions. The exact mechanism by which E2 modulates all these responses deserves further investigation.

Acknowledgments

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