Ovarian Interleukin-1 Receptor Antagonist in Rats: Gene Expression, Cellular Localization, Cyclic Variation, and Hormonal Regulation of a Potential Determinant of Interleukin-1 Action[1]

Shahar Kol,[3] Barry W. Donesky,[4] Kristina Ruutiainen-Altman, Izhar Ben-Shlomo,[5] Minoru Irahara,[6] Motomu Ando,[7] Richard M. Rohan,[8] and Eli Y. Adashi[2,9]

Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, University of Maryland School of Medicine, Baltimore, Maryland 21201

ABSTRACT

It is hypothesized that the intraovarian interleukin (IL)-1 system plays a prominent role in the regulation of follicular development and ovulation. A central component of the intraovarian IL-1 system is the IL-1 receptor antagonist (IL-1RA), a protein acting as a pure IL-1 receptor antagonist and one for which intracellular (icIL-1RA) and secretory (sIL-1RA) varieties have been described. It was the objective of this study to explore rat ovarian IL-1RA gene expression, to establish the identity and relative abundance of its alternative transcripts, to study its cellular localization, to determine its cyclic variation, and to assess its hormonal regulation. Protected IL-1RA cDNA fragments corresponding to either sIL-1RA or icIL-1RA were barely detectable in untreated whole ovarian tissue of immature rat origin. However, sIL-1RA transcripts reached a maximal value (3.3-fold increase over untreated control values; $p < 0.05$) 12 h after hCG administration (time of projected ovulation). In situ hybridization localized IL-1RA to mural, antral, and cumulus granulosa cells. Modestly intense staining was also apparent in oocytes. The basal pattern of sIL-1RA expression by cultured whole ovarian dispersates was characterized by a spontaneous increase to a peak value at 4 h. The early (4 h) sIL-1RA burst proved IL-1-, nitric oxide-, and protein biosynthesis-independent. However, treatment with IL-1β led to a secondary sIL-1RA peak at 48 h, an effect that was substantially reversed by IL-1RA. This stimulatory effect of IL-1β on IL-1RA expression proved relatively specific, and nitric oxide independent, but contingent upon de novo protein biosynthesis. The in vitro expression of icIL-1RA was barely detectable. Taken together, these in vivo and in vitro observations 1) document the rat ovary as a site of IL-1RA (sIL-1RA > cIL-1RA) expression, 2) localize the relevant transcripts to the granulosa cell, 3) disclose peak expression at the time of ovulation, and 4) establish IL-1 dependence.

[2]Correspondence: Eli Y. Adashi, Division of Reproductive Sciences, Departments of Obstetrics and Gynecology, University of Utah Health Science Center, 546 Chipeta Way, Mailbox #20, Salt Lake City, UT 84108. FAX: 801 585 9256; e-mail: eadashi@hsc.utah.edu
[3]Current address: Department of Obstetrics and Gynecology, Rambam Medical Center, Haifa, Israel.
[4]Current address: 979 E. 3rd Street, Suite 2000, Chattanooga, TN 37421.
[5]Current address: Department of Obstetrics and Gynecology, Haemek Medical Center, Afula, Israel.
[6]Current address: Department of Obstetrics and Gynecology, Tokushima University, Tokushima 770, Japan.
[7]Current address: Department of Obstetrics and Gynecology, Kyorin University, Tokyo, Japan.
[8]Current address: Children's Hospital, Boston, MA 02115.
[9]Current address: Division of Reproductive Sciences, Department of Obstetrics and Gynecology, University of Utah Health Sciences Center, Salt Lake City, UT 84108.

INTRODUCTION

It has been hypothesized that ovulation may constitute a cyclic inflammatory-like process [1] and that gonadotropin-inducible intraovarian interleukin (IL)-1 [2], an established mediator of inflammation [3], may play a central role in this regard [4]. At this time, four independent but complementary lines of evidence support this hypothesis. First, the ex vivo provision of IL-1β has been shown to bring about ovulation and to synergize with LH in this respect [5, 6]. Second, the addition of an IL-1 receptor antagonist has been shown to attenuate LH-supported ovulation ex vivo [7] and in vivo [8]. Third, the expression of some components of the intraovarian IL-1 system (e.g., IL-1α, IL-1β, and the type I IL-1 receptor) appears to be enhanced in vivo during a narrow periovulatory window [2, 9–14]. Fourth, IL-1β has been shown to induce a host of ovulation-associated phenomena in vitro such as the promotion of prostaglandin production [15–18], the stimulation of hyaluronic acid biosynthesis [19], the induction of collagenase activity [20], the modulation of plasminogen activator activity [21], and the activation of nitric oxide synthase [22–24].

A potential component of the intraovarian IL-1 system is the IL-1 receptor antagonist (IL-1RA), a protein acting as a pure IL-1 receptor antagonist [25] for which intracellular (icIL-1RA; subtypes I and II) and secretory (sIL-1RA) varieties have been described [26–31]. Although the precise physiologic role of IL-1RA remains uncertain, it is generally thought of as an attenuator or terminator of IL-1 action [32–38]. Indeed, macrophage-derived IL-1RA has been shown to be released in a temporally distinct and in fact delayed fashion relative to IL-1, thereby raising the possibility that IL-1RA may be charged with the dampening or neutralization of residual bioactive IL-1 [39]. Although the ovarian expression of IL-1α and IL-1β, as well as the type I IL-1 receptor, is enhanced only during a narrow periovulatory window [2, 9–14], little is known of temporal expression pattern of ovarian IL-1RA. It was the objective of this study to explore the rat ovary as a site of IL-1RA gene expression, to establish the identity and relative abundance of its alternative intracellular (icIL-1RA) and secretory (sIL-1RA) transcripts, to establish its cellular localization, to determine its cyclic variation, and to assess its hormonal regulation.

