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Effects of Adrenalectopmy on the Stress-Induced Changes in Ovarian Sympathetic Tone in the Rat

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A 3-wk period of stress promotes the development of ovarian cysts in rats apparently mediated by increased sympathetic nerve activity and ovarian steroid secretion. After 11 wk of stress, these parameters are indistinguishable from nonstressed control rats. To study adrenal contribution, we adrenalectomized rats and studied the effect of 3-wk of cold/restraint stress (1.5 h/d) on them compared to intact animals. Adrenalectomy (ADX) increased ovarian norepinephrine (NE) release, the content of β-adrenergic receptors (ßAR) and basal, but not isoproterenol (Iso)-induced, androgen secretion. Stress to intact animals increased NE release, decreased βAR content, and Iso-induced, but not basal, androgen secretion from the ovary. ADX did not modify the response to stress. We propose a tonic inhibition by the adrenal gland on nerve activity of ovarian nerves. Stress overrides this inhibitory effect, and nerve activity downregulates βAR, decreasing ovarian steroid secretion.

Key Words: Sympathetic nerves; ovary; stress; adrenal.

Introduction

Much evidence exists to support the view that sympathetic nerves participate in the regulation of ovarian function *(1)*. These nerves—mainly originating in the celiac ganglion—project to the ovary by two routes: (1) The plexus nerve, which provides the innervation to blood vessels and (2) The superior ovarian nerve, which innervates ovarian secretory cells of the follicle *(2)*. The physiological role for sympathetic nerves in the control of ovarian function is supported by:

1. The changes in norepinephrine (NE) release and β-adrenergic receptor (βAR) concentration of the rat ovary during estrous cycle *(3,4)*,
2. The demonstration that activation of βAR is coupled to progesterone and androgens secretion from granulosa and theca-interstitial cells of the ovary, respectively *(5,6)*, and
3. The demonstration that chronic increase in ovarian NE release induced by estradiol valerate administration or by stress procedures is a principal component in the development of ovarian cyst in the rat *(7–9)*.

We recently found that 3 wk of chronic (cold and restraint) stress to rats activates sympathetic nerves to the ovary and promotes the development of ovarian cysts *(9)*. However, prolonged periods of stress (11 wk) were no longer effective in maintaining the increased levels of ovarian NE release and the ovarian cysts found at 3 wk of stress. The hypothesis was is that a compensatory mechanism driven by the adrenal gland could be responsible for the decrease in ovarian nerve activity found at 11 wk of stress. In the present work, we studied the changes produced by adrenalectomy on the increase in ovarian sympathetic nerve activity and steroid secretion induced by stress. As a marker of ovarian neuroendocrine function, we measured the amount of NE and its release from the ovary, the βAR concentration, and its coupling to steroidogenic response.

Results

The Effect of ADX on Stress-Induced Changes in Luteinizing Hormone (LH) Plasma Levels, Ovarian NE Content, and Dopamine-β-Hydroxylase (DβH) and NE from the Celiac Ganglion

A decrease in mean LH plasma levels in ADX, stress and ADX-stress rats was found. A decrease in ovarian NE content and in DβH activity in the celiac ganglia were found at 3 wk ADX (Table 1). Although stress produced an increase in NE content of the celiac ganglia (where NE neurons projecting to the ovary originate), no change in NE content at the ovary was found (Table 1). Stress applied to ADX rats recovered the decreased amount of NE in the ovary, but increased NE content and recovered DβH activity at the celiac ganglion.

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Table 1

Effects of ADX in Changes in LH Plasma Levels, NE, and DβH in Celiac Ganglion and the Ovary Induced by Chronic Stress to Rats*a*

