Chronic Intermittent Cold Stress Activates Ovarian Sympathetic Nerves and Modifies Ovarian Follicular Development in the Rat[1]

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ABSTRACT

We studied the effects of a chronic intermittent cold stress regime on sympathetic nerve activation and ovarian physiology. This paradigm (4°C for 3 h/day, Monday–Friday, for 3 or 4 wk) does not affect basal plasma levels of corticosterone. After 3 wk of stress, we detected a decrease in noradrenaline (NA) in the ovary, but after 4 wk, this ovarian neurotransmitter concentration increased over that of unstressed control rats. To analyze whether this effect on NA is preceded by an activation of the neurotrophic factor system responsible for growth and survival of sympathetic neurons, we measured both nerve growth factor (NGF) (by enzyme immunoassay) and the intraovarian levels of its low affinity receptor mRNA (by reverse transcription-polymerase chain reaction). The activation of sympathetic nerves was followed by an increase in NGF concentration without affecting the ovarian levels of either NGF or the mRNA of its receptor. Interestingly, follicular development changed during the stress procedure; after 3 or 4 wk of stress, we found a decrease in preantral healthy follicles without a compensatory increase in atresia. Concomitantly with the increase in NA and NGF in the ovary, we observed that a new population of follicles with hypertrophied thecal cell layers appeared after 4 wk of stress. These results suggest that chronic stress, through an intraovarian neurotrophin-mediated sympathetic activation, produces changes in follicular development that could lead to an impairment of reproductive function.

ovary, stress

INTRODUCTION

Much evidence suggests that psychological stress is prevalent in patients with polycystic ovary syndrome [1, 2]. Although these data do not suggest that stress is the only factor that participates in the etiology of the disorder, they do suggest that stress could be an important factor. Studies on the role of the neurotransmitters involved in the reproductive response to stress have mainly concentrated on the central neurotransmitters that affect GnRH secretion, the LH/FSH ratio, and hence ovulation [3]. Neurotransmitters impinging on hypothalamic endocrine neurons are not, however, the only neural influences on the ovary. Another pathway, more directly related to stress as a primary stimulus, has received considerable interest. Evidence has been accumulated establishing that the mammalian ovary's sympathetic innervation, which originates in the central nervous system [4, 5], participates in the control of ovarian function [6, 7]. The activity of these sympathetic nerves is believed to be associated with follicular development, steroid secretion, and ovulation [8, 9], and strong support for this view comes from experiments in which permanent loss of the sympathetic innervation, caused either by passive or active neonatal immunosympathectomy, resulted in delayed follicular development, reduced steroidal response to gonadotropins, and marked irregularities of the estrous cycle [10, 11]. Our previous studies using both pharmacological doses of estradiol valerate [12] and chronic combined cold and restraint stress [13] on rats were the first to demonstrate that hyperactivation of sympathetic nerves is directly associated with the aberrant follicular development that precedes a polycystic ovary. After chronic restraint stress was applied, the increased sympathetic activity returned to control levels after 3 wk [14], the minimal time necessary to complete a follicular cycle [15]. This reversion is likely due to restraint stress-induced secretion of corticosterone, a well-known inhibitor of sympathetic nerve activity [16], including that in the ovary [17]. To investigate the effects of chronic activation of ovarian sympathetic nerves on follicular development, the present study employed a stressor previously shown to not affect the corticotropic axis [18] as restraint stress does [19]. In addition, we studied the effect of chronic intermittent cold stress on the mediation of nerve growth factor (NGF), the neurotrophic factor responsible for growth and survival of sympathetic neurons.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats from a Universidad de Chile stock were used. Rats weighing 200 g were maintained in individual cages at 23°C in a 12L:12D regimen (lights off from 1900 to 0700 h) with food and water ad libitum. Only rats having regular 4-day estrous cycles were used for the study. Twenty-seven rats were divided into three groups of 9 rats each, with group a being the control, group b being the 3-wk stressed rats, and group c being the 4-wk stressed rats. No changes in estrous cycling in any experimental group was found. All animal procedures were performed using protocols previously approved by the Institutional Ethic Committee of Faculty of Chemical and Pharmaceutical Sciences, Universidad de Chile, and experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction.

