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The rat intraovarian interleukin (IL)-1 system: cellular localization, cyclic variation and hormonal regulation of IL-1β and of the type I and type II IL-1 receptors

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Abstract

An increasing body of evidence supports the possibility that intraovarian interleukin (IL)-1 plays an intermediary role in the periovulatory cascade. To gain further insight into the intraovarian IL-1 hypothesis, we studied the cellular localization cyclic variation and hormonal regulation of IL-1β, as well as of the type I and type II IL-1 receptors (IL-1R) in immature rats. In situ hybridization localized IL-1β and type I IL-1R transcripts to the granulosa cell compartment, the innermost layers of the theca interna and to the oocyte of the untreated immature ovary. Molecular probing of whole ovarian material in the course of a simulated estrous cycle revealed a progressive preovulatory increase in IL-1β and type I IL-1R transcripts to an in vivo peak at the time of ovulation (3.0- and 2.5-fold increases over untreated controls; $P < 0.05$). Comparable efforts to localize and probe for type II IL-1R transcripts failed to elicit a detectable signal. The basal in vitro expression pattern of IL-1β and type II IL-1R transcripts by whole ovarian dispersates revealed an early (4 h) spontaneous increase to a peak (2.1- and 5.8-fold increases over time 0; $P < 0.05$) followed by a gradual decline to a 48 h nadir. Treatment of whole ovarian dispersates with the IL-1 receptor antagonist (IL-1RA) or with IL-1β failed to alter the initial (4 h) burst of IL-1β or of type II IL-1R expression thereby suggesting IL-1-independence. Treatment with hCG proved equally ineffective. However, longer-term treatment of whole ovarian dispersates with IL-1β produced a significant secondary increase (5.9-fold over time 0; $P < 0.05$) in IL-1β (but not type II IL-1R) transcripts by 48 h. This IL-1 effect was completely blocked by co-treatment with IL-1RA thereby suggesting mediation via a specific IL-1 receptor. Qualitatively comparable but quantitatively reduced results obtained for isolated granulosa cells. The basal in vitro expression pattern of type I IL-1R transcripts by whole ovarian dispersates revealed a progressive spontaneous increase (3.1-fold increase overall) over the 48 h culture. Treatment with IL-1β produced a significant ($P < 0.05$) increase (5-fold) in type I IL-1R transcripts by 48 h, an effect which was completely blocked by co-treatment with IL-1RA. Taken together, these observations: (1) localize IL-1β and its type I receptor to granulosa cells, the innermost layers of the theca interna and to the oocyte; (2) confirm their periovulatory in vivo expression pattern; (3) document their expression by untreated cultured whole ovarian dispersates; and (4) demonstrate their in vitro responsiveness to receptor-mediated/IL-1-driven autocrine amplification. The type II IL-1R was undetectable in vivo, its in vitro expression pattern proving IL-1- and hCG-independent. The periovulatory expression pattern of

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IL-1β and its receptor (type I) is compatible with the notion that the intraovarian IL-1 system may play an intermediary role in the ovulatory process.

Keywords: Follicle; Ovary; Ovulation; Granulosa; Cytokines

1. Introduction

A growing body of direct and indirect evidence supports the notion that intraovarian interleukin (IL)-1β may constitute an intermediary in the ovulatory process (Ben-Shlomo and Adashi, 1994). First, the ex vivo provision of IL-1β has been shown to bring about ovulation and to synergize with LH in this regard (Brannstrom et al., 1993a; Takehara et al., 1994). Second, the addition of an IL-1 receptor antagonist (IL-1RA) has been shown to attenuate LH-supported ovulation under both ex vivo (Peterson et al., 1993) and in vivo (Simon et al., 1994a) circumstances. Third, IL-1β has been shown to induce a host of ovulation-associated phenomena such as the promotion of ovarian prostaglandin production (Nothnick and Page, 1990; Kokia et al., 1992; Pitzel et al., 1993; Brannstrom et al., 1993b; Townson and Pate, 1994), the stimulation of ovarian hyaluronic acid biosynthesis (Kokia et al., 1993), the induction of ovarian collagenase activity (Hurwitz et al., 1993), the modulation of ovarian plasminogen activation (Bonello et al., 1995; Hurwitz et al., 1995) and the activation of ovarian nitric oxide synthase (Ellman et al., 1993; Ben-Shlomo et al., 1994a,b; Bonello et al., 1996).