	Control	ADX	Stress	ADX-stress
Ovary weight	44 ± 3	45 ± 6	42 ± 3	35 ± 2
(mg)	(n = 10)	(n = 10)	(n = 10)	(n = 10)
NE content				
(NE ng/mg ovary)	0.08 ± 0.01	0.05 ± 0.01*b*	0.08 ± 0.02	0.12 ± 0.02
	(n = 7)	(n = 4)	(n = 6)	(n = 6)
(NE ng/ganglion)	75 ± 11	65 ± 6	151 ± 20*c*	113 ± 13*c*
	(n = 4)	(n = 4)	(n = 4)	(n = 3)
DβH activity				
(nmol/h/ganglion)	238 ± 22	149 ± 16*b*	252 ± 17	217 ± 8
	(n = 3)	(n = 4)	(n = 4)	(n = 5)
LH (ng/mL)	20 ± 4.7	5.0 ± 0.2^c	5.2 ± 1.0^c	3.9 ± 1.2^c
	(n = 7)	(n = 5)	(n = 4)	(n = 3)

*a*All animals were sacrified during diestrus. Animals were stressed during 3 wk, and for ADX rats, stress procedure began 1 d after surgery. LH plasma levels were determined by RIA and expressed as ng/mL using rat LH-RP-1 as standard. NE and DβH were determined by radioenzymatic assay. DßH activity is expressed as nmol octopamine formed/h/ganglion. Results are expressed as mean value ± SEM of the number of experiment shown in parentheses.

*b*p < 0.05 vs control.

*c*p < 0.01 vs control.

The Effect of ADX on Stress-Induced Changes in Ovarian NE Release, βAR Content, Progesterone, and Androgen Secretion from the Ovary

Total NE measured in whole ovarian homogenates decreased 3 wk post-ADX. When ovarian halves were provided with [^{3}H]NE, the radioactive marker is incorporated to the nerve terminal and could be released following electrical stimulation. In the intact and adrenalectomized animals, this ability was further enhanced by stress In rats, ovarian NE release was increased 3 weeks following ADX (Fig. 1A). A further increase in NE release occurred after 3 wk of stress and also when the stress procedure was applied to adrenalectomized rats. Although adrenalectomized rats presented an increased number of ovarian βAR, three weeks of stress decreased the number of βAR when it was applied either to normal or ADX rats (Fig 1B). The increased βAR number found in ADX rats was correlated with an increase in basal, but not in isoproterenol-induced androgen and progesterone secretion from the ovary in vitro (Fig. 2). The decreased number of receptors found in stressed (control and ADX) rats was correlated with a decrease in isoproterenol-induced progesterone and androgens release from the ovary in vitro. Basal secretion of progesterone and androgens from the ovary of stressed (control and ADX) rats was higher than control.

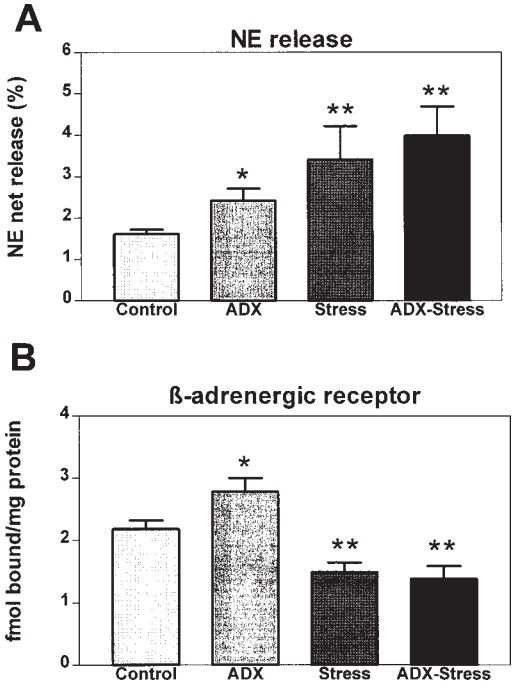

Fig. 1. Release of newly incorporated (^{3}H)NE (panel **A**) and βAR (panel **B**) from the ovaries of rats after 3 wk of ADX, 3 wk of stress (Stress), and under the combined effect of ADX and stress (ADX-Stress). Panel A: Bars correspond to the total amount of NE released after 1 min of transmural stimulation (80 V, 2 ms length, 10 Hz) minus the basal release without stimulation. Results are expressed as the percent fractional release and represent the mean ± SEM of 4 individual experiments/group. * p < 0.05 vs control and ** p < 0.02 vs control. Panel B: βAR concentration is expressed as fmol ^{3}H-dihydroalprenolol bound/mg protein and represents the mean ± SEM of n = 4 experiments.