Rats used for the cold stress procedure were transported, in their cages, to a 4°C cold room and remained there for 3 h each day, Monday–Friday, for 3 or 4 wk. Control rats were moved to a location near the cold room, then returned to the animal room after 3 h.

One hour after the last stress session, control and experimental rats

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were killed by decapitation because this procedure has been demonstrated to have the least effect on corticosterone (CORT) plasma levels [20]. Rats were transported, one by one, from an adjoining room to the sacrifice location.

Trunk blood was collected and its serum was stored at −20°C for later corticosterone, progesterone, and androstenedione determination. The ovaries were collected and immediately frozen at −80°C, with the exception of four left-side ovaries that were fixed for morphological studies.

Morphology

Fresh ovaries were immersed in Zamboni fixative, imbedded in paraffin, cut in 8-μm sections, and stained with hematoxylin and eosin. For morphometric analysis, we used the criteria presented before by Lara et al. [21], but we counted each slice instead of one of every five as we reported previously. We analyzed for the presence of preantral, antral, atretic, precystic, and cystic follicles according to Lara et al. [21]. Briefly, preantral (mainly secondary) follicles were defined as follicles without any antral cavity and with two or more layers of granulosa cells. Atretic follicles were defined as those follicles with more than 5% of cells that had pyknotic nuclei in the largest cross-section and showed shrinkage and an occasional breakdown of the germinal vesicle. Antral follicles were counted when the nucleus of the oocyte was visualized. Cystic follicles were defined as those follicles devoid of oocytes and displaying a large antral cavity, an enlarged thecal cell layer, and a thin (mostly monolayer) granulosa-cell compartment that contained apparently healthy cells.

Corticosterone, Androstenedione, and Progesterone Assays

Serum corticosterone levels were determined by RIA following the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO), as previously described [22]. Intra- and interassay variations were less than 5%; the minimal detectable value of CORT was 0.3 μg/ml serum. Serum (25 μl/sample) androstenedione was determined by enzyme immunoassay following the manufacturer's instruction (Alpco Diagnostic, Windham, NH). Intra- and interassay variations were less than 6%; the minimal detectable value of androstenedione was 0.02 ng/ml serum. Progesterone plasma levels were determined by a Microparticle Enzyme Immunoassay (Ortoclinical Diagnostic, Amersham, UK). Intra- and interassay variations were less than 5%; the minimal detectable value of progesterone was 0.1 ng/ml serum.

RNA Preparation and Semiquantitative Reverse Transcription-Polymerase Chain Reaction

Ovarian total RNA was extracted using Trizol Reagent (Gibco BRL, Gaithersburg, MD) and treated for 30 min at 37°C with RNAase-free DNAase RQ1 (Promega, Madison, WI) to eliminate any potential genomic DNA contamination. Reverse transcription was performed on 5 μg of total RNA per sample, with a regime of 22°C for 10 min and then 42°C for 45 min using 1.6 mM NTPs, 10 mM DTT, 176 nM random hexamers (Gibco BRL), 25 U RNAsin (Promega), 125 U reverse transcriptase (Gibco BRL), and first strand buffer, in a final volume of 30 μl. Dilutions of the RT reaction were incubated with 1 U of DNA Taq polymerase (Promega), 0.8 mM dNTPs, 25 pmol of each primer in a final volume of 50 μl. For each sample, the same reverse transcription (RT) reaction was used to measure the level of specific mRNA by polymerase chain reaction (PCR) but in separate tubes to avoid competition between primers. The PCR was programmed for 35 cycles (NGF), 31 cycles (p75 NGFR) or 21 cycles (β-actin) and consisted of denaturation at 94°C for 1 min, annealing at 60°C for 60 sec, and extension at 72°C for 1 min using a DNA thermal cycler (MJ Research Inc., Watertown, MA). The PCR oligonucleotide primers were previously published [22–24]. To verify that the mRNA samples were not contaminated with genomic DNA, -actin primers were designed from a codogenic sequence of the β-actin gene, which spans an intronic sequence and generates either a 351-base pair (bp) fragment or an 809-bp fragment for cDNA or genomic DNA, respectively. All RT-PCR and PCR reactions included the use of water instead of template as negative controls. RT-PCR products were electrophoresed in 1.0% agarose gels, stained with ethidium bromide, and photographed. Band intensities were measured with the UN-SCAN-IT program (Silk Scientific, Orem, Utah) and normalized to that of the corresponding β-actin bands.