As is true for every intraovarian IL-1 system, the intraovarian IL-1 system is comprised of two ligands (IL-1α and IL-1β), two receptors (type I and type II IL-1R) and an IL-1RA. Although the mammalian ovary has been shown to be a site of IL-1β expression (Hurwitz et al., 1991a, 1992; Brannstrom et al., 1994; Polan et al., 1994; Simon et al., 1994b), precise information on its cyclic variation and hormonal regulation remains incomplete. Similarly, limited information is available as to the cellular localization and hormonal regulation of ovarian type I and type II IL-1 receptors (Deyerle et al., 1992; Simon et al., 1994b; Scherzer et al., 1995; Wang et al., 1997). It was the purpose of this communication to characterize in some detail the cellular localization, cyclic variation and hormonal regulation of rat ovarian IL-1β as well as of its type I and type II IL-1 receptors. Special emphasis has been placed on the regulation of the intraovarian IL-1 system inclusive of the cellular mechanism(s) underlying these phenomena.

2. Materials and methods

2.1. Animals

Immature Sprague–Dawley female rats from Zivic–Miller Laboratories (Zelienople, PA) were sacrificed by CO_2 asphyxiation on day 25 of life. The project was approved by the Instititional Animal Care and Use Committee.

2.2. Hormones and reagents

Recombinant human IL-1β (2×10^7 units/mg) was generously provided by Drs Errol B. De Souza and C.E. Newton, DuPont-Merck Pharmaceutical Co. (Wilmington, DE). A recombinantly-expressed preparation of the naturally occurring human IL-1 receptor antagonist (IL-1RA) was generously provided by Dr Daniel E. Tracey, The Upjohn Co. (Kalamazoo, MI). Highly-purified human chorionic gonadotropin (hCG; CR-127, 14900 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). Tumor 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 (serum-free), penicillin-streptomycin solution, l-glutamine, trypan blue stain and bovine serum albumin (BSA) were from Life Technologies, Inc. (Grand Island, NY). Collagenase (Clostridium Histolyticum; CLS type I; 144 U/mg) was from Worthington Biochemical Corp. (Freehold, NJ). DNAse (bovine pancreas), aminoguanidine hemisulfate salt (AG), RNase A and cycloheximide (CHX) were from Sigma Chemical Co. (St. Louis, MO). Alkaline phosphatase-conjugated anti-digoxigenin polyclonal antibody Fab fragments, digoxigenin-UTP, nitroblue tetrazolium and 5-bromo-4-chloro-3-inodyl-phosphate were from Boehringer Mannheim (Indianapolis, IN). T7 RNA polymerase, pGEM2 and other molecular biology grade reagents were from Promega (Madison,

WI). The RNA marker set kit was from Ambion (Austin, TX).

2.3. Tissue culture procedures

Whole ovarian dispersates or isolated granulosa cells were prepared and maintained as previously described (Hernandez et al., 1988; Kokia et al., 1992). Briefly, cells were prepared by collgenase/DNase dispersion, cultured (37°C, 5% CO_2, 95% air) in McCoy's 5a medium (modified, without serum), supplemented with l-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cell culture intended for RNA extraction were incubated (1.5×10^6 cells/3 ml) for up to 48 h.

2.4. Nucleic acid probes

The rat IL-1β cDNA was provided in PUC8 by Dr A. Shaw of Glaxo (Geneva, Switzerland). A 222 bp *Pst*I fragment of the original cDNA was sub-cloned into pGEM2. T7-driven transcription of the *Eco*RI-linearized plasmid yielded a 272 nt riboprobe which upon hybridization was projected to generate a 222 nt protected fragment (Hurwitz et al., 1991b).

Rat type I and type II IL-1 receptor probes were generated as previously described (Scherzer et al., 1995). Briefly, reverse transcription was performed with 1 μg of total RNA and the reaction products amplified with trans-species oligonucleotide primer sets. In order to generate a plasmid clone suitable for riboprobe synthesis, the PCR products were ligated into a pCR1000 vector. The expected lengths for each of the probes and the resultant protected fragments are 374 and 307 nt (type I IL-1R) and 444 and 355 nt (type II IL-1R).

The ribosomal protein large 19 (RPL19) probe was generated as previously described (Scherzer et al., 1995). The resultant transcription was projected to generate 283 or 234 nt probes capable of protecting a 194 or 153 nt segment, respectively.

{α-^{32}P} UTP-labeled RNA size markers were generated by using a marker set kit as the DNA template.