MATERIALS AND METHODS

Animals

Immature Sprague-Dawley female rats from Zivic-Miller Laboratories (Zelienople, PA) were killed by CO_2 asphyxiation. The project was approved by the Institutional Animal Care and Use Committee.

Hormones and Reagents

A recombinantly expressed preparation of the naturally occurring human IL-1RA was generously provided by Dr. Daniel E. Tracey, The Upjohn Co. (Kalamazoo, MI). Recombinant human IL-1β (2 × 10⁷ U/mg) was generously provided by Drs. Errol B. De Souza and C.E. Newton, DuPont-Merck Pharmaceutical Co. (Wilmington, DE). Equine CG was from Sigma Chemical Co. (St. Louis, MO). Highly purified hCG (CR-127, 14 900 IU/mg) was generously provided by Dr. R.E. Canfield through the Center for Population Research, NICHHD, NIH (Bethesda, MD). Activin A was a gift from Dr. Jennie Mather, Genentech (South San Francisco, CA). Endothelin-1 (ET-1) was from Peninsula Laboratories, Inc. (Belmont, CA). Transforming growth factor α (TGFα) was from Oncogene Science (Uniondale, NY). Tumor necrosis factor α (TNFα) was a generous gift from Dr. Dawson, National Biological Standard Board, (Hertfordshire, UK). Insulin-like growth factor-I (IGF-I) was from Bachem (Torrance, CA).

McCoy's 5a medium (without serum), penicillin-streptomycin solution, L-glutamine, trypan blue stain, Moloney Murine Leukemia Virus reverse transcriptase, and BSA were from Life Technologies (Grand Island, NY). Collagenase (*Clostridium histolyticum*; CLS type I; 144 U/mg) was from Worthington Biochemical (Freehold, NJ). DNase (bovine pancreas), RNase A, aminoguanidine hemisulfate salt (AG), cycloheximide (CHX), and S-nitroso-*n*-acetyl-penicilamine (SNAP) were from Sigma Chemical Co; pBS vector was from Stratagene (La Jolla, CA). Random primers (pdN6) and RNase T1 were from Pharmacia (Piscataway, NJ). AmpliTaq polymerase was from Perkin Elmer (Norwalk, CT). Alkaline phosphatase-conjugated anti-digoxigenin polyclonal antibody *Fab* fragments, digoxigenin-UTP, nitro blue tetrazolium, and 5-bromo-4-chloro-3-inodyl-phosphate were from Boehringer-Mannheim (Indianapolis, IN). Other molecular biology grade reagents were from Promega (Madison, WI). RNA size marker kit was from Ambion (Austin, TX).

Tissue Culture Procedures

Whole ovarian dispersates were prepared and maintained as previously described [40].

Nucleic Acid Probes

The rat IL-1RA probe was generated by reverse transcription followed by the polymerase chain reaction (PCR). The cDNA was synthesized from macrophage-derived total RNA (1 μg) using random primers (pdN6) and Moloney Murine Leukemia Virus reverse transcriptase. Polymerase chain reactions were performed with AmpliTaq polymerase in the recommended reaction mixture. Primers were selected from the published sequence [41] using Primer Designer software (Scientific and Educational Software, State Line, PA). The sense strand primer was 5′-AGCGAATTCCTT-GACACAAGACAGGCACA-3′, and the antisense strand primer was 5′-ATAGTCGACCAGTCACCTAATCTC TCTCC-3′. These primers span the putative alternative splice acceptor site in the icIL-1RA isoform [26, 27]. The resultant 286-base pair (bp) PCR product was cloned into the *Eco*RI and *Bam*HI sites of a pBS vector using the restriction sites incorporated into the 5′ ends of the sense and antisense strand primers, respectively. The resultant plasmid was sequenced and found to conform to the previously published rat IL-1RA cDNA sequence [41]. After digestion

with *Hin*dIII and transcription with T7 RNA polymerase, a 300-nucleotide (nt) full-length riboprobe was generated, which was projected to protect 267-nt and 224-nt segments of the secretory and intracellular IL-1RA mRNA, respectively. The construct was further verified using tissue RNA with established preference for one of the two subtypes: macrophage and liver for sIL-1RA and small bowel and skin for icIL-1RA (see Fig. 1). It is recognized that the riboprobe used in this study does not distinguish between the type I icIL-1RA [26, 27] and the type II [31] variety. The RPL19 probe was generated as previously described [11]. [α-³²P]UTP-labeled RNA size markers were generated using the Ambion kit according to instructions.

RNA Extraction

Total RNA of cultured cells and of tissues was extracted with RNAZOL-B (Tel Test, Friendswood, TX) according to the manufacturer's protocol.

RNase Protection Assay

Linearized DNA templates were transcribed with T7 RNA polymerase to specific activities of 800 Ci/mmol [α-³²P]UTP (IL-1RA) or 160 Ci/mmol [α-³²P]UTP (RPL19). The riboprobes were gel purified in an effort to eliminate transcribed products shorter than full length. The assay was performed as previously described [42].

Gels were exposed to XAR film (Eastman Kodak, Rochester, NY) for varying lengths of time with intensifying screens. To generate quantitative data, gels were also exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA). The resultant digitized data were analyzed with ImageQuant Software (Molecular Dynamics). The hormonally independent RPL19 mRNA signal was used to normalize the IL-1RA mRNA data for possible variation in RNA loading. Specifically, the net protected IL-1RA signal (respective background subtracted) to net RPL19 signal ratio was calculated for each sample.

In Situ Hybridization

Nonradioactive digoxigenin-driven in situ hybridization was carried out as previously described [11]. Slides labeled with the corresponding sense probes did not yield positive signals. The specificity and sensitivity of the assay with respect to the antisense probes were also validated by identifying signal-positive and signal-negative follicles within the same ovary and by assessing for labeling in duplicate samples.

