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Chronic-Intermittent Cold Stress in Rats Induces Selective Ovarian Insulin Resistance[1]

Mauricio Dorfman,[3] Victor D. Ramirez,[3,4] Elisabet Stener-Victorin,[5] and Hernan E. Lara[2,3]

Laboratory of Neurobiochemistry,[3] Department of Biochemistry and Molecular Biology, Faculty of Chemistry and Pharmaceutical Sciences, Universidad de Chile, Santiago 838-0492, Chile
Molecular Integrative Physiology,[4] Illinois University at Urbana-Champaign, Urbana, Illinois 61801
Institute of Neuroscience and Physiology,[5] Department of Physiology, Sahlgrenska Academy, Göteborg University, SE-405 30 Göteborg, Sweden

ABSTRACT

In rat ovary chronic cold stress increases sympathetic nerve activity, modifies follicular development, and initiates a polycystic condition. To see whether there is a relationship between the previously described changes in follicular development and metabolic changes similar to those in women with polycystic ovary, we have studied the effect of chronic cold stress (4°C for 3 h/day, Monday to Friday, for 4 wk) on insulin sensitivity and the effect of insulin on sympathetic ovarian activity. Although cold-stressed rats ate more than the controls, they did not gain more weight. Insulin sensitivity, determined by hyperinsulinemic-euglycemic clamp, was significantly increased in the stressed animals. Insulin in vitro increased the basal release of norepinephrine from the ovaries of control rats but not from those of stressed rats, suggesting a local neural resistance to insulin in stressed rats. The levels of mRNA and protein for IRS1 and SLC2A4 (also known as GLUT4), molecules involved in insulin signaling, decreased significantly in the ovaries but not in the muscle of stressed rats. This decrease was preferentially located in theca-interstitial cells compared with granulosa cells, indicating that theca cells (the only cells directly innervated by sympathetic nerves) are responsible for the ovarian insulin resistance found in stressed rats. These findings suggest that ovarian insulin resistance produced by chronic stress could be in part responsible for the development of the polycystic condition induced by stress.

catecholamines, insulin, insulin resistance, ovary, polycystic ovary, stress

INTRODUCTION

Psychological and physiological stressors can disturb neuroendocrine, reproductive, and metabolic functions [1–4]. Stress-induced neuroendocrine alterations include activation of the sympathetic nervous system. Previously, we showed that 4 wk of chronic intermittent cold stress caused ovarian morphology alterations, such as the development of follicles presenting a thickened theca cell layer [5]. When the time period of intermittent cold stress was increased to 8 wk, the ovary had a typical polycystic ovary morphology [6]. Changes in insulin sensitivity have been demonstrated both in chronic stress models [7] and polycystic ovary syndrome (PCOS) [8], one of the most common endocrine disorders in premenopausal women [9]. Insulin resistance accompanied by a mild compensatory hyperinsulinemia is a common feature of woman with PCOS, but we do not know whether the PCOS-like follicular changes following cold stress are related to changes in insulin resistance.

Ovarian sympathetic nerves participate in the control of ovarian function [10]. The activity of these sympathetic nerves is believed to be associated with follicular development, steroid secretion, and ovulation [11, 12]. These effects of norepinephine (NE) are probably mediated by adrenergic receptors located in the ovary [13–15]. We and others have demonstrated that ovarian sympathetic innervation is a potential factor involved in PCOS (a characteristic recently confirmed to occur in women with PCOS [16–18]). Activation of the sympathetic nervous system, determined by NE release, has been demonstrated in aortic slices incubated with insulin [19]. It is therefore possible that insulin modifies NE release in ovarian nerves.

Insulin is among the intraovarian factors that control local glucose utilization and steroidogenesis [20, 21]. In ovarian cells from women with PCOS, insulin resistance extends to polycystic ovaries and affects the insulin metabolic pathway [20, 22]. However, steroidogenesis by PCOS granulosa cells remains responsive to insulin in patients with peripheral insulin resistance [23]. We suggest that chronic-intermittent cold stress in rats might modify the sensitivity to insulin of the ovarian sympathetic nerves and/or other ovarian cells and thus explain the changes in follicular development found previously when rats were exposed to chronic cold stress [5]. We have studied the effect of insulin on ovarian adrenergic activity to determine whether there is a novel interaction between insulin and ovarian sympathetic function.

MATERIALS AND METHODS

Animals

Adult female Sprague Dawley rats from Universidad de Chile stock were used. Rats (200 g) were kept individually in cages at 23°C in a 12L:12D regimen (lights off from 1900 h until 0700 h) with food and water ad libitum. Only rats having regular 4-day estrous cycles were used for the study. The animals were randomly divided into two experimental groups: cold stress (4°C for 3 h/day, Monday to Friday, for 4 wk) and control (maintained at 23°C). Chronic-intermittent cold stress represents an example of physical stress [1]; with this protocol there is no change in plasma corticoids [5, 24]. The stage of cyclicity was determined by microscopic analysis of the predominant cell type in vaginal smears obtained daily (Monday to Friday) in both control and cold-stressed rats. A total of 61 rats were used: 15 in the euglycemic-hyperinsulinemic clamp experiment, 18 for NE-release experiments, 10 in mRNA expression, 10 in Western blot analysis, and 8 for secretion of steroids by the ovary. All animal procedures were performed using protocols previously

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[2]Correspondence: H.E. Lara, Department of Biochemistry and Molecular Biology, Faculty of Chemistry and Pharmaceutical Sciences, Universidad de Chile, P.O. Box 233, Santiago-1, Chile.
FAX: 56 2 737 8920; e-mail: hlara@ciq.uchile.cl

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approved by the Institutional Ethics Committee of the Faculty of Chemical and Pharmaceutical Sciences, Universidad de Chile.

Metabolic Measurement

Measurements of body weight and food intake were obtained every day (Monday to Friday) from 1 wk before the start of the experimental studies until the last day of stress protocol in control and cold-stressed rats.