2.5. RNA extraction

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

2.6. RNase protection assay

Linearized DNA templates were transcribed with T7 RNA polymerase to specific activities of 800 Ci/mmol {α-^{32}P}UTP (IL-1β, type I IL-1R and type II IL-1R) or 160 Ci/mmol {α-^{32}P}UTP (RPL19). The riboprobes were gel-purified as described (Kol et al., 1996) in an effort to eliminate transcribed products that are shorter than the full length probes. The assay was performed as previously described (Lowe et al., 1987). Gels were exposed to XAR film (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 Image Quant Software (Molecular Dynamics, Sunnyvale, CA). The hormonally-independent RPL19 mRNA signal was used to normalize the IL-1β type I IL-1R and type II IL-1R mRNA data for possible variation in RNA loads. Specifically, the net protected signal (respective background subtracted) to net RPL19 ratio was calculated for each sample and gene of interest.

2.7. In situ hybridization

Technology conformed to that previously described (Scherzer et al., 1995). Briefly, ovaries were removed, dissected free of surrounding tissues, fixed in 10% buffered formalin and embedded in paraffin wax. Sections (4–6 μm thick) were baked overnight at 60°C, after which wax was removed, pepsin was applied and slides were fixed with 2.7% paraformaldehyde. Riboprobes were synthesized with digoxigenin-UTP and were added in hybridization buffer to the samples. Slides were heated for 95°C for 15 min and hybridized at 47°C overnight. Rinsed slides were then incubated with alkaline phosphatase-conjugated anti-digoxigenin polyclonal antibody Fab fragments. Unbound antibody was removed and bound antibody detected by incubation with nitroblue tetrazolium and NBT/BCIP.

2.8. 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) determined by ANOVA and Student's *t*-test. Statistical values were calculated using Statview 512+ for MacIntosh (Brair Power, Calabasas, CA).

3. Results

3.1. Cellular localization of IL-1β and type I IL-1 receptor transcripts: in situ hybridization studies

To establish the identity of the ovarian cell population responsible for IL-1β and type I IL-1R gene ex-

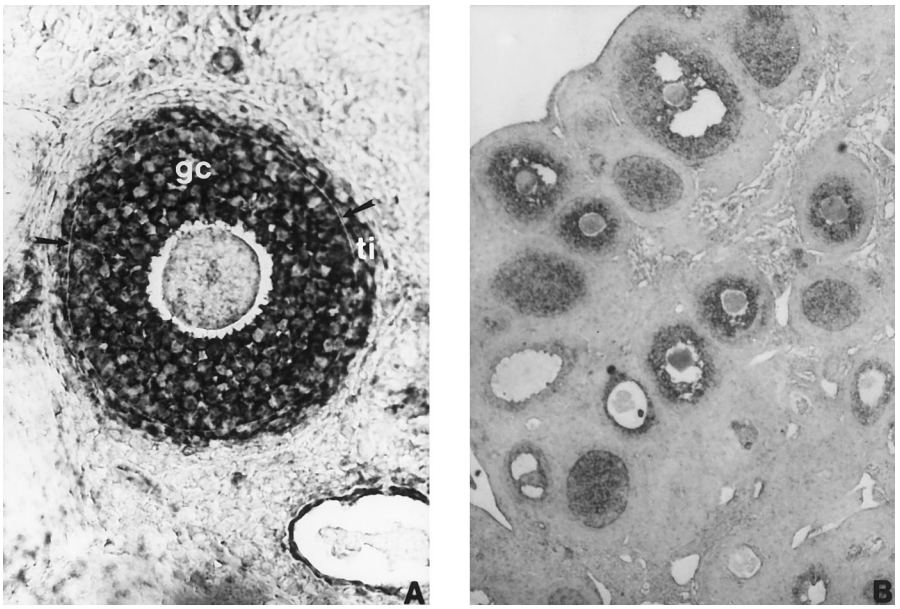

Fig. 1. Cellular localization of IL-1β transcripts: in situ hybridization studies. Ovaries from intact 25-day old rats were processed for in situ hybridisation using a digoxigenin-labeled rat IL-1β antisense riboprobe as described. (A) A 400-fold magnification of a section through an early antral follicle. 'gc' denotes the granulosa cell layers. 'ti' denotes the theca-interna cell layers. The black arrows point to the basement membrane. (B) A 100-fold magnification of a section through an immature ovary. Incubation with sense probe proved negative (not shown).

pression, ovaries were obtained from intact 25-day old rats and processed for in situ hybridization as described. As shown (Figs. 1 and 2), IL-1β and type I IL-1R transcripts were localized to the granulosa cell (mural, antral and cumulus) compartment and to the innermost (Brannstrom et al., 1993a; Ben-Shlomo and Adashi, 1994) cell layers of the theca interna. Modestly intense labeling was also apparent in oocytes. Efforts to detect type II IL-1R transcripts in the ovary failed to elicit a detectable signal.