Data Analysis

Except as noted, each experiment was replicated a minimum of three times. Data points are presented as mean ± SE, and statistical significance (Fisher's protected least-significance difference) was determined by ANOVA and Student's *t*-test. Statistical values were calculated using Statview 512+ for MacIntosh (Brain Power, Inc., Calabasas, CA).

RESULTS

Tissue Distribution of IL-1RA Transcripts in the Immature Rat: RNase Protection Analysis

To evaluate the relative expression of IL-1RA in different organs and to validate the construct described in *Ma-*
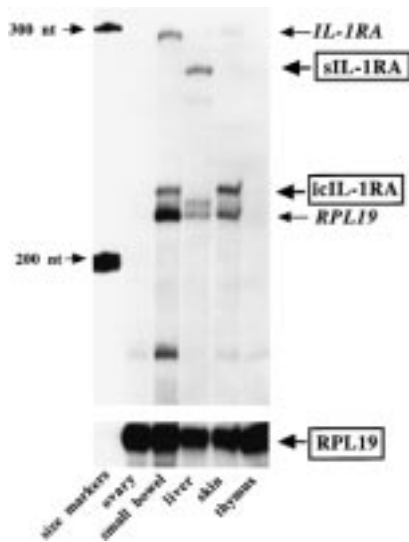
FIG. 1. Tissue distribution of IL-1RA transcripts in the immature rat. Total RNA (20 μg) extracted from the indicated tissues of intact untreated immature rats was subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1RA and RPL19. The intensity of the signals was quantified as described. The full-length riboprobes are in italics and protected fragments are boxed.

terials and Methods, total RNA samples from untreated immature rat ovary, small bowel, liver, skin, and thymus were hybridized with an antisense riboprobe corresponding to rat IL-1RA. As shown in Figure 1, a prominent protected fragment corresponding to the icIL-RA variant(s) was noted in skin and small bowel samples. These findings are consistent
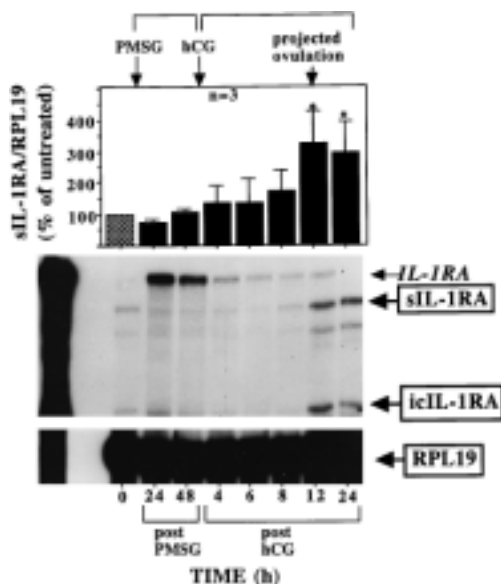

FIG. 2. IL-1RA gene expression: effects of follicular maturation, ovulation, and corpus luteum formation. Intact, 25-day-old rats were primed with 15 U eCG (PMSG on figure). Ovulation was triggered 48 h later with 15 U hCG. The animals were killed at the indicated time points, and total ovarian RNA was extracted and subjected to a solution hybridization RNase protection assay with antisense riboprobes corresponding to rat IL-1RA and RPL19. The intensity of the signals was quantified as described. The bar graph (top) depicts the mean ± SE of 3 experiments, the data being normalized to the value obtained for the untreated state (Time 0). A representative autoradiograph is shown (middle and lower panels). The full-length IL-1RA riboprobe is in italics; protected fragments are boxed. *$p < 0.05$ vs. Time 0 (untreated animals).

with reported IL-1RA expression in cultured human keratinocytes and with the isolation of the icIL-1RA cDNA from inflamed rabbit colon [26, 27]. It is recognized that the riboprobe used in this study does not distinguish between the type I icIL-1RA [26, 27] and the type II [31] variety. In contrast, liver RNA was mainly associated with the sIL-RA variant. The signals corresponding to sIL-RA or icIL-1RA for either the ovary or the thymus were barely detectable. These findings suggest that, in relative terms, untreated whole ovarian tissue is not a major site of IL-1RA gene expression.

IL-1RA Gene Expression: Effects of Follicular Maturation, Ovulation, and Corpus Luteum Formation

To assess IL-1RA gene expression in the course of a simulated estrous cycle, 25-day-old rats were initially primed with 15 IU of eCG. Ovulation was triggered 48 h later with 15 IU hCG. The animals were killed at the indicated time points, the ovaries were snap frozen in −70°C, and total RNA was extracted and subjected to a solution hybridization RNase protection assay with antisense riboprobes corresponding to IL-1RA and RPL19. As shown in Figure 2, protected fragments corresponding to sIL-1RA were apparent virtually throughout the experiment. The steady state level of sIL-1RA transcripts peaked (3.3-fold increase over untreated control values; $p < 0.05$) 12 h after hCG administration (time of projected ovulation). A slight decrease was noted 12 h later (still a 3-fold increase over untreated control values; $p < 0.05$). The signal corresponding to icIL-1RA was too faint for meaningful quantification in some (but not all) experiments, but it appeared to mostly parallel its secretory counterpart.

Cellular Localization of IL-1RA Transcripts: In Situ Hybridization Studies

To establish the identity of the ovarian cell population(s) responsible for IL-1RA gene expression, ovaries were obtained from intact 25-day-old rats and processed as previously described [11]. As shown in Figure 3, IL-1RA transcripts localized to mural, antral, and cumulus granulosa cells. Meaningful staining was confined to within the basement membrane. Modestly intense staining was also apparent in oocytes.