Noradrenaline Determination by HPLC

Frozen ovaries were homogenized in 300 μl of 0.2 N perchloric acid using a glass-glass homogenizer, centrifuged at 15 000 × *g* for 10 min, and 50 μl of the supernatant was subjected to concentration in alumina and quantified by HPLC with electrochemical detection [25] with dihydroxybenzyl amine (DHBA, 2000 pg in 20 μl) added as internal standard. Briefly, the 50-μl samples were mixed with 50 mg of activated alumina in 1 ml of Tris (1.5 M, pH 8.3–8.5) and 2% EDTA. The alumina was rinsed thoroughly with water and noradrenaline (NA) was eluted with 100 μl of 0.2 N perchloric acid, which was centrifuged, and 20 μl of the supernatant was injected into a Waters HPLC system with a C18 reverse phase column (Lichrosphere, 60 RP-Select B, Merck, Darmstadt, Germany) and an electrochemical detector (Waters 464). The mobile phase contained 0.1 M NaH_2PO_4, 0.42 mM octyl-sulphate, 0.02% EDTA, and 1.5% acetonitrile (pH 2.5) with a flow rate of 0.9 ml/min. The potential of the amperometric detector was set at 0.7 V. Under these experimental conditions, retention time was 4 min for NA and 10 min for the DHBA used to correct for procedural recovery.

NGF Quantification

NGF was measured using Promega's NGF E-max ImmunoAssay System. Each ELISA 96-well plate was coated overnight at 4° C with an anti-NGF polyclonal antibody (pAb; 1:1000; 100 μl/well) in the carbonate coating buffer (0.05 M sodium bicarbonate/carbonate, pH 9.7). On the following day, the contents of the wells were removed and the plates were washed with Tris-buffered saline with Tween (TBST; 20 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.05% v/v Tween-20). After that, they were incubated at room temperature (RT) for 1 h in block and sample buffer (200 μl/well). Two columns on the ELISA plates were designated for the NGF standard curve as provided by the supplier. The final concentrations within the plates were 0–500 pg/ml. Ovarian tissue samples were weighed, homogenized in 1:4 Dulbecco phosphate buffered saline (DBPS, pH 7.35), and centrifuged for 15 min (13 000 rpm). The samples to be assayed were prepared as follows. A 200-μl aliquot of the tissue sample was added to the well and three successive 1:2 dilutions were made. Sample or standard was incubated for 6 h and washed and incubated overnight at 4°C with anti-NGF mAb. At the end of the procedure, plates were washed and incubated with anti-rat IgG and HRP conjugate for 2.5 h at room temperature. After the incubation, a tetramethylbenzidine and the peroxidase substrate solution was added to each well, and the plates were shaken for 5–10 min at RT; the reaction was terminated by adding 1 N hydrochloric acid to the wells. The optical density of the reaction product was read on a microplate reader at 450 nm, and values were normalized per gram of tissue assayed.

Statistics

Differences between control and experimental groups were analyzed with the Student *t*-test, and comparisons between several groups were made by one-way analysis of variance, followed by the Student-Newman-Keuls multiple comparison test for unequal replication.

RESULTS

Effect of Cold Stress on Corticosterone, Androstenedione, and Progesterone Serum Levels

No changes in corticosterone serum levels were found among control rats and 3- or 4-wk stress rats or between the two stress procedures (mean value ± SEM of five rats in each experimental condition was 3.5 ± 1.5 μg/100 ml for controls vs. 6.2 ± 2.0 μg/100 ml or 5.5 ± 1.0 μg/100 ml for 3 and 4 wk of stress, respectively). These results confirm previous data [18] that show that a similar protocol of chronic and intermittent cold stress causes no change in the corticosterone plasma levels, and supports the reports of nonactivation of the hypothalamus-pituitary-adrenal axis with this stress procedure. On the contrary, a progressive decrease in androstenedione serum levels was found, reaching a significant decrease after 4 wk of stress (0.49 ± 0.12 ng/ml for controls vs. 0.36 ± 0.12 ng/ml after 3 wk and 0.19 ± 0.06 ng/ml after 4 wk of stress, with $P < 0.05$ vs. control levels, but not within 3 and 4 wk of stress; results are the mean value ± SEM of five animals in each group). Progesterone serum levels increased after 4 wk but not at 3 wk of stress (26.1 ± 4.7 ng/ml for controls vs. 23.5 ±