Discussion

We found that ADX produces an increase in NE release from the sympathetic nerve terminals of the ovary and also an upregulation of the ovarian βAR. Although it is difficult to dissect, the decrease in DβH activity and no increase in

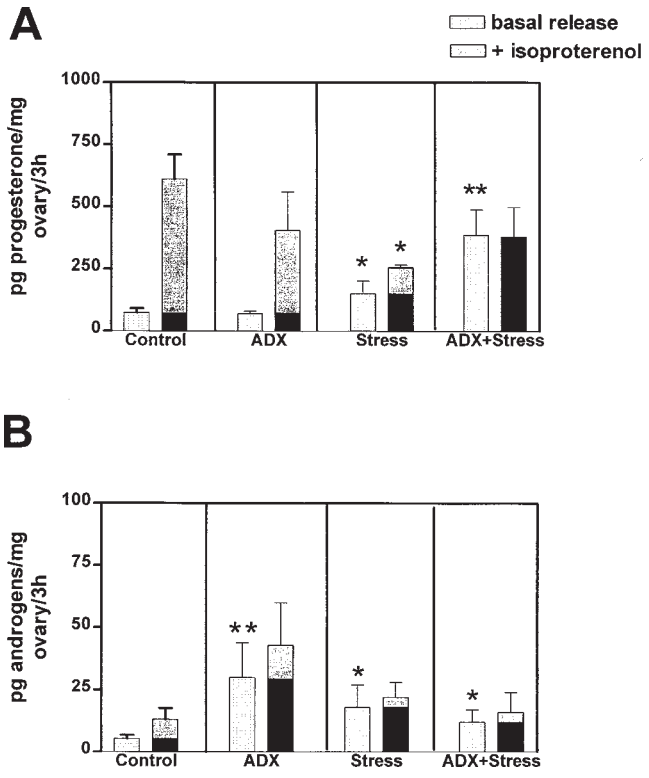

Fig. 2. Isoproterenol-induced secretion of progesterone (panel **A**) and androgens (panel **B**) from the ovaries of rats after 3 wk of ADX, 3 wk of stress (Stress) and under the combined effect of ADX and stress (ADX-Stress). The ovaries were halved and incubated for 3 h in Krebs Ringer bicarbonate buffer alone (basal) or isoproterenol (10 μ*M*). The amount of steroids secreted into the incubation medium was measured by RIA. Each bar represents the mean value ± SEM of four independent observations/group. Net release of steroids corresponds to the value of secretion in the presence of Iso minus the basal secretion (represented as a black area under each bar). * $p < 0.05$ vs same condition as control; ** $p < 0.02$ vs same condition as control.

NE content at the celiac ganglion after ADX suggest that the changes in noradrenergic activity found in the ovary could be locally produced by the ovary. Because of the location as a constitutive protein in the membrane of NE-storing vesicles, the activity of DβH (the enzyme that catalyzes the conversion of dopamine to NE) has been widely used as a vesicular marker *(10,11)*. Thus, changes in the activity of the enzyme might represents changes in the amount of NE vesicles being produced and transported from the neuronal body to the nerve terminal. Thus, the decrease in DβH activity found at the celiac ganglion could represents be a reflex of an increased axonal flow to the nerve terminal. To maintain the increased NE release at the ovary without affecting the expression of NE-synthesis enzymes at the celiac ganglion level, an increased supply of neurotransmitter and storage vesicles to the nerve terminal is needed *(12)*. The increase in ovarian ß-adrenergic receptor in spite of an the increased NE release that could produce a downregulation of receptors, could also represent a direct effect of an adrenal product (absent after ADX) on theca-interstitial cells (the ovarian cells in close synaptic contact to nerve cells) of the ovary. Corticoids of adrenal origin have been identified as potential down regulators of ovarian βAR *(13)*. Thus the increased androgen secretion in ADX rats could be the result of the tonic increase in NE release acting on the increased amount of βAR. Although we cannot rule out a direct effect of hormones from the hypothalamic-pituitary axis that could be modified by ADX or stress, we found low LH plasma levels (a possible activator of NE release, *[3]*) in ADX and stressed rats (data not shown). We do not, however, have data for a direct effect of prolactin or adrenocorticotropic hormone (ACTH) on ovarian NE release. The incapability to respond to neurogenic (isoproterenol) stimulation could be the result of a tonic increase in the release of NE from the ovary of ADX rats that maximizes basal secretion of progesterone and androgens, thus decreasing the ability making it difficult to release more steroids under the neurogenic stimulation.