IL-1RA Gene Expression by Untreated Cultured Whole Ovarian Dispersates: Basal Expression Pattern

To explore basal ovarian IL-1RA gene expression under in vitro circumstances, whole ovarian dispersates were cultured for the duration indicated (up to 48 h) in the absence of any treatment. At the conclusion of the culture period, media were removed, and total RNA was extracted and subjected to an RNase protection assay with antisense riboprobes corresponding to rat IL-1RA and RPL19. As shown in Figure 4 (right panels), sIL-1RA transcripts were first noted at the time of plating (Time 0). The latter appeared to increase spontaneously to a peak value by 4 h of culture (14-fold increase over Time 0; $p < 0.05$). Thereafter, however, IL-1RA transcripts declined to a 48-h nadir. The intensity displayed by protected fragments corresponding to icIL-1RA (Fig. 4) appeared to parallel that noted for sIL-1RA transcripts. However, in other experiments, the signals corresponding to icIL-1RA were too faint for meaningful quantification.
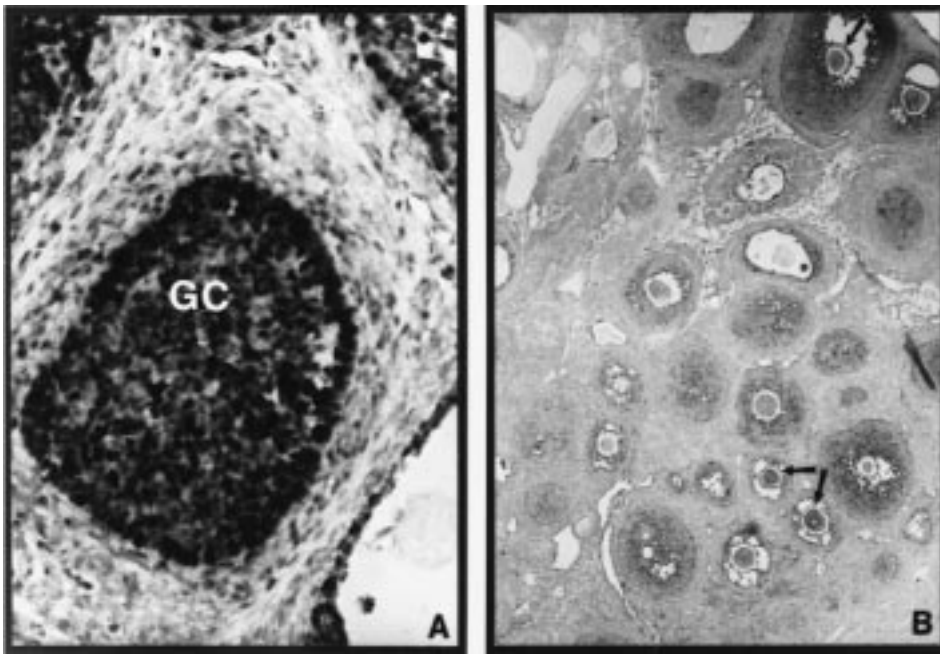
FIG. 3. Cellular localization of IL-1RA transcripts: in situ hybridization studies. Ovaries were obtained from untreated 25-day-old rats and processed for in situ hybridization using a digoxigenin-labeled rat IL-1RA antisense riboprobe as described. **A**) A 400-fold magnification of a section through a preantral follicle. GC, granulosa cells. **B**) A 100-fold magnification of a section through an untreated ovary. The black arrows point to positively stained oocytes. Published at 84%.
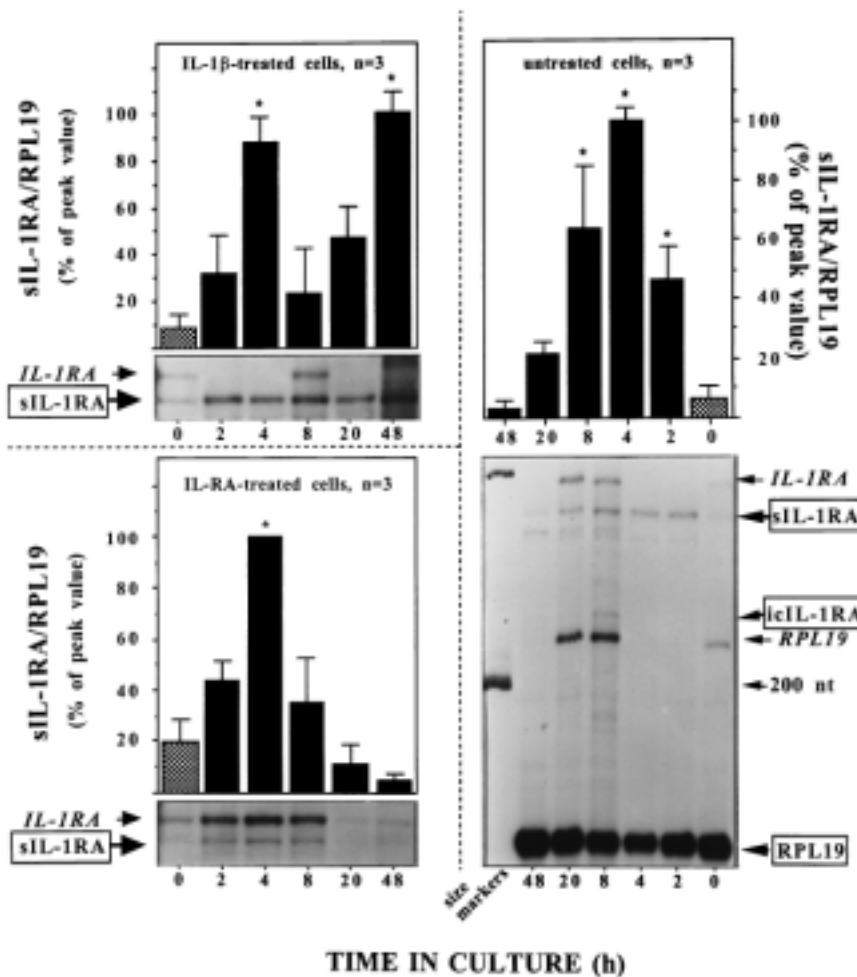