Euglycemic-Hyperinsulinemic Clamp

Nonfasted rats were subjected to a euglycemic-hyperinsulinemic clamp, as described previously [25], 24 h after the last cold stress event to measure peripheral insulin sensitivity. The clamp was performed in the diestrus phase to avoid effects of variations in the estrous cycle. Briefly, rats were anesthetized throughout the entire duration of the experiment, typically 1.5 h from the onset of the administration of anesthesia. Rats received intraperitoneal administration of thiopental sodium (120 mg/kg body weight), and the body temperature was maintained at 37°C with a heating blanket. Catheters were inserted into the left carotid artery for blood sampling and into the right jugular vein for glucose and insulin infusions. Respiration was maintained by tracheotomy. A baseline blood glucose sample was determined directly with an Accu-chek Comfort Glucometer (Accu Chek, Roche). Insulin (100 U/ml; Humalog; Eli Lilly, West Ryde, Australia), together with 0.2 ml albumin and 10 ml of 0.9% NaCl, was infused at 24, 16, and 12 mU/min per kg body weight for 1, 2, and 3 min, respectively, followed by 8 mU/min per kg body weight for the rest of the clamp. Euglycemia (6.0 mM) was maintained by administration of a 30% (w/v) glucose solution in 0.9% NaCl. The glucose infusion rate was guided by glucose concentration measurements every 5 min. At the end of the clamp, blood samples were taken to determine plasma insulin concentrations. The mean glucose infusion rate was normalized for body weight and calculated at steady state (after approximately 60 min).

Epinephrine and NE Determination by HPLC-Electrochemical Detection

Serum samples (100 µl) from control and stressed rats were concentrated first in activated alumina and quantified with a Waters HPLC with electrochemical detection (Waters 464) using dihydroxybenzylamine as an internal standard. Experimental details were as previously described [5, 26, 27].

Release of NE from Rat Ovary

Norepinephrine release was assessed as described previously [28], with some modifications. Rats were killed 24 h after the last cold stress event, and the ovaries were rapidly removed through an abdominal midline incision, preincubated for 20 min in Krebs bicarbonate buffer (pH 7.4), gassed with O_2/CO_2 (95:5), and then incubated for 30 min at 37°C with 2 µCi [^{3}H]-NE (specific activity 74.9 Ci/mmol; Perkin-Elmer, Boston, MA). The diffusion of ^{3}H-NE is homogeneous within the ovary. The uptake occurs in cells with specific transporters located mainly in sympathetic nerve endings and also in granulosa cells, as described recently in the rat and human ovaries [29, 30]. Radioactivity not retained by the tissue was washed out by further incubation for 60 min in Krebs bicarbonate free of [^{3}H]-NE. For the study of spontaneous and induced release of [^{3}H]-NE, each pair of ovaries was randomly separated and transferred to different incubation wells containing 1.5 ml Krebs bicarbonate buffer. The ovaries were moved every 2 min to the next well. After 6 min, the ovaries were stimulated by depolarization with an isotonic medium containing 80 mM KCl in place of NaCl. The tissues were subjected to a second stimulation period using the same protocol as described above, but now the incubation buffer in one of the series of wells was supplemented with insulin (10 U/L). Previously, the ovaries were incubated for 30 min in the presence of insulin to allow time for the hormone to diffuse into the ovary, and then they were transferred to the wells for the second stimulation. See Figure 1 for details. At the end of the experiment the ovaries were weighed and homogenized in 1.5 ml of 0.4 M $HClO_4$. The resulting suspension was centrifuged at 15 000 × *g* for 10 min, and the supernatant containing the [^{3}H]-NE was collected. Portions (0.6 ml) from each incubation well and from the tissue homogenate were counted for radioactivity (52% efficiency) in a Tri-Carb Liquid Scintillation Analyzer 1600TR (Packard Instruments, Meriden, CT); with a 72.5% efficiency for ^{3}H. The overflow of radioactivity was calculated and expressed as a percentage of fractional release (percentage of total radioactivity present in the ovary at any moment). The total amount of neurotransmitter released was calculated as the area under the curve after stimulation minus that for basal efflux.

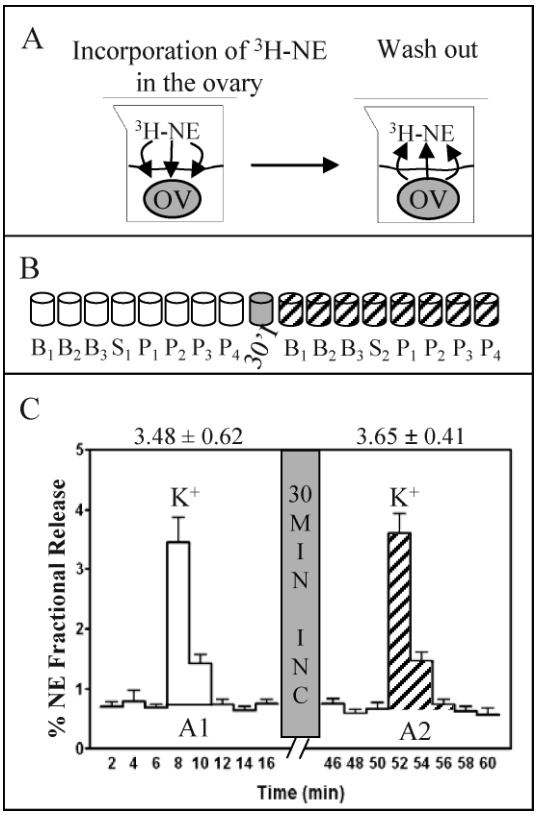

FIG. 1. Schematic overview of the procedure used to study basal and stimulated NE release. **A**) The ovaries (OV) were first preincubated with [^{3}H]-NE and washed to eliminate nonincorporated [^{3}H]-NE. **B**) The experimental protocol to perform two consecutive stimuli by depolarization with a medium containing 80 mM KCl. B1–3, Basal release without high KCl; S, stimulation in the presence of high KCl; P1–4, poststimulation without high KCl; 30′ I, 30-min incubation with or without insulin. **C**) A typical result of an experiment. Numbers above profiles (A_1 and A_2) correspond to the total amount of [^{3}H]-NE released during stimulation minus basal release.