Chronic cold and restraint stress by itself increases NE activity in the ovary *(9)*. Both stress procedures have also been previously shown to activate the adrenal response in catecholamine release *(14)*. In the case of the ovary, the effect could be the result of an increased firing rate of sympathetic nerves within the ovary. This is supported by the increase in NE concentration at the celiac ganglion level and the increased release of NE at the nerve terminals of the ovary. Control and ADX rats under stress conditions presented increased levels of NE release and low levels of βAR. Probably the increase in NE release produced a ligand-dependent downregulation of receptors; this would contrast with upregulation found in ADX rats without stress. This last suggestion would was also be supported by the absence of isoproterenol-induced progesterone and androgen secretion we observed even though the system was functional, as illustrated by the higher secretory activity of in spite of the higher secretory activity found in both steroidal hormones under basal (nonstimulated) conditions. We recently found that 3 h of stress/d for a 3-wk duration produced an increase in the basal and isoproterenol-induced androgen secretion from the ovary *(9)*. Although the magnitude of stress used in the present work (1.5 h/d instead of 3 h/d) was sufficient to induce chronically an increase of androgen release (1) it was not enough to facilitate the neurogenic-induced release of androgens from the ovary or (2) because of the decreased βAR concentration, the potentiation of NE release we found after 3 wk of stress was not sufficient to stimulated the androgen release from the ovary in ADX rats.

In conclusion, we found that ADX produced an increase in ovarian NE release, upregulation of βAR, and an increase in androgen release from the ovary. The further increase in NE release found in stressed rats (whether control or ADX) appeared to downregulate βAR, resulting in a decrease in isoproterenol-induced, but not in basal secretion of andro-

gens from the ovary. Because androgens participate in the development of ovarian cysts in rats, the present observations could explain why prolonged stress of control rats reversed the polycystic condition.

Experimental Procedures

Animals

Virgin adult cycling Sprague-Dawley rats (200–220 g) were used. They were allowed free access to pelleted food and tap water and were housed (2 rats/cage) in quarters with controlled temperature (22°C) and photoperiod (lights on from 0700 to 1900 h). Only animals exhibiting regular 4-d estrous cycles were used for the experiments. Estrous cyclicity was monitored by daily vaginal smears obtained between 1000 and 1200 h. The experimental procedures were previously approved by the Institutional Review Committee of Facultad de Ciencias Químicas y Farmacéuticas. We used two experimental groups (control and adrenalectomized rats) of 20 rats each. Adrenalectomy was performed surgically via a dorsal incision under ether anaesthesia. ADX rats were maintained with 0.9% NaCl drinking water and fresh vegetables in addition to their pelleted food. Two ADX and two ADX-stress rats died during the experimental procedure. In each group, half served as controls, whereas the others were exposed to a combined cold and restraint stress procedure as previously described *(9)* with minor modification (1.5 h/d instead of 3 h). Both types of stress produce increased activation of the sympathoadrenal system *(9,14–18)*. The stress procedure began 1 d after adrenalectomy. Control and ADX rats were placed in a restriction cage and were kept for 1.5 h in a cold room at 4°C for 5 d each week (Monday to Friday). The procedure was continued for 3 wk. We choose this time schedule, because previous observations in our laboratory showed that under this stress schedule, rats undergo a phase in which they lose their capability to ovulate in a cyclic manner. If stress is continued up to 11 wk, there was a recovery of their ovulating cycling activity *(9)*. We have recently published the effect of a 3-h stress procedure, for 3 and 11 wk, on the sympathetic innervation of the ovary *(9)*. In that publication, we proposed that corticoid from the adrenal gland could decrease ovarian sympathetic activity. Because increased nerve activity could mask a subsequent increase in sympathetic activity following the stress procedure, in this study, we decreased the length of stress exposure in order to protect the ovary from the possible increase in nerve activity developed after ADX. After 3 wk of stress, the rats were killed by decapitation, total blood was collected for LH assay, and ovaries and celiac ganglia were rapidly removed. Ovaries for measurement of NE release or steroid secretion were immediately transferred to Krebs-bicarbonate buffer for preincubation. Ovaries and celiac ganglia for NE determination and or DβH activity were frozen at –80°C.