FIG. 4. Secretory IL-1RA gene expression by untreated cultured whole ovarian dispersates: time- and IL-1-dependence. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for up to 48 h in the presence of the indicated treatments. Total cellular RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1RA and RPL19. The intensity of the signals was quantified as described. **Right panels**) Mean ± SE of 3 experiments carried out without any treatment (bar graph and representative autoradiograph). **Upper left**) Mean ± SE of 3 experiments carried out with IL-1β (50 ng/ml) as treatment (a representative autoradiograph is shown at the base of the bar graph). **Lower left**) Mean ± SE of 3 experiments carried out with IL-1RA (5 μg/ml) as treatment (a representative autoradiograph is shown at the base of the bar graph). In all the autoradiographs, full-length riboprobes are in italics and protected fragments are boxed. Data were normalized relative to the peak value in each individual experiment.
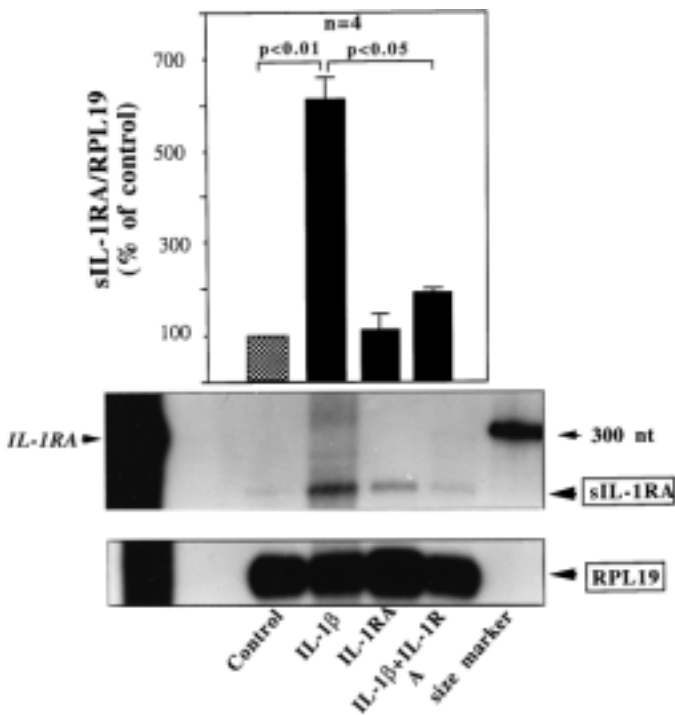
FIG. 5. Secretory IL-1RA gene expression by cultured whole ovarian dispersates: IL-1 receptor mediation. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for 48 h in the absence or presence of IL-1β (50 ng/ml), with or without IL-1RA (5 μg/ml). The resultant RNA samples were subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1RA and RPL19. The bar graph depicts the mean ± SE of 4 experiments, and beneath it is a representative autoradiograph (full-length IL-1RA probe in italics; protected fragments are boxed). In each individual experiment data were normalized relative to control.

Secretory IL-1RA Gene Expression by Cultured Whole Ovarian Dispersates: IL-1 Dependence

Given the apparent concordance in the temporal in vivo expression patterns of intraovarian IL-1β [2] and IL-1RA (Fig. 2), we undertook to assess a possible effect of IL-1β on sIL-1RA gene expression. Whole ovarian dispersates were cultured for the duration indicated (up to 48 h) in the presence of IL-1β. As shown in Figure 4 (top left), treatment with IL-1β was without apparent effect on the spontaneous early (4 h) sIL-1RA peak (a 10-fold increase over Time 0; $p < 0.05$). However, treatment with IL-1β reversed the subsequent decline, thereby resulting in a secondary sIL-1RA peak at 48 h (11.5-fold increase over Time 0; $p < 0.05$), an effect characterized by an approximate ED_{50} of 1.5 ng/ml (not shown). These data suggest that subject to the limitations of the RNase protection assay and the frequency of sampling, the transcriptional effect of exogenously provided IL-1β becomes apparent ≤ 20 h into the culture period.

To explore the possibility that the early spontaneous (4 h) increase in sIL-1RA gene expression (Fig. 4, right panels) may be mediated by endogenously produced IL-1β, whole ovarian dispersates were cultured for the duration indicated (up to 48 h) in the presence of IL-1RA, thereby creating an "IL-1β vacuum." As shown in Figure 4 (lower left), treatment with IL-1RA was without significant effect on the early (4 h) sIL-1RA peak (5-fold increase over Time 0; $p < 0.05$), thereby further suggesting that the early burst in sIL-1RA expression in vitro is IL-1β independent.
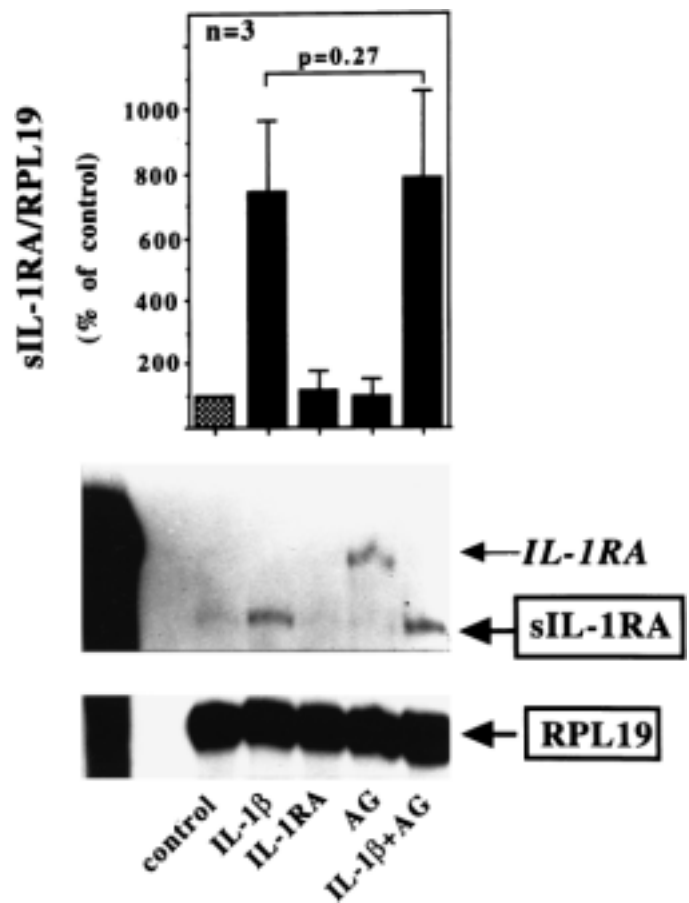

