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ESTROGENIC REGULATION OF NADPH-DIAPHORASE IN THE SUPRAOPTIC AND PARAVENTRICULAR NUCLEI UNDER ACUTE OSMOTIC STRESS

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Abstract—Estrogen receptors (ERs) α and β are involved in the regulation of the nitrergic system in the supraoptic (SON) and paraventricular (PVN) nuclei under basal conditions. In this study we have assessed whether ERs are also involved in the modulation of the nitrergic system in the SON and PVN under acute systemic hypertonic conditions. Adult ovariectomized rats received a single injection of either estradiol, a selective ERα agonist, a selective ERβ agonist, a selective ERα antagonist, a selective ERβ antagonist or vehicle. Twenty-four hours later, animals received one i.p. injection of 1.5 M NaCl to induce osmotic stress and were sacrificed after two additional hours. The number of NADPH-diaphorase-positive cells in the SON and PVN was determined. Their number in the SON was not affected by NaCl administration, whereas in the four PVN subdivisions it was decreased after NaCl administration. Estradiol and the ERα agonist prevented the action of NaCl in the four subdivisions of the PVN. In contrast, the inhibition of ERα enhanced the effect of NaCl, inducing a further decrease in the number of NADPH-diaphorase-positive cells. Moreover, the ERβ agonist enhanced and the ERβ antagonist blocked the effect of NaCl on the number of NADPH-diaphorase-positive neurons in the SON and in the medial magnocellular subdivision of the PVN. These findings indicate that estradiol regulates the nitrergic system in the SON and PVN under acute osmotic stress conditions, but the effects specifically depend on the anatomical subregions and different ERs.

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Abbreviations: ANOVA, analysis of variance; CVO, circumventricular organs; ERs, estrogen receptors; NADPH, nicotinamide adenine dinucleotide phosphate; nNOS, neural isoform of nitric oxide synthase; NO, nitric oxide; OVLT, organum vasculosum laminae terminalis; PaDC, dorsal parvocellular; PaMM, medial magnocellular; PaLM, lateral magnocellular; PaV, parvocellular; PBS, phosphate-buffered saline; PVN, paraventricular nucleus; SFO, subfornical organ; SON, supraoptic nucleus; TRIS, tris(hydroxymethyl)aminomethane.

Key words: estrogen receptors, estrogen receptor ligands, nitric oxide, paraventricular nucleus, supraoptic nucleus, water balance.

INTRODUCTION

The supraoptic (SON) and the paraventricular hypothalamic (PVN) nuclei are involved in the regulation of different relevant physiological responses (Swanson and Sawchenko, 1980; Higuchi and Okere, 2002; Engelmann et al., 2004), having a critical role in the control of body fluid homeostasis (Mueller et al., 2006; Heesch et al., 2009). The magnocellular neurons in the SON and the PVN, responsible for the release of arginine-vasopressin and oxytocin, are sensitive to changes in intracellular Na^+ concentration and osmolarity in mammals and other vertebrates (Verney, 1958; Leng et al., 1982; Ramieri and Panzica, 1989; Voisin et al., 1997).

Nitric oxide (NO) plays a relevant role in the control of fluid balance homeostasis (Calapai et al., 1992, 1994). The neural isoform of NO synthase (nNOS) enzyme, responsible for the formation of NO, has the same distribution and properties of the enzyme NADPH-diaphorase (Dawson et al., 1991). Therefore histochemistry for NADPH-diaphorase is considered a reliable method to identify the neurons that produce NO (Dawson et al., 1991; Hope et al., 1991; Vincent and Kimura, 1992; Rodrigo et al., 1994). In rodents, NADPH-diaphorase activity and nNOS immunoreactivity have been detected in magnocellular neurons of the SON and the PVN and in the circumventricular organs (CVOs), such as the subfornical organ (SFO) and the organum vasculosum laminae terminalis (OVLT), structures involved in osmoregulation (Dawson et al., 1991; Kadowaki et al., 1994; Rodrigo et al., 1994; Bhat et al., 1995; Cork et al., 1998; Liu et al., 1998; Gotti et al., 2005). Under conditions of osmotic stress (water deprivation), the number of NADPH-diaphorase cells as well as the nNOS synthase gene expression and the production of NO increases in the SON and the PVN (Kadowaki et al., 1994; Ueta et al., 1995; Srisawat et al., 2004; Gillard et al., 2007; Ryu et al., 2008).

Gonadal hormones play an important role in the regulation of the brain nitrergic system (Panzica et al., 2006). Specifically, in rodents, nNOS expression is

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modulated throughout the estrous cycle in brain structures involved in the control of reproductive behavior, such as the medial preoptic area, the arcuate nucleus, the ventromedial hypothalamic nucleus, the bed nucleus of the stria terminalis (Sica et al., 2009), the bed nucleus of the accessory olfactory tract (Collado et al., 2003), the anteroventral subdivision of the amygdala (Carrillo et al., 2007), as well as the hippocampus (Gotti et al., 2009). In all of these brain regions, nNOS cell number is increased when estradiol levels rise during the estrous cycle. Regulation of nitrergic system by gonadal hormones has been also demonstrated in the SON and the PVN. Estradiol, via estrogen receptors (ERs) α and β, regulates NADPH-diaphorase activity in the SON and the PVN. In the SON, the number of NADPH-diaphorase-positive cells is decreased by ERα and ERβ agonists and increased by ERα and ERβ antagonists. In contrast, the activation of ERα increases the number of NADPH-diaphorase-positive cells in the PVN subnuclei (parvocellular, PaV; lateral magnocellular, PaLM; medial magnocellular, PaMM; dorsal parvocellular, and PaDC), while the activation of ERβ has the opposite effect (Grassi et al., 2012).