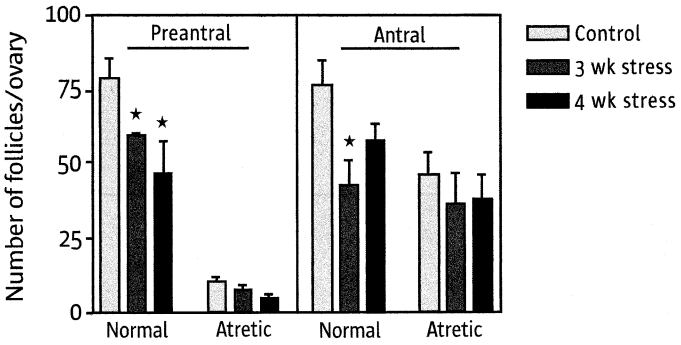

FIG. 1. Changes in the number of normal preantral (secondary), atretic preantral, normal antral, and atretic antral follicles in controls and after 3 or 4 wk of cold stress. Results are expressed as the mean value ± SEM of the number of follicles in one ovary from four rats in each group. *$P < 0.05$ vs. control value.

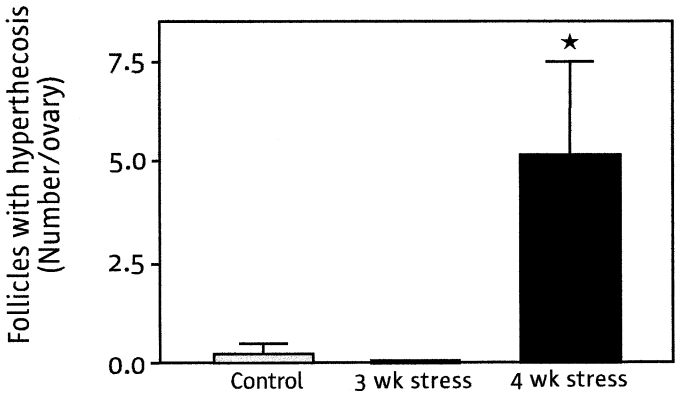

FIG. 2. Increase in antral follicles with a hypertrophied thecal cell layer after 4 wk of stress. The figure represents the number of follicles with a hypertrophied thecal cell layer per ovary. Results are the mean value ± SEM of four ovaries from different rats in each experimental condition. *$P < 0.05$ vs. control.

1.9 ng/ml after 3 wk and 60.0 ± 13.8 ng/ml after 4 wk of stress, with $P < 0.05$ vs. control levels; results are the mean value ± SEM of five animals in each group). No differences in estradiol serum levels were found among any experimental conditions (data not shown).

Effect of Cold Stress on Morphological Aspects of the Ovary

After 3 and 4 wk of stress, a morphological examination of the ovaries showed a decrease in the total population of follicles (210 ± 20 follicles for controls vs. 145 ± 10 and 150 ± 5 follicles per ovary after 3 and 4 wk of stress, respectively; $P < 0.05$ for 3 and 4 wk vs. controls; mean value ± SEM of four individual rats in each experimental condition). This population included healthy as well as atretic preantral and antral follicles. To determine whether this change was prevalent in one type of follicle, we quantified each population of follicles per ovary (Fig. 1). There was a decrease in normal preantral secondary follicles after both 3 and 4 wk of stress compared with control rats, but the number of normal antral follicles decreased after 3 wk and recovered to control levels after 4 wk of stress. No changes in the number of atretic follicles after 3 or 4 wk of stress were found. Although the number of normal antral follicles after 4 wk of stress had returned to values similar to control, nonstressed animals, a new population of follicles with an enlarged theca cell layer—and not included in the normal antral population of follicles—was clearly different from the normal antral follicles (Fig. 2). In Figure 3, the appearance of these follicles is shown. Theca cells from normal antral follicles (Fig. 3C) are aligned parallel to one another and form a radial arrangement of elongated fibroblast-like cells around the entire follicle, but theca cells from follicles of stressed rats (Fig. 3D) presented a polygonal-type structure with the aspect of hypertrophied differentiated theca cells, leading to an increase in the thickness of the theca layer.