FIG. 6. Secretory IL-1RA gene expression by cultured whole ovarian dispersates: nitric oxide dependence. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for 48 h in the absence or presence of IL-1β (50 ng/ml), with or without AG (0.4 mM). The resultant RNA samples were subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1RA and RPL19. The bar graph depicts the mean ± SE of 3 experiments, and beneath it is a representative autoradiograph (the full-length IL-1RA probe in italics; protected fragments are boxed). In each individual experiment data were normalized relative to control.

To further evaluate the dependence of ovarian sIL-1RA transcripts on IL-1β, whole ovarian dispersates were cultured for 48 h in the presence of IL-1β, with or without human recombinant IL-1RA. As shown in Figure 5, and in keeping with earlier observations (Fig. 4, top left), treatment with IL-1β (but not IL-1RA) produced a secondary sIL-1RA peak (6-fold increase over untreated control values; $p < 0.01$). Importantly, this effect was substantially ($p < 0.05$) reversed by IL-1RA. These observations support the view that the ability of IL-1β to up-regulate sIL-1RA transcripts is IL-1 receptor mediated.

Secretory IL-1RA Gene Expression by Cultured Whole Ovarian Dispersates: Nitric Oxide Dependence

To determine whether the effect of treatment with IL-1β on sIL-1RA gene expression is contingent upon endogenously produced nitric oxide, whole ovarian dispersates were cultured for 48 h in the presence of exogenously provided IL-1β, with or without AG, an established inhibitor of the inducible variety of ovarian nitric oxide synthase [22–24]. As shown in Figure 6, a "nitric oxide vacuum" was without significant effect on either the spontaneous or the IL-1β-induced expression pattern of sIL-1RA. A 48-h application of SNAP, an established nitric oxide generator
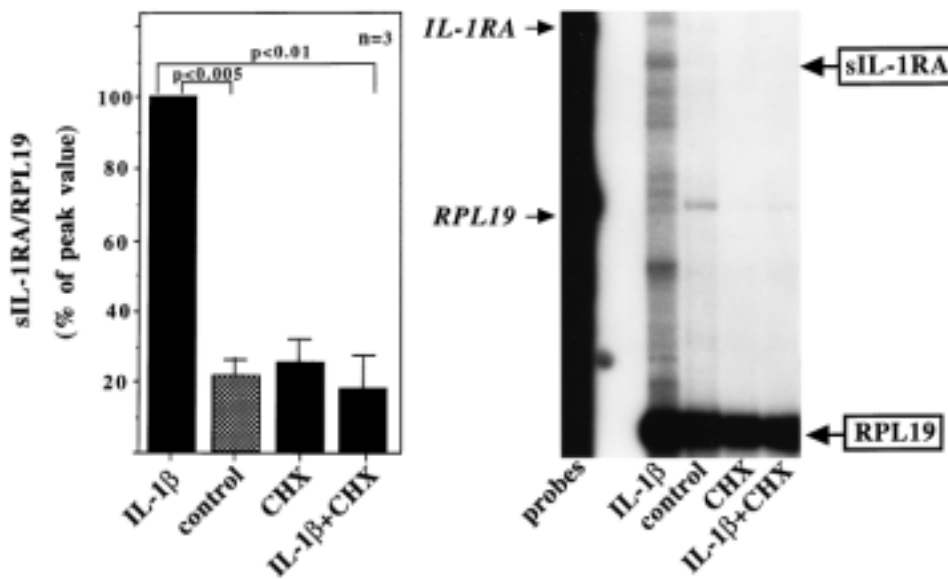
FIG. 7. Secretory IL-1RA gene expression by cultured whole ovarian dispersates: protein synthesis dependence. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for 48 h in the absence or presence of IL-1β (50 ng/ml), with or without CHX (0.1 μg/ml). The resultant RNA samples were subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1RA and RPL19. **Left**) Mean ± SE of 3 experiments. **Right**) A representative autoradiograph (full-length riboprobes in italics, protected fragments are boxed). Data were normalized relative to the peak value in each individual experiment.

[24], failed to alter the steady state levels of sIL-1RA transcripts (not shown). These observations suggest that the stimulatory effect of IL-1β on sIL-1RA expression is nitric oxide independent. Blockade of nitric oxide synthase activity was also without effect on the early (4 h) spontaneous burst of sIL-1RA expression (not shown).