Release of NE

The experimental procedure was as previously described *(3,19)*. Ovaries, removed through an abdominal midline incision, were cut in half. Two halves, one from each ovary, were stored together at –80°C for NE determination. The other two halves were preincubated for 20 min in Krebs-bicarbonate buffer, pH 7.4, and incubated for 30 min at 37°C with 2 mCi of [^{3}H]NE (SA 40.1 Ci/mmol, New England Nuclear, Boston, MA). No differences in NE content and release between halves has been found. After washing (to remove any radioactivity not incorporated), the two halves were transferred to a thermoregulated superfusion chamber and perifused at a flow rate of 2.5 mL/min. One-minute fractions were collected; after 3 min, the ovaries were subjected to a train of monophasic electrical pulses (80 V, 10 Hz, 2 ms, 1 min). After the stimulation, four 1-min samples of perfusion buffer were collected (for details see *[19]*). At the end of the experiment, the halves were homogenized in 0.4 *M* $HClO_4$, the homogenate was centrifuged (15,000*g*,10 min), and [^{3}H]NE remaining in the tissue was determined by scintillation counting. Overflow of radioactivity was calculated as a percentage of the fractional release and expressed as net release, i.e., the total NE released after 1 min of stimulation minus the spontaneous NE release from the ovary under a nonstimulated condition. The method used here permits us to mimic the physiological in vivo conditions of nerve activity in vitro by using train of electrical pulses at a frequency normally used by sympathetic nerves (10 Hz) in vivo and represents the methodology of choice to study the activity of sympathetic nerves in vitro without the contribution of other putative regulators present in plasma *(3,19)* that could mask results.

Steroid Response to βAR

Ovaries were halved. Halves were incubated in vitro in 2 mL Krebs-Ringer bicarbonate buffer, pH 7.4, for 3 h at 37°C *(5,20,21)* with D,L-isoproterenol-HCl (10^{-5} *M*; Sigma Chemical Co, St Louis, MO), or without drug (basal). Progesterone and androgen released into the incubation medium were measured by radioimmunoassay *(5,21)*.

Plasma LH Levels

LH was measured by RIA using kits provided through the NIDDK National Pituitary Agency. Results are expressed in terms of the rat LH RP-1 standard preparation.

Determination of NE and Protein

The remaining ovarian halves from NE release experiments and the celiac ganglia were each homogenized in 0.2 *M* $HClO_4$. The suspensions were centrifuged (15,000*g*, 10 min), and catecholamines present in the supernatant were determined by a specific radioenzymatic method *(22)* as previously described *(3,19)*. Pellets were dissolved in 1 *M* NaOH, and protein content was determined *(23)* with bovine serum albumin (BSA) as standard.

βAR Determination

Membranes from whole ovaries were prepared as described *(3,8)*. In brief, the tissues were homogenized in 0.02 *M* Tris/HCl, 0.25 *M* sucrose, pH 7.4 and the homogenates centrifuged at 30,000*g* for 20 min. The resulting pellets were washed and resuspended in 0.02 *M* Tris-HCl, 10 m*M* $MgCl_2$, pH 7.4 (assay buffer), and used in the radioreceptor assay. The assay was carried out with 2 n*M* [^{3}H]dihydroalprenolol (SA 92.0 Ci/mmol, New England Nuclear) and 20–30 mg of membrane protein in a total volume of 0.2 mL. Nonspecific binding was assessed in tubes containing 10^{-4} *M* DL-propanolol. Samples were incubated for 30 min at 37°C. Binding was terminated by the addition of 10 vol of assay buffer and vacuum filtration through Whatman GF/C fiber glass filters as described *(3)*. Radioactivity retained by the filters was determined by liquid scintillation counting.