Secretion of Steroids by the Ovary

Ovaries from control and stressed rats were halved and incubated immediately in 2 ml Krebs-Ringer bicarbonate buffer, pH 7.4, for 3 h at 37°C with or without insulin (10 IU/L). The experimental design was such that two halves were used simultaneously. Progesterone, androstenedione, and estradiol secreted into the incubation medium were measured by enzyme immunoassay following the manufacturer's instructions (Alpco Diagnostics, Windham, NH). Intraassay and interassay variations were less than 11%, 7%, and 10% for progesterone, androstenedione, and estradiol respectively. The minimal detectable value for progesterone was 2.5 pg; for androstenedione it was 1.25 pg; and for estradiol it was 0.5 pg.

Separation of Granulosa Cells and the Residual Ovary

Granulosa cells were collected as described previously [31, 32]. Briefly, ovaries were punctured with a needle, and the cell suspension was carefully expressed into Krebs bicarbonate buffer. The cells were transferred to a 1.5-ml plastic tube, pelleted by centrifugation at 250 × *g*, and washed three times with Krebs bicarbonate buffer. Both the suspension of granulosa cells and the rest of the ovary (residual ovary) were used for extraction of total RNA and measurement of *Irs1* and *Slc2a4* mRNA expression, as described below. To assess the purity of the preparation we measured mRNA expression (real-time PCR) for the FSH receptor in the granulosa cell fraction and in the residual ovary (the FSH receptor is only expressed in granulosa cells [33]). More than 99% of the *Fshr* mRNA was present in the granulosa cell fraction.

Real-Time PCR

Total RNA was extracted as described by Chomczynski and Sacchi [34] from tibialis muscle, whole ovary, and granulosa and theca-interstitial cells

TABLE 1. Primer information for rat *Irs1*, *Irs2*, *Slc2a1*, *Slc2a4*, *Fshr* and *18s*.

Name	Sequence (5′-3′)	Gene accession no.
Irs1		
Forward	GGA AGC CAT GGA CAA ACG GAG TAG	NM_012969
Reverse	TCT GGG CCA TAG TAG CAT TCT CAG	
Irs2		
Forward	CCC TGT ACT TGC GGT GGT GGA G	XM_001076309
Reverse	CTG AGT GAT GAG GCT GGG TAT GAC	
Slc2a1		
Forward	CCT ACC GCC AGC CCA TCC TCA T	NM_138827
Reverse	CCA CGA CGA ACA GCG ACA CCA C	
Slc2a4		
Forward	CGT CCT CCT GCT TGG CTT CTT CAT	NM_012751
Reverse	CCA CCA TTT TGC CCC TCA GTC ATT	
Fshr		
Forward	CAT CAC TGT GTC CAA GGC CA	BC 137991
Reverse	TGC GGA AGT TCT TGG TGA AAA	
18s		
Forward	TCA AGA ACG AAA GTC GGA GG	NM_X01117
Reverse	GGA CAT CTA AGG GCA TCA CA	

obtained as described above. Total RNA (5 μg) was subjected to reverse transcription at 42°C for 60 min using 1.6 mM dinucleotide triphosphates, 10 mM dithiothreitol, 176 nM random hexamers (Invitrogen, Carlsbad, CA), 25 units RNaseOUT (Invitrogen), 125 units of reverse-transcriptase SuperScriptII (Invitrogen, Carlsbad), and first-strand buffer, in a final volume of 30 μl. The reaction was terminated by heating the samples at 75°C for 10 min. A standard real-time PCR reaction mix was prepared containing the following components:

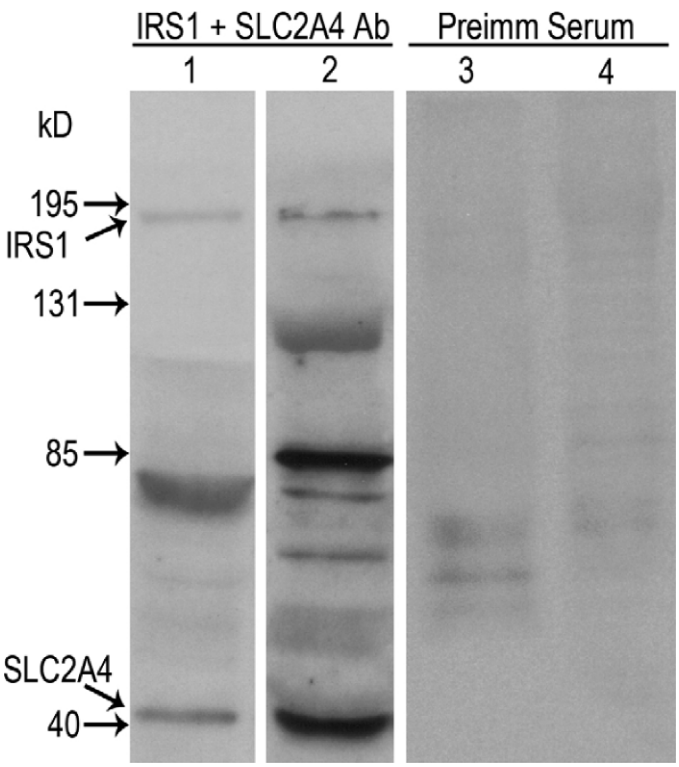

FIG. 2. Example of a typical Western blot analysis of IRS1 and SLC2A4 in ovary and tibialis muscle. To check the nonspecific binding we first incubated an ovarian and a tibialis muscle sample with a mix of IRS1 and SLC2A4 antibodies (Ab) to show both specific signals simultaneously (lane 1 for the ovary and lane 2 for tibialis muscle). In the blot to the right we incubated other aliquots of the same samples with rabbit preimmune (Preimm) serum instead of the primary antibodies, but at the same concentration, and they were developed in the same way as with primary antibodies (lane 3 for the ovary and lane 4 for tibialis muscle). Arrows represent the localization of the standard protein (kDa, labeled as kD) and of IRS1 and SLC2A4.