IL-1β-Induced sIL-1RA Gene Expression by Cultured Whole Ovarian Dispersates: Protein Synthesis Dependence

To determine whether IL-1β-induced sIL-1RA gene expression is contingent upon an intact protein synthesis machinery, whole ovarian dispersates were cultured for 48 h in the presence of IL-1β, with or without CHX. As shown in Figure 7, treatment with CHX led to complete blockade of the ability of IL-1β to up-regulate sIL-1RA transcripts. Importantly, RPL19 transcripts remained unaffected, thereby arguing against an overt general toxic effect of CHX on the cellular transcription system. These observations suggest that IL-1β-induced sIL-1RA gene expression requires de novo protein biosynthesis. In contrast, CHX proved

without effect on the early (4 h) spontaneous burst of sIL-1RA expression (not shown).

Secretory IL-1RA Gene Expression by Cultured Whole Ovarian Dispersates: Specificity Studies

To determine whether the modulation of ovarian sIL-1RA gene expression is IL-1 specific, whole ovarian dispersates were cultured for 48 h in the presence of ovarian agonists other than IL-1β, specifically TNFα, IGF-I, activin A, TGFα, or ET-1. As shown in Figure 8, treatment with none of these established agonists (with the exception of IL-1β) affected the steady state levels of ovarian sIL-1RA transcripts. These observations suggest that the ability of IL-1β to up-regulate sIL-1RA gene expression is not shared by a series of cytokines and growth factors.

DISCUSSION

Naturally occurring IL-1RA, a 22-kDa protein, was first reported in 1985 by Arend [43–45]. In all, two forms of IL-1RA have been cloned: a secretory variant (sIL-1RA),
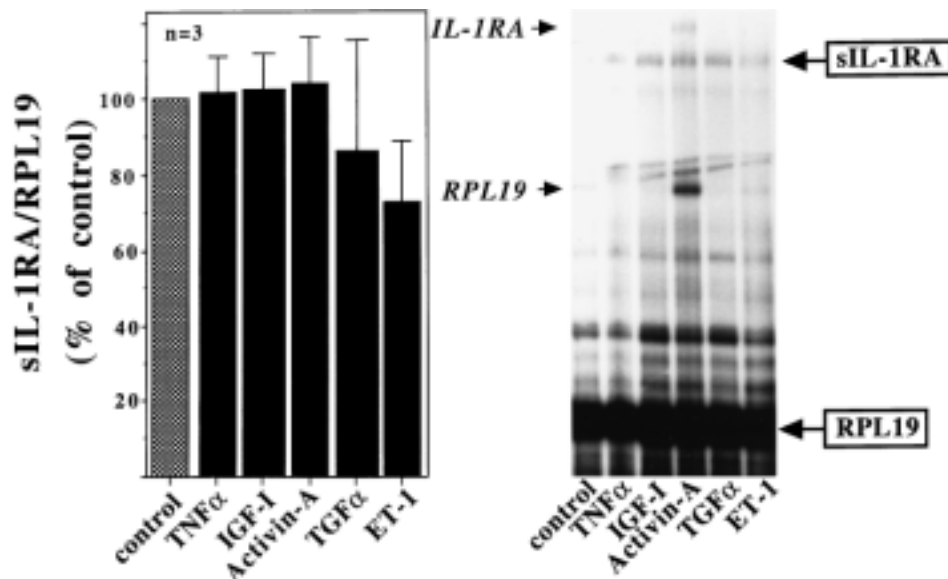

FIG. 8. Secretory IL-1RA gene expression by cultured whole ovarian dispersates: specificity studies. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for 48 h in the absence or presence of TNFα (30 ng/ml), IGF-I (50 ng/ml), activin A (50 ng/ml), TGFα (10 ng/ml), or ET-1 (10^{-8} M). The resultant RNA samples were subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1RA and RPL19. **Left**) Mean ± SE of 3 experiments. **Right**) A representative autoradiograph (full-length riboprobes in italics, protected fragments are boxed). Data were normalized relative to control in each individual experiment.

expressed mainly in monocyte-derived cell populations, and an intracellular form (icIL-1RA; subtypes I and II), expressed mainly by epithelial cells [26–31]. The latter is believed to subserve a defensive intracellular role in "environmentally" exposed cells (mainly keratinocytes). Functionally, IL-1RA is a pure competitive inhibitor of both IL-1α and IL-1β action by way of binding to type I IL-1 (but not type II) cell surface receptors, but without inducing a cellular effect [25, 46]. Expectedly, IL-1RA has generated considerable interest as a potential therapeutic agent in the context of sepsis [36], rheumatoid arthritis [34, 38], glomerulonephritis [37], graft vs. host reaction [32], and inflammatory bowel disease [27].

The identification of the rat granulosa cell of untreated rats as a site of ovarian IL-1RA expression is in agreement with a previous study wherein use was made of macrophage-free human granulosa cells [9]. However, this does not eliminate the possibility of other ovarian cell types as a source of perovulatory IL-1RA. Our observation suggests that the rat granulosa cell might serve as the primary source for various components of the IL-1 system including IL-1β (unpublished results) and its type I and type II receptors [11]. Thus, the rat ovary is a site of IL-1 production [2, 14], reception [11], and action [2, 5, 7, 8, 15, 18–24].

Initial efforts at establishing, by RNase protection assay, the ovarian expression of IL-1RA revealed that, as compared to other tissues, intact whole ovarian material from untreated immature rats is not a major site of IL-1RA gene expression (Fig. 1). It is likely that this observation reflects the highly compartmentalized nature of ovarian IL-1RA expression, whose virtually exclusive localization to the membrana granulosa and cumulus layer was revealed by in situ hybridization technology (Fig. 3). Interestingly, sIL-1RA expression by untreated and IL-1-treated cultured whole ovarian dispersates proved vigorous when probed by the same RNase protection technology.