Effect of Stress on Ovarian NA and NGF Content

The analysis of ovarian NA concentration showed that an initial decrease in NA concentration after 3 wk of stress

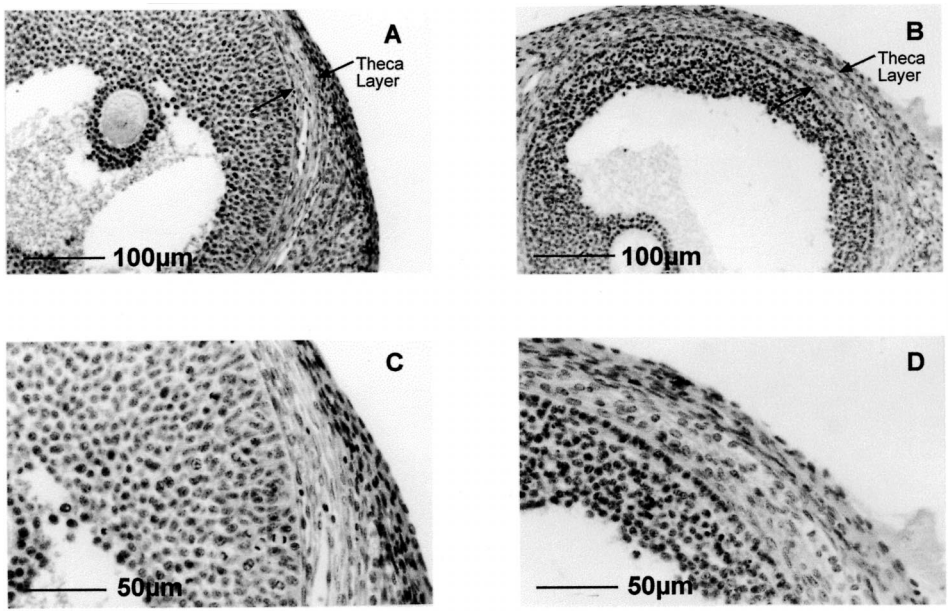

FIG. 3. Morphological aspect of a control antral follicle (**A**, **C**) compared with a hypertrophied follicle of a 4-wk stressed rat (**B**, **D**). The bar indicates the magnification of the picture.

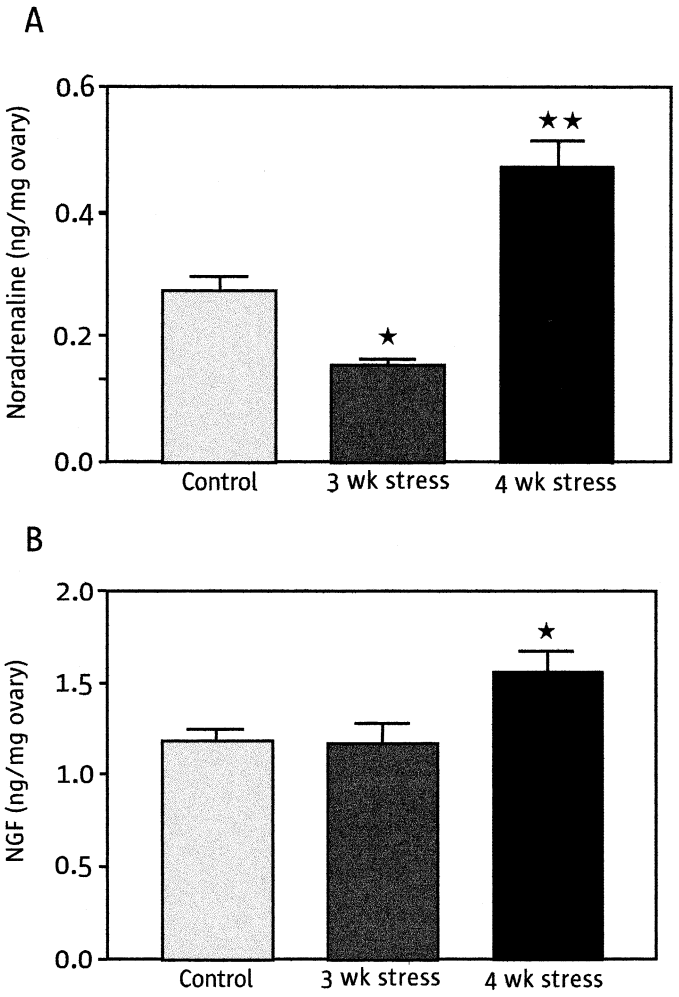

FIG. 4. Changes in the noradrenaline (**A**) and NGF (**B**) concentration in the ovary from control and stressed rats. Bars are mean value ± SEM of at least four individual animals per experimental group. *, $P < 0.05$ vs. control; **, $P < 0.01$ vs. control.