15 μl Platinum SYBR Green PCR Super Mix UDG (Invitrogen), 2.4 mM $MgCl_2$, 0.16 μM each primer, and 2 μl cDNA.

For specific gene amplification, a standard protocol of 40 cycles was used in the MJ Research PT-200 (MJ Research Inc., Watertown, MA) after initial polymerase activation at 95°C for 10 min; primer-specific amplification and quantification cycles were run at 60°C for 15 sec and 72°C for 20 sec. The fluorescence intensity of the double-strand specific SYBR Green I, reflecting the amount of PCR product actually formed, was read at the end of each elongation step after previous melting curve analyses to determine the melting points of the PCR products. Then, the amounts of specific initial template mRNA were calculated by determining the time point at which the linear increase of sample PCR product started relative to the corresponding points of a standard curve obtained by serial dilution of known copy numbers of the corresponding control tissue. To evaluate specific amplification, a final melting curve was created (72°C–95°C) under continuous fluorescent measurement.

The *Irs1*, *Irs2*, *Slc2a1* (previously known as *Glut1*), and *Slc2a4* (previously known as *Glut4*) primers were designed according to data from GenBank, and *Fshr* primers were obtained from the literature [35] (Table 1). To normalize the quantification of *Irs1*, *Irs2*, *Slc2a1, Slc2a4,* and *Fshr* mRNAs, ribosomal *18s* mRNA was measured in each protocol. Amounts of *18s* mRNA were determined using a commercially available RT primer pair (Ambion, Austin, TX). Amplification of 18s RNA was performed in a different tube to avoid interference with the amplification of the mRNAs. Reaction tubes lacking RT were used as PCR controls. The RT-PCR products were separated on 2.0% agarose gels, stained with ethidium bromide, and photographed digitally.

Western Blot Analysis

Proteins were extracted from ovaries, tibialis, granulosa cell fraction, and theca-interstitial fraction by homogenization in a glass homogenizer using 200 μl lysis buffer (Tris-HCl 50 mM, NaCl 50 mM, EGTA 1 mM, EDTA 1 mM, and Triton 1%; pH 7.4) supplemented with Protease Inhibitor Cocktail (Sigma, St. Louis, MO), PMSF 35 mM and DTT 0.4 mM. Total lysate in the supernatant fluid was obtained by microcentrifuge at 16 750 × *g* for 10 min at 4°C. Total protein was measured using the Lowry method [36]. Proteins (50 μg) from each sample were resolved through 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose filters by electroblotting. Filters were blocked for 1 h at room temperature with TBST (20 mM Tris, 137 mM NaCl, and 0.1% Tween-20, pH 7.6) containing 5% dry milk. Blots were incubated with anti-IRS1 (C-20; 1:100 dilution; Santa Cruz Biotechnology Inc.), anti-SLC2A4 (GLUT4; H-61; 1:100 dilution; Santa Cruz, Biotechnology) and anti-β-TUBULIN (3F3-G 2; 1:1000 dilution; Santa Cruz Biotechnology) antibody overnight at 4°C in TBST. Filters were washed (three times, 7 min each time) with TBST and incubated with anti-rabbit (IRS1, SLC2A4) or anti-mouse (β-TUBULIN) antibody for 1 h at room temperature. The detection was performed using the ECL plus Western blotting detection system (Amersham Biosciences). Band intensities were measured with the UN-SCAN-IT program (Silk Scientific, Orem, UT) and normalized to that of the corresponding β-TUBULIN (TUBB) bands. To check the nonspecific binding we first incubated an ovarian and a tibialis muscle sample with a mix of IRS1 and SLC2A4 antibodies to show both specific signals simultaneously (Fig. 2, lanes 1 for the ovary and 2 for tibialis muscle). In a parallel blot we incubated other aliquot of the same

TABLE 2. Animal model characterization.[a]

Parameter	Control rats	Stressed rats
Weight gain (g)	35.3 ± 2.6	35.2 ± 3.8
Food intake (g/day) in week before stress	20.0 ± 0.6	21.1 ± 0.9
Food intake (g/day) in fourth week of stress	19.5 ± 0.5	23.5 ± 0.9*
Glucose infusion rate (mg/kg/min)	34.5 ± 2.4	45.4 ± 3.1*
Glucose (mmol/L)[b]	6.20 ± 0.21	5.82 ± 0.42
Insulin (μg/L)[b]	2.36 ± 0.60	1.54 ± 0.34
Epinephrine (pg/μl)[b]	17.61 ± 5.45	27.45 ± 12.90
Norepinephrine (pg/μl)[b]	4.75 ± 1.68	5.31 ± 1.97
Adrenal catecholamines (μg/mg adrenal)	0.897 ± 0.048	0.929 ± 0.075

[a] The values represent the mean ± SEM of at least four rats in each condition.
[b] Measured in plasma samples at the end of the treatment.
* $P < 0.05$ vs. control.

samples with rabbit preimmune serum (instead of the primary antibodies) at the same concentration as with the primary antibody and developed in the same way as for primary antibodies (Fig. 2, lanes 3 and 4). Although there are many nonspecific bindings, there was a unique signal for both primary antibodies at the predicted size that was not present when the primary antibody was replaced with preimmune serum.