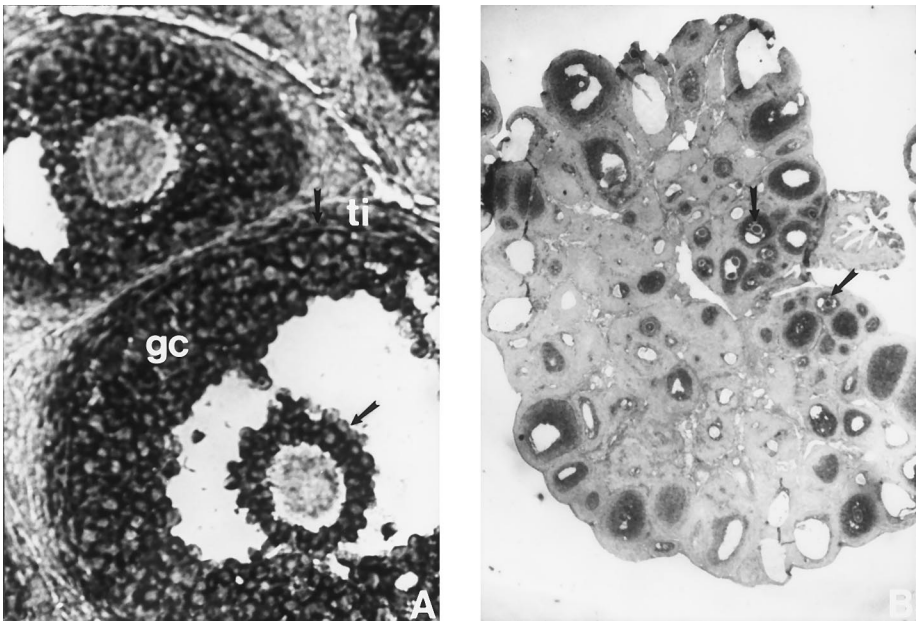

Fig. 2. Cellular localization of type I IL-1R transcripts: in situ hybridization studies. Ovaries were obtained from intact 25-day-old rats and processed for in situ hybridization using a digoxigenin-labeled rat type I IL-1R antisense riboprobe as described. (A) A 400-fold magnification of a section through an antral follicle. 'gc' denotes the granulosa cell layers. 'ti' denotes the theca-interna cell layers. The upper black arrow points to the basement membrane. The lower black arrow points to positively-stained cumulus cells. (B) A 100-fold magnification of a section through an immature ovary. The black arrows point to stained oocytes. Incubation with sense probe proved negative (not shown).