was followed by an increase over control values after 4 wk (Fig. 4A). No changes in NGF concentration were found after 3 wk of stress, but a clear increase in the peptide was found at 4 wk of stress (Fig. 4B)

Effect of Stress on Ovarian NGF mRNA and Its Low Affinity Receptor

To study the relationship between NGF and its low affinity receptor (p75 NGFR)—the most abundant intraovarian neurotrophic receptor present in theca cells and colocalized with the peptide [26]—we measured the relative amount of the mRNAs encoding both the peptide and its receptor by semiquantitative RT-PCR. Though we did not perform real-time PCR, for greater certainty of the values, we did establish the linear range of initial concentration to perform the analyses (Fig. 5A). As shown in Fig. 5B, the increase in the intraovarian concentration of NGF found after 4 wk of stress was not accompanied by a similar increase in its mRNA or in the mRNA for its low affinity receptor, p75 NGFR.

DISCUSSION

Herein we demonstrate that a chronic intermittent cold stress protocol produces changes in follicular development of the rat ovary and that these changes are associated with a neurotrophin-dependent sympathetic nerve activation.

The primary effect of stress appears to be a central activation of sympathetic nerves expressed as an initial decrease in ovarian NA content that could be explained by an increased outflow of catecholamines from the ovary. In support of this, we have previously found that NA content in the cat ovary decreased after the induction of ovulation with gonadotropins [27]. The close correlation found between the decrease in total tissue NA and the NA stored in intracellular vesicles of sympathetic nerve terminals of the ovary (analyzed by differential centrifugation and iso-osmotic gradients) permitted us to suggest that the decrease in NA content was the result of an increased release of NA from the tissue. Because these changes are characteristically seen after a sympathetic discharge [27], their occurrence in the rat ovary strongly suggest an increased activity of the ovarian nerves after 3 wk of stress. In contrast, the increase in NA found after 4 wk of stress could be the result of a compensatory mechanism mediated by the neurotrophic effect of NGF at the celiac ganglion level, where it is able to stimulate transcription of NA's biosynthetic enzymes, as was demonstrated to occur in sympathetic ganglia [28] and as we also previously found in the rat ovary during the estradiol valerate-induced polycystic ovary [21]. In this last case, estradiol acts directly to stimulate NGF and p75NGFR gene expression due to the presence of estradiol-responsive

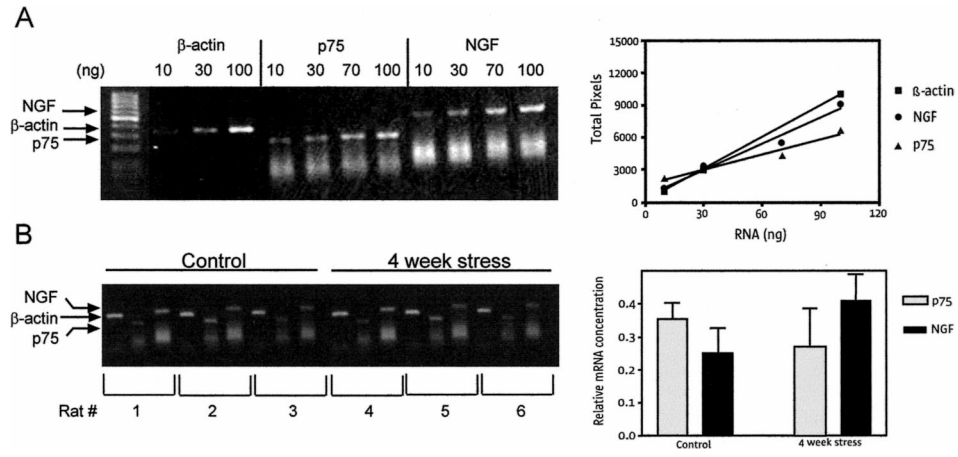

FIG. 5. Changes in the mRNAs for NGF and p75 NGFR at 3 or 4 wk of stress. **A**) The linear range of RT-PCR for β-actin, p75 NGFR, and NGF mRNAs, as expressed from the initial amount of total RNA used for the assay. On the right side is shown the densitometric analysis of the gel shown on the left. **B**) The gel for the individual samples (60 ng total RNA each) from controls and 4-wk stressed rats. On the right side is shown the densitometry quantification of the gel. Numbers represent the mean value ± SEM from three individual ovaries for each experimental condition.