Statistical Analysis

Differences between control and experimental groups were analyzed with Student *t*-test, and comparisons between several groups were made by one-way ANOVA, followed by the Student-Newman-Keuls multiple comparison test for unequal replication. The level of significance was set at $P < 0.05$.

RESULTS

Metabolic and Hormonal Characteristics of Intermittent Cold Stress Rats

Because of physiological adaptation, chronic intermittent exposure to stress promotes only minor changes in hormonal levels and metabolic parameters. During cold stress there were no changes in estrous cycling activity in either group (data not shown). The slope of the weight gain curve and the weight gain, measured between the first and last days of the procedure, were similar in cold-stressed and control rats. However, during the fourth week of stress the animals increased their daily food intake (Table 2). Stressed rats ate 20% more than control rats during the last week of stress. During the fist, second, and third weeks of stress there was no significant change in food intake. To investigate whether rats with cold stress were insulin resistant, we measured the peripheral insulin sensitivity by an euglycemic-hyperinsulinemic clamp. In this technique plasma glucose is maintained at a steady state within the euglycemic range while plasma insulin levels are elevated to a desired plateau. The higher the glucose infusion rate needed to maintain euglycemia, the greater the insulin sensitivity. As depicted in Table 2, the insulin sensitivity was significantly higher in intermittent cold stress rats. At steady state, the plasma glucose level was ~6 mmol/L, and the plasma insulin level was 95.1 ± 7.3 mU/L. The results obtained during the evaluation of the metabolic and hormonal parameters are summarized in Table 2.

Effect of Insulin on NE Release from the Ovary

We have previously characterized the incorporation of [^{3}H]-NE into ovarian nerve terminals and its release in response to depolarization with a medium containing 80 mM KCl [28].

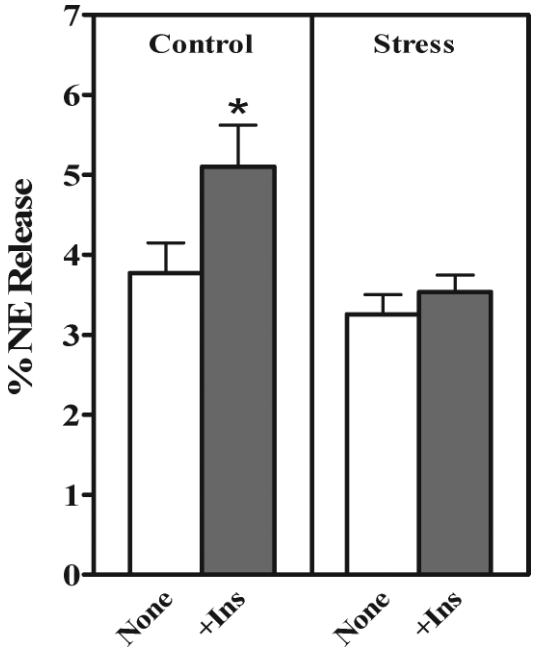

FIG. 3. Effect of insulin (10 U/L) on the basal release of [^{3}H]-NE in control and stress rats. Ovaries were incubated without insulin (None) or with insulin (+Ins) for 30 min. Results are expressed as a percentage of the fractional release and represent the mean ± SEM of at least four individual experiments per group. *$P < 0.05$ vs. control.

Insulin did not modify the NE release induced by high concentrations of K^+ in control and stressed rat ovaries. The ratio between the total amount of radioactivity released during the first stimulation (A1, without insulin) and the second stimulation (A2, with insulin) compared with the A2:A1 ratio for two successive stimulations without insulin were similar (1.19 ± 0.10 A2:A1 with insulin and 1.20 ± 0.14 without insulin in the second stimulus, mean value ± SEM, n = 9 experiments per group). In the ovaries of stressed rats, the A2:A1 ratio was 1.35 ± 0.21 with insulin in the second stimulus and 1.22 ± 0.14 in the absence of insulin (mean value ± SEM, n = 9 experiments per group). Basal fractions obtained each 2 min did not change the NE release induced by insulin in both experimental groups. Probably, 2 min was not enough time for insulin diffusion to the whole tissue. Additional studies using a time course will answer the question. Chi et al. [19] demonstrated that insulin produces an increase of NE release from aortic strips after 30 min of incubation. Therefore, we tested whether insulin increased the basal NE release from the ovary after 30 min of incubation. Results showed that the ovaries from control rats incubated with insulin released significantly more [^{3}H]-NE than ovaries incubated only with Krebs bicarbonate buffer ($P < 0.05$). Insulin failed to stimulate the release of NE from the ovaries of cold-exposed rats (Fig. 3); the slow time scale for an insulin effect on NE release from ovarian nerves suggests an indirect action of the hormone.

The same dose of insulin (10 U/L) used in the NE release experiments was used to determine whether insulin modifies secretion of ovarian steroids. Table 3 shows the levels of progesterone, androstenedione, and estradiol obtained after incubation of the ovaries with insulin. No changes were found in control and stressed rats. The same tissue, however, responded to 2.5 IU hCG, a dose previously reported to induce steroid secretion form the ovary [14].

TABLE 3. Effect of insulin (10 U/L) on steroids secretions from ovaries in vitro.[a]

Treatment	Progesterone (ng/mg ovary)	Androstenedione (pg/mg ovary)	Estradiol (pg/mg ovary)
Control			
Krebs	0.96 ± 0.30	23.12 ± 3.28	24.62 ± 3.92
Krebs + insulin	0.88 ± 0.20	25.46 ± 3.18	31.98 ± 11.06
Stress			
Krebs	1.02 ± 0.10	28.98 ± 6.36	36.34 ± 12.72
Krebs + insulin	0.86 ± 0.54	32.04 ± 5.64	18.31 ± 3.38

[a] Results are expressed as mean ± SEM of five individual ovaries for each experimental condition.

Expression of IRS1 and SLC2A4 in Tibialis Muscle and Ovary

To ascertain whether changes in peripheral insulin sensitivity and in the ovary of chronic cold-stressed rats were tissue specific, we measured expression of IRS1, IRS2, SLC2A1, and SLC2A4 in the muscle and ovary from control and stressed rats, evaluating the protein level by Western blot analysis using specific antibodies, and the mRNA level by real-time PCR. The amounts of *Irs1* and *Slc2a4* were significantly lower in rat ovaries exposed to chronic cold stress ($P < 0.05$; Fig. 4, A and B), whereas the mRNA level in the skeletal muscle was unchanged (Fig. 4, C and D). The same situation occurred with the protein levels of IRS1 and SLC2A4 (Fig. 5). The mRNA expressions of *Irs2* and *Slc2a1* in the ovary and muscle did not change (data not shown). The fact that the mRNA and the amounts of IRS1 and SLC2A4 were decreased in the ovary (and both molecules are involved in the intracellular signaling of insulin action) raised the possibility of different cells being involved in insulin resistance in the ovary. To elucidate whether granulosa cells or the residual ovary, principally theca cells, are involved in the lack of response of NE release from the whole ovary after insulin stimulation, we isolated these cells from control and cold-stressed ovaries and measured IRS1 and SLC2A4 expression. The mRNA level of *Fshr* was used to determine the purity of the fractions. The ratio *Fshr*:*18s* was 3.83 ± 1.16 for the granulosa cell fraction and 0.038 ± 0.009 for the residual ovary. The residual ovary showed the same magnitude of reduction in *Irs1* and *Slc2a4* mRNA levels as in the whole ovary (Fig. 6, C and D). However, *Irs1* and *Slc2a4* in the isolated granulosa cells did not differ between the study groups (Fig. 6, A and B). The amount of SLC2A4 was decreased in the residual ovary but not in the granulosa fraction (Fig. 7, C and D). Interestingly, the amount of IRS1 protein was significantly lower in both granulosa and the residual ovary (Fig. 7, A and B).

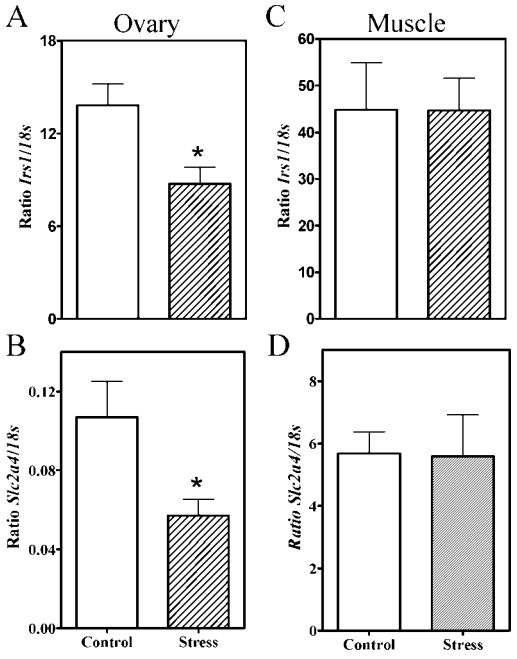

FIG. 4. Changes in the mRNAs for *Irs1* (**A** and **B**) and *Slc2a4* (**C** and **D**) in the ovary and muscle of control and stress rats. To normalize the *Irs1* and *Slc2a4* mRNA quantification by real-time PCR, in each protocol ribosomal *18s* mRNA was measured. The data represent the mean ± SEM of five individual ovaries and muscles for each experimental condition. *$P <$ 0.05 vs. control.

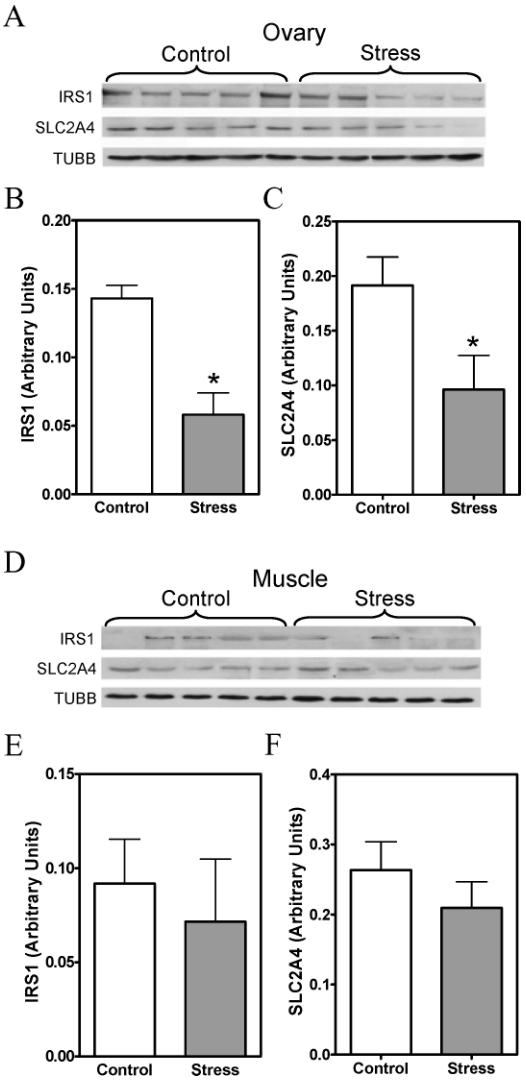

FIG. 5. Effect of cold stress on the protein levels of IRS1 and SLC2A4 in ovary (**A**–**C**) and muscle (**D**–**F**). **A**) Western blot for IRS1, SLC2A4, and β-TUBULIN (TUBB) in the ovary. **B** and **C** represent the quantification of the respective bands. **D**) Western blot for IRS1, SLC2A4, and β-TUBULIN (TUBB) in muscle. **E** and **F** represent the quantification of the respective bands. Band intensities were measured with the UN-SCAN-IT program and normalized to that of the corresponding β-TUBULIN (TUBB) bands. Data are presented as means ± SEM of five individual ovaries and muscles for each experimental condition. *$P <$ 0.05 vs. control.

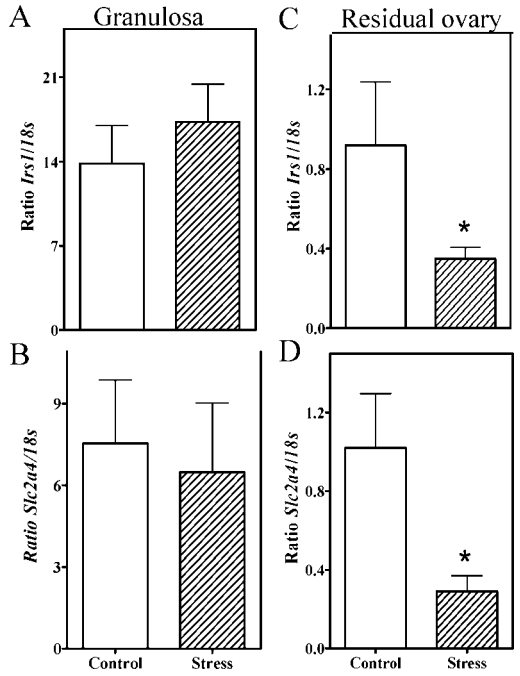

FIG. 6. Changes in the mRNAs for *Irs1* and *Slc2a4* in granulosa cells (**A** and **B**) and the residual ovary (**C** and **D**) of control and stress rats. To normalize the *Irs1* and *Slc2a4* mRNA quantification by real-time PCR, in each protocol ribosomal *18s* mRNA was measured. The data represent the mean ± SEM of five individual ovaries for each experimental condition. $^*P < 0.05$ vs. control.

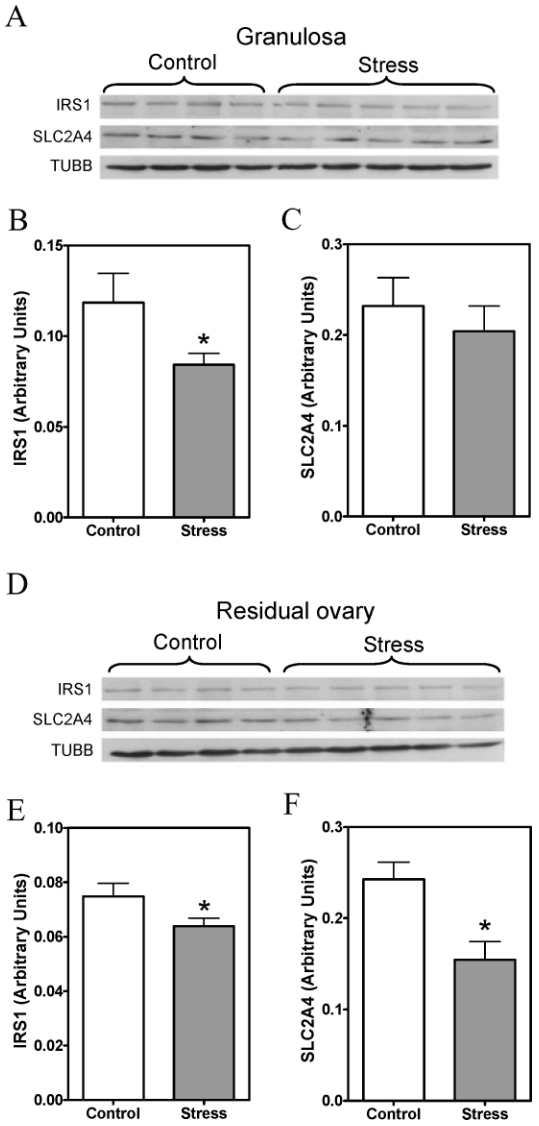

FIG. 7. Effect of cold stress on the protein levels of IRS1 and SLC2A4 in granulosa cells (**A**–**C**) and in residual ovary (**D**–**F**). **A**) Western blot for IRS1, SLC2A4, and β-TUBULIN (TUBB) in granulosa cells. **B** and **C** represent the quantification of the respective bands. **D**) Western blot for IRS1, SLC2A4, and β-TUBULIN (TUBB) in residual ovary. **E** and **F** represent the quantification of the respective bands. Band intensities were measured with the UN-SCAN-IT program and normalized to that of the corresponding β-TUBULIN (TUBB) bands. Data are presented as means ± SEM of four individual ovaries for control and five ovaries for stressed rats. $^*P < 0.05$ vs. control.

DISCUSSION

The present study suggests that chronic stress develops an ovary-specific insulin resistance, thus opening up the possibility that stress-induced disruption of insulin signaling in ovary could be an initial event, in part responsible for inducing changes in ovarian physiology.

We demonstrated previously that chronic intermittent cold stress results in changes in follicular development of the rat ovary that include theca cell hypertrophy and a premature luteinization of the follicles [5], and that these changes were associated with sympathetic nerve activation. Some of these characteristics are seen in the PCOS in women [17, 18]. Growing evidence also points to an association of PCOS with insulin resistance and compensatory hyperinsulinemia [37]. The proposed link between hyperinsulinaemia and PCOS is a direct effect of insulin on ovarian steroidogenesis [38]. There are, however, no studies on the effect of insulin on the function of the sympathetic nervous system in the ovary.

Women with PCOS exhibit decreased insulin sensitivity in 50%–90% of cases [39–41], whereas we found that rats exposed to intermittent chronic cold stress have increased peripheral insulin sensitivity.