DβH Activity

DβH, the final enzyme of NE biosynthesis, is used as a biochemical marker for the presence of NE storage vesicles and, therefore, as a measure of the potential for releasing capacity to release NE *(24)*. Aliquots (0.2 mL) of celiac ganglia homogenates were used. The enzyme activity was determined by the enzyme-catalyzed hydroxylation of ^{3}H-tyramine to ^{3}H-octopamine as described *(10,24)*. This product was chemically oxidized to ^{3}H-p-hydroxybenzaldehyde and selectively extracted in toluene.

Statistics

Differences between control and experimental groups were analyzed with Student's *t*-test. When percentages were compared (release experiments), data were normalized by arc-sine transformation before statistical evaluation. Comparisons between several groups were made by one-way analysis of variance, followed by the Student-Newman-Keuls multiple-comparison test for unequal replications.

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References

1. Ojeda, S. R. and Lara, H. E. (1989) In: *The Menstrual Cycle and Its Disorders*. Pirke, K. M., Wuttke, W., and Scheiwerg, U., eds. Springer-Verlag, Berlin, pp. 26–32.
2. Burden, H. W. (1985) In: *Catecholamines as Hormones Regulators*. Ben-Jonathan, N., Bahr, J. M., and Weiner, R. I., eds. Raven Press, New York, pp. 261–278.
3. Ferruz, J., Barria, A., Galleguillos, X., and Lara, H. E. (1991) *Biol. Reprod.* **45,** 592–597.
4. Jordan, A. W. (1981) *Biol. Reprod.* **24,** 245–248.
5. Aguado, L. I., Petrovic, S. L., and Ojeda, S. R. (1982) *Endocrinology* **110,** 1124–1132.
6. Hernandez, E. R., Jimenez, J. L., Payne, D. W., and Adashi, E. Y. (1988) *Endocrinology* **122,** 1592–1602.
7. Lara, H. E., Ferruz, J. L., Luza, S., Bustamante, D., Borgez, Y., and Ojeda, S. R. (1993) *Endocrinology* **133,** 2690–2695.
8. Barria, A., Leyton, V., Ojeda, S. R., and Lara, H. E. (1993) *Endocrinology* **133,** 2696–2703.
9. Paredes, A., Galvez, A., Leyton, V., Ararena, G., Fiedler, J. L., Bustamante, D., and Lara, H. E. (1998) *Endocrine* **8,** 309–315.
10. Lara, H. E. and Belmar, J. (1989) *Neurochem. Int.* **15,** 445–454.
11. Viveros, O. H., Arqueros, L., Connett, R. J., and Kirshner, N. (1969) *Mol. Pharmacol.* **5,** 60–68.
12. Fried, G., Terenius, L., Holkfelt, T., and Goldstein, M. (1985) *J. Neurosci.* **5,** 450–458.
13. Aguado, L. I. and Ojeda, S. R. (1986) *Biol. Reprod.* **34,** 45–50.
14. Pacak, K., Palkovits, M., Yadid, G., et al. (1998) *Am. J. Physiol.* **275,** R1247–155.
15. Bhatnagar, S., Mitchell, J. B., Beitto, K., et al. (1998) *Am. J. Physiol. Behav.* **57,** 633–639.
16. Mansi, J. A. and Drolet, G. (1997) *Am. J. Physiol.* **273,** R3813–R820.
17. Kvetnansky, R. and Mikulaj, L. (1970) *Endocrinology* **87,** 738–743.
18. Viau, V. and Meaney, M. J. (1991) *Endocrinology* **129,** 2503–2511.
19. Ferruz, J., Ahmed, C. E., Ojeda, S. R., and Lara, H. E. (1992) *Endocrinology* **130,** 1345–1352.
20. Aguado, L. I. Ojeda, S. R. (1984) *Endocrinology* **114,** 1944–1946.
21. Lara, H. E., McDonald, J. K., Ahmed, C. E., and Ojeda, S. R. (1990) *Endocrinolgy* **127,** 2199–2209.
22. Saller, C. and Zigmond, M. A. (1978) *Life Sci.* **23,** 1117–1130.
23. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254.
24. Lara, H. E. and Belmar, J. (1991) *Biol. Reprod.* **44,** 752–759.