elements in both genes [29, 30]. The stress procedure used in the present work did not modify either estradiol serum levels compared with rats at the same stage of the estrous cycle at the time of sacrifice nor estrous cyclicity of the rats, as monitored by daily vaginal lavages, suggesting that there was no effective disturbance in estrogen plasma levels through the estrous cycle, as there are with other procedures that activate sympathetic nerves [13]. Thus, the increase in NGF found in the ovary is not mediated by hormonal stimulation of NGF transcription but rather is a compensatory response to an increased outflow of NA from the nerve terminal, as has been previously demonstrated to occur in sympathetic ganglia [28]. The fact that independent of changes in estrogens and corticosterone plasma levels we still induced nerve activation with a concomitant increase in NGF concentration suggests that the ovary of adult, as do other organs sympathetically innervated, also maintains a close relation between nerve activity and neurotropic factor as a mechanism to control both growth and survival of the neurons, as we have previously found to occur after the administration of antibodies against NGF to neonatal rats [11].

The cold stress procedure did cause significant changes in the development of ovarian follicles. The decreased number in preantral secondary follicles (60–150 μm in diameter) found at 3 and 4 wk of stress could be responsible for the decrease in antral follicles found at 3 wk of stress. This situation was, however, reversed after 4 wk of stress because the number of antral follicles not only recovered to control values but a new population of follicles also appeared, with enlarged theca cell layers that, according to morphological characteristics, seem to derive from antral follicles. It has recently been demonstrated that, during the first stage of follicular development (i.e., the transition between primordial to secondary follicles), intraovarian NGF participates both in the differentiation of mesenchymal cells to form the primary follicle [31] and in the proliferation and differentiation of granulosa cells to form secondary follicles that present specific receptors to and respond to FSH [32]. Because adult rats have already defined their populations of primordial follicles during the first days after birth, the possibility exists that the increased levels in ovarian NGF found in the ovary of adult rats after 4 wk of stress could promote the maturation of a population of secondary follicles (even more decreased in number). This effect, in addition to the increased sympathetic tone (as stress does), move this population of follicles toward the antral follicle pool to permit the recovery of the antral follicles found after 4 wk of stress. Further support of the idea that the activities of the nerves are important in promoting the development of follicles comes from experiments showing that permanent destruction of sympathetic nerves decreased the number of antral preovulatory follicles [33].

Intraovarian NGF has been involved in proliferation and differentiation of theca cells of the follicles [24]. The low affinity receptor and NGF peptide are mainly located in ovarian thecal cells [26]; thus, any changes found in the whole ovary essentially represent what is occurring in this cellular compartment. The appearance of a new population of antral follicles with a thicker layer of theca cells in addition to the increased progesterone serum levels found in the 4-wk stressed rats support the concept that this population of follicles could then represent luteinized follicles that will not ovulate. Because of the decreased capacity to produce androgens and no changes in follicular atresia, they could represent follicles arrested at the preovulatory stage with no participation of the increased levels of NGF in the steroidogenic response of theca cells, as has been demonstrated to occur in the luteinized cells [24]. Therefore, the increase in NGF could be the response of a neurally stimulated ovary after a period of chronic stimulation by stress, leading to development of theca cell hypertrophy and a premature luteinization of the follicles. Because some of these characteristics are seen in the polycystic ovary syndrome in humans, we can hypothesize that chronic stress via a chronic sympathetic activation (probably through β-adrenergic receptors) could represent an etiological factor of the polycystic ovary. We have recently found experimental support for this explanation because direct stimulation of β-adrenergic receptors by in vivo administration of isoproterenol (a β-adrenergic agonist) induced the appearance of follicular cyst in the rat [34].

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