Physiological adaptation is an important component for maintaining homeostasis when homoeothermic animals are exposed to a cold environment. The first response is to reduce heat loss under an energy-sparing process. The subsequent response involves an increased energy demand that is reflected eventually in weight loss or increased food intake. In results similar to ours, Gasparetti et al. [42] showed that rats exposed to 8 days of continuous cold stress evinced an early fall in body weight, increase in daily food intake, and a much more efficient glucose uptake during a glucose tolerance test. The stress procedure used in the present work, although it did not affect body weight, did cause a significant increase in food intake during the last week. Because stressed rats did not change their growth curve during the treatment and did not gain more weight than controls, we suggest that the balance between food consumption and energy spend was maintained. Activation of the sympathetic nervous system may stimulate increased thermogenesis and diminished response to satiety signals at the level of the central nervous system [43].

The increase of insulin sensitivity found in these rats raises the possibility that the ovaries from stressed rats will respond more than those from control rats when incubated with insulin. Sympathetic nervous activity was modified by insulin in control rats in a way similar to that demonstrated in incubation of aorta [19]. In contrast, insulin did not increase basal NE release from ovaries of stressed rats, suggesting insulin resistance in ovarian sympathetic nerves or, possibly, in

another type of ovarian cell if insulin acts indirectly. Granulosa cells from women with PCOS exhibit insulin resistance; this may affect glucose uptake [20]. The amount of insulin that increased the release of NE did not affect steroid hormone secretion in vitro, indicating selectivity of this hormone. Previous studies using ovarian granulosa cells from patients with PCOS and insulin resistance have shown that insulin maintains its stimulatory effect on ovarian steroidogenesis regardless of an impaired metabolic action [22]. In contrast, in our study using the whole ovary we did not demonstrate an effect of insulin in steroid secretion. Probably, it would have been necessary to coincubate the ovary with gonadotropins in order to see an effect in steroidogenesis as it has been demonstrated to occur in cultures of granulosa cells [44].

Insulin receptor signaling has been implicated in the regulation of female reproductive function, in part through its action in the ovaries. Insulin receptor substrate (IRS) proteins mediate the effects of the insulin receptor on cellular and whole-body physiology, including reproduction [45]. IRS1 does not seem to be an important protein for reproductive function, because mice that lack *Irs1* are only mildly affected in this concern. In contrast, female *Irs2*-null mice are infertile [46], and when this gene is lacking only in the central nervous system mice have minimal reproductive defects [47]. Accordingly, in the present study we found neither changes in *Irs2* mRNA levels nor severe changes in the reproductive function after 4 wk of stress. When we measured the expression of IRS1 and SLC2A4 in the ovary and tibialis muscle to assess tissue-specific regulation of the insulin signaling pathway in stressed rats, we found significant decreases in the expression of *Irs1* and *Slc2a4* and IRS1 and SLC2A4 in ovaries from stressed rats. This finding is consistent with studies in which decreased mRNA expression of *Irs1* and *Slc2a4* in adipose tissue from PCOS women with insulin resistance was demonstrated [48, 49]. But when we analyzed which cells of the ovary were responsible for this selective ovarian insulin resistance in the cold-stressed animals we found that theca-interstitial cells had decreased mRNA expression of *Irs1* and *Slc2a4*, and not granulosa cells, as described by Wu et al. [20] studying women with PCOS. The decrease in the mRNA level of IRS1 and SLC2A4 in theca-interstitial cells was accompanied by a similar decrease in their proteins, supporting the insulin resistance in these cells. Although the level of *Irs1* and *Slc2a4* mRNA did not change in granulosa cells, the amount of IRS1 protein decreased. This molecule is at the first step in signaling transduction of insulin and involves both mitogenic and metabolic pathways. The fact that IRS1 decreased in granulosa cells without changing the metabolic pathway activated by insulin opens up the possibility of a decrease in the mitogenic activity mediated by insulin.

We suggest that an increase in NE outflow from ovarian nerve terminals of stressed rats (described previously in this model [5]) could be responsible for the changes in *Irs1* and *Slc2a4* mRNA and the level of IRS1 and SLC2A4 protein found in the theca-interstitial cells, because these cells are the only intraovarian cells directly innervated by sympathetic nerve terminals [12]. Although granulosa cells present β-adrenergic receptors, sympathetic nerve fibers do not reach granulosa cells through basal membrane of the follicle [12, 17]. In support of this, Jones and Dohm [50] also found that infusion of adrenaline after medullectomy decreases SLC2A4 transcription, suggesting that catecholamines decrease SLC2A4 expression as expected in the ovaries of stressed animals.

We propose that insulin could be a local regulator of noradrenergic activity in the ovary. In the ovary of the control rats the effect of insulin on the ovarian NE release occurred only after a long time, suggesting an indirect action of the hormone that is not visualized in stress condition. Jones and Dohm [50] showed that intravenous infusion of epinephrine in adrenodemedullated animals decreased SLC2A4 gene transcription by 60%. In a similar way, when nerve activity increases as a consequence of stress, NE released could induce a compensatory mechanism at theca-interstitial cells (the cells directly innervated by sympathetic nerves), decreasing the expression of the molecules participating in signal transduction signals and thus inhibiting the effect of insulin on NE release from nerve terminals.

The decreased responsiveness to insulin could affect other ovarian functions, such as follicular development. Insulin resistance in granulosa cells could be responsible for the delayed follicular development found in stressed rats [5]. There is differential expression of the insulin receptor at different stages of follicular development [51], and thus the ovarian insulin resistance during cold stress could be at least in part responsible for the aberrant follicular development characteristically seen in rats with PCO following stress exposure.

We propose that chronic intermittent cold stress as a physical stress activates ovarian NE nerve terminals, leading to insulin resistance in theca-interstitial cells, and through the alteration on ovarian morphology and function could be in part responsible for the etiology of PCOS induced by stress. These data could have pathological relevance to humans, where it has been demonstrated recently that PCOS is associated with increased sympathetic tone [18] and could respond to a local response to insulin independently of the general metabolic changes in the organism.

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