Special note must be made of the 4-h "burst" of sIL-1RA transcripts in cultured whole ovarian dispersates. This reproducible IL-1-independent phenomenon has previously been documented for IL-1α, IL-1β, and the type II IL-1 receptor (unpublished results). Although the precise molecular and cellular mechanism(s) underlying this phenomenon remain unknown, it is tempting to speculate that the dispersion and plating of ovarian cells may constitute a self-limited injurious process leading to an adaptational response akin to inflammation. To the extent that ovulation constitutes an inflammatory-like process [1], the interim burst in the expression of IL-1α, IL-1β, type II IL-1 receptor, and IL-1RA may represent a crude simulation of intraovarian events reminiscent of ovulation.

To evaluate the possible dependence of ovarian sIL-1RA on IL-1β, whole ovarian dispersates were cultured for 48 h in the absence or presence of IL-1β (Fig. 4). Treatment with IL-1β proved up-regulatory to ovarian sIL-1RA transcripts (secondary sIL-1RA peak), thereby strongly suggesting IL-1 dependence. The IL-1β effect appeared to be receptor mediated in that the concurrent presence of IL-1RA abolished the IL-1β effect (Fig. 5). Presumably, only the type I IL-1 receptor was involved [47], since the type II receptor does not appear to engage in signaling but rather serves as a stationary binding protein [48]. Moreover, ovarian transcripts corresponding to the type I IL-1 receptor are more abundant than those for the type II receptor [11]. Taken together, these observations support the view that ovarian IL-1RA transcripts are IL-1 dependent and that the IL-1 effect under study is receptor mediated. In this respect,

our findings are in keeping with those reported for several extraovarian sites [49]. These findings establish IL-1RA as yet another rat ovarian "endpoint" of IL-1 action apart from the induction of prostaglandin biosynthesis [15–18], the promotion of proteoglycan formation [19], the induction of collagenase activity [20], the modulation of plasminogen activator activity [21], the activation of nitric oxide synthase [22–24], the causation of morphogenic/cytotoxic changes [50], and the perturbation of FSH action [51–56].

The apparent periovulatory increase in ovarian IL-1RA transcripts suggests a role for this principle during this critical time. On the one hand, intuitive reasoning might have predicted a midcycle decrease in IL-1RA expression thereby enhancing the net IL-1 impact. Such view would be compatible with the hypothesis that IL-1 plays a central intermediary role in the ovulatory process [4]. On the other hand, it is possible that the midcycle enhancement of ovarian IL-1RA expression is designed to "buffer" what might otherwise be an unrestrained IL-1 effect. Indeed, given the documented ability of IL-1 to self-amplify by way of a positive feedback loop [2], an in-built attenuating mechanism may well be in order. It may be relevant that IL-1RA peaks later than IL-1β [2], thereby allowing it to exercise its potential "dampening" action.

In an effort to better characterize the IL-1 effect, we set out to examine the possible intermediary role of nitric oxide and of protein synthesis. Although the role of nitric oxide in ovarian physiology remains to be defined, relevance to the ovulatory process has been proposed [57]. Given the proposed role of IL-1 in the ovulatory process and its established ability to activate the inducible variety of ovarian nitric oxide synthesis [22–24], we set out to examine the role of nitric oxide in IL-1 hormonal action. Our findings (Fig. 6) revealed that the generation of a nitric oxide vacuum is without significant effect on IL-1 action. On the other hand, blockade of protein biosynthesis resulted in complete abrogation of IL-1 hormonal action. Although the nature of the obligatory protein remains unknown, it is tempting to speculate that the synthesis of the type I IL-1 receptor is at play.

Studies at the transcript levels suggest that the ability of IL-1 to up-regulate sIL-1RA transcripts is characterized by an ED_{50} of 1.5 ng/ml. This concentration of IL-1β is higher than required for the induction of secretory PLA_2 (0.3 ng/ml; PLA: phospholipase A) or nitric oxide generation (0.7 ng/ml)—comparable to that required for the induction of cytosolic PLA_2 (2 ng/ml), of prostaglandin endoperoxide synthase-2 (2 ng/ml), or of Glut1 and 3 (2 and 3 ng/ml) transcripts—but is far lower than that required for the induction of IL-1β (6 ng/ml) or type I IL-1 receptor (10 ng/ml) (unpublished results). To the extent that IL-1 may play a role in the ovulatory cascade, these observations suggest that the induction of IL-1RA may constitute one of the relatively early events in the sequence leading to follicular rupture. Such arrangement may well be designed to enable IL-1RA to control the runaway positive feedback loop of IL-1. Thus, the initial IL-1 signal might induce all the components necessary for ovulation, the induction of IL-1RA preventing sufficient accumulation of IL-1 activity for the further activation of IL-1β expression.

However, null mutants of both the IL-1β [58] and the type I IL-1 receptor [59] genes feature a normal reproductive phenotype. Although these observations could be taken to argue against the intraovarian IL-1 hypothesis, consideration must be given to the possibility that the chronicity of the experimental paradigm allowed yet-to-be-defined

compensatory mechanisms to mask possible ovulatory dysfunction. In addition, one cannot as yet rule out the possibility that there may exist additional yet-to-be-defined IL-1 receptors that may provide the necessary substrate in the context of genetically destructed IL-1β and type I IL-1 receptor genes. Thus, although the null mutants clearly establish that IL-1 does not constitute an obligatory intermediary in the ovulatory process, a possible participatory role for IL-1 in mammalian ovulation cannot as yet be entirely ruled out.

In summary, our present in vivo and in vitro observations document the rat ovary as a site of IL-1RA expression, localize the relevant transcripts to the granulosa cell, disclose peak expression during a narrow periovulatory window, and establish IL-1 dependence. This latter effect of IL-1 proved receptor mediated and gonadotropin- and nitric oxide-independent, but contingent upon de novo protein biosynthesis.

ACKNOWLEDGMENTS

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