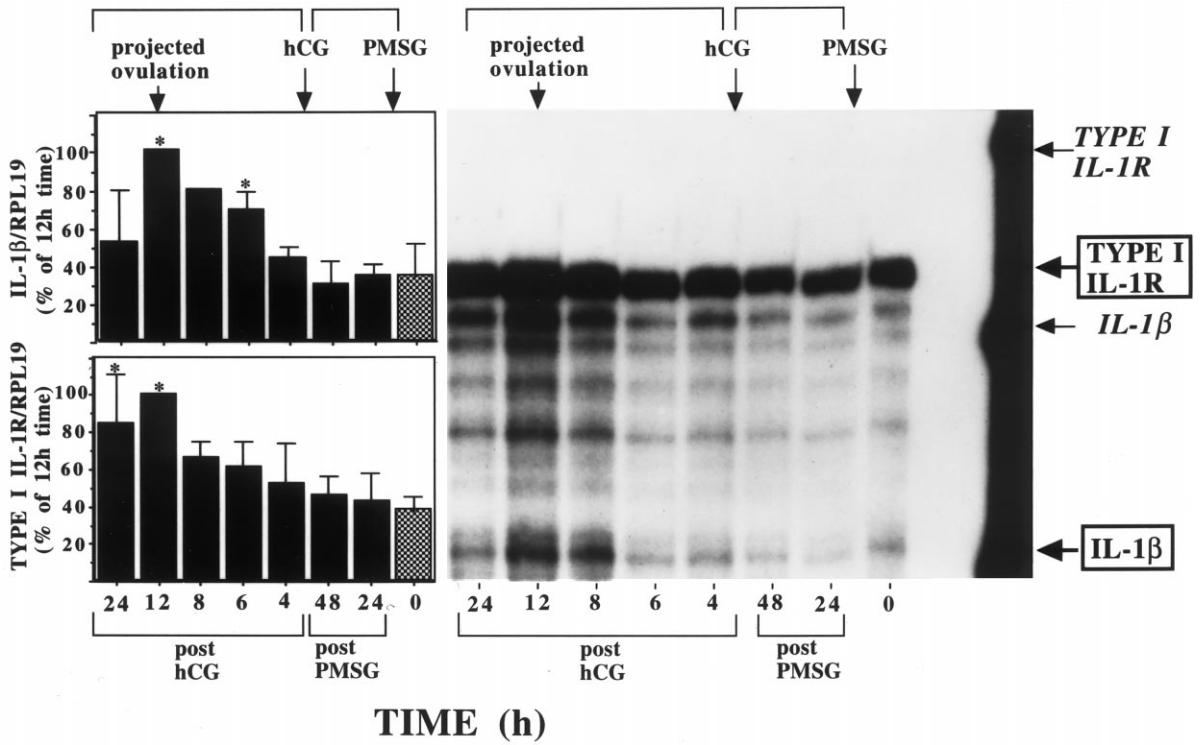

Fig. 3. Ovarian IL-1β and type I IL-1R gene expression: effects of follicular maturation, ovulation and corpus luteum formation. Immature rats were initially primed with 15 IU of PMSG. Ovulation was triggered 48 h later with 15 IU of hCG. The animals were sacrificed at the indicated time points, the ovaries snap-frozen in −70°C, total RNA extracted and subjected to a solution hybridization RNase protection assay using antisense, riboprobes corresponding to rat IL-1β type I IL-1R and RPL19. The intensity of the signals was quantified as described. The bar graphs depict the mean ± SE of three experiments. In each individual experiment, data were normalized relative to the 12 h peak. The right panel depicts a representative autoradiograph. Protected fragments are depicted in bold-faced letters. The full-length riboprobes are depicted in italics. $^{*}P < 0.05$.

3.2. Ovarian IL-1β and type I IL-1 receptor gene expression: effects of follicular maturation, ovulation and corpus luteum formation

To assess IL-1β and type I IL-1 receptor gene expression in the course of a simulated estrous cycle, 25-day-old rats were initially primed with 15 IU of PMSG. Ovulation was triggered 48 h later with 15 IU of hCG. The animals were sacrificed at the indicated time points, the ovaries snap-frozen at −70°C, total RNA extracted and subjected to a solution hybridization RNase protection assay with antisense riboprobes corresponding to rat IL-1β, type I IL-1R and RPL19. As shown (Fig. 3), protected fragments corresponding to IL-1β and type I IL-1R were apparent throughout the experiment. The in vivo expression of IL1β increased progressively and substantially to a peak (3-fold increase over untreated controls, $P < 0.05$) 12 h after hCG administration (time of projected ovulation). A comparable pattern was noted for type I IL-1 receptor transcripts for which a 2.5-fold increase ($P < 0.05$) was documented. Thereafter, transcripts corresponding to both genes displayed a modest decrease. Efforts to detect type II IL-1R transcripts in the ovary failed to elicit a detectable signal.

3.3. IL-1β gene expression by cultured whole ovarian dispersates: time- and IL-1-dependence

To characterize the basal expression pattern of ovarian IL-1β in vitro, 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, total RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1β and RPL19. As shown (Fig. 4, left panel), IL-1β transcripts were noted at the time of cell plating (time 0). Thereafter, note was made of a spontaneous increase to a 4 h peak (2.1-fold increase over time 0, $P < 0.05$) followed by a gradual decline to a 48 h nadir. Treatment with IL-1β (Fig. 4, right panel) was without effect on the 4 h peak but produced a marked increase in IL-1β expression at 48 h (a 5.9-fold increase as compared with time 0, $P < 0.05$). Qualitatively comparable but quantitatively reduced results obtained for isolated granulosa cells (not shown). An identical outcome has previously been reported for other end points of IL-1 (Kokia et al., 1992; Hurwitz et al., 1993; Kokia et al., 1993; Ben-Shlomo et al., 1994a), a phenomenon attributable to cell–cell cooperation. These in vitro data document