Although these findings indicate that the number of NADPH-diaphorase-positive cells in the SON and the PVN is regulated by estradiol through ERα and ERβ, it is unknown whether this regulation is altered in conditions of osmotic stress. Under these conditions, magnocellular neurons suffer different structural and functional changes, including modifications in synaptic function (Kim et al., 2011), transcriptional activity (Lafarga et al., 1998; Arima et al., 1999; Berciano et al., 2002) and expression of ERs (Somponpun and Sladek, 2003, 2004). Since these modifications may alter the response of magnocellular neurons to estradiol, it is important to determine the regulation exerted by ERα and ERβ on the nitrergic system in the SON and the PVN neurons under conditions of osmotic stress. Therefore, in the present study we have assessed the effect of different ERα and ERβ ligands on the number of NADPH-diaphorase-positive cells in the SON and the PVN of adult female ovariectomized rats under acute osmotic stress.

EXPERIMENTAL PROCEDURES

Animals and experimental treatments

Wistar albino female rats from our in-house colony were kept on a 12:12-h light–dark cycle and received food and water *ad libitum*. Animals were handled in accordance with the guidelines published in the in accordance with the guidelines presented in the ''NIH Guide for the care and use of laboratory animals'', the principles presented in the ''Guidelines for the Use of Animals in Neuroscience Research'' by the Society for Neuroscience, and following the European Union legislation (86/609/EEC) and the Spanish Government Directive (R.D. 1201/2005). Experimental procedures were approved by our Institutional Animal Use and Care Committee. Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum necessary.

Female rats were bilaterally ovariectomized at the age of 3 months under isoflourane anesthesia. They were then housed in plastic cages and randomly assigned to the different treatments. Seven days after surgery four rats per group received one i.p. injection of vehicle (corn oil), 17β-estradiol (Sigma, Madrid, Spain; 50 μg/kg), the selective ERα agonist-PPT (4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol; BiogenCientifica, Madrid, Spain; 1 mg/kg), the selective ERβ agonist-DPN (2,3-bis(4-hydroxyphenyl)-propionitrile; BiogenCientifica; 1 mg/kg), the selective ERα antagonist-MPP (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride; BiogenCientifica; 1 mg/kg), or the selective ERβ antagonist-PHTPP (4-[2-phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol; BiogenCientifica; 1 mg/kg). Doses of ER ligands were based on previous studies *in vivo* (Waters et al., 2009; Santollo et al., 2010; Grassi et al., 2012).

Twenty-four hours after the injection of the estrogenic compounds or the vehicle, the animals received one i.p. injection of 1.5 M NaCl to induce salt load. Two hours after salt load induction all the animals were deeply anesthetized with pentobarbital (Normon Veterinary Division, Madrid, Spain, 50 mg/kg) and perfused through the left cardiac ventricle with 50 ml of saline solution (0.9% NaCl) followed by 250 ml of fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were quickly removed and immersed for 4–6 h at 4 °C in the same fixative solution and then rinsed with phosphate buffer. Brains were placed for 72 h in a 30% sucrose solution in phosphate-buffered saline (PBS), frozen in liquid isopentane at −35 °C, and stored in a deep freezer at −80 °C until sectioning. Brains were serially cut in the coronal plane at 25-μm thickness with a cryostat. The plane of sectioning was oriented to match the drawings corresponding to the transverse sections of the rat brain atlas of Paxinos and Watson (1986). Sections were collected in a cryoprotectant solution (Watson et al., 1986) at −20 °C. Every fourth section (one section every 100 μm) was histochemically stained for NADPH-diaphorase.

NADPH-diaphorase histochemistry

The presence of NADPH-diaphorase, a specific marker for NO-producing neurons (Dawson et al., 1991; Hope et al., 1991; Vincent and Kimura, 1992; Rodrigo et al., 1994) was detected by histochemistry performed on free-floating sections. Before the reaction, the sections collected in the cryoprotectant solution were washed for 30 min at room temperature in PBS 0.01 M, pH 7.3–7.4 and then overnight at 4 °C in phosphate buffer 0.1 M, pH 7.4. The following day, free-floating sections were washed at first in tris(hydroxymethyl)aminomethane (TRIS) buffer 0.1 M, pH 8.0 for 15 min at room temperature, then in TRIS buffer, pH 8.0 containing 0.5% Triton X-100 for 10 min. The sections were incubated for 30 min at 37 °C in a solution of 0.8 mM nitrobluetetrazolium (Sigma) and 1 mM reduced NADPH (Sigma) in TRIS buffer, 0.1 M, pH 8.0, containing 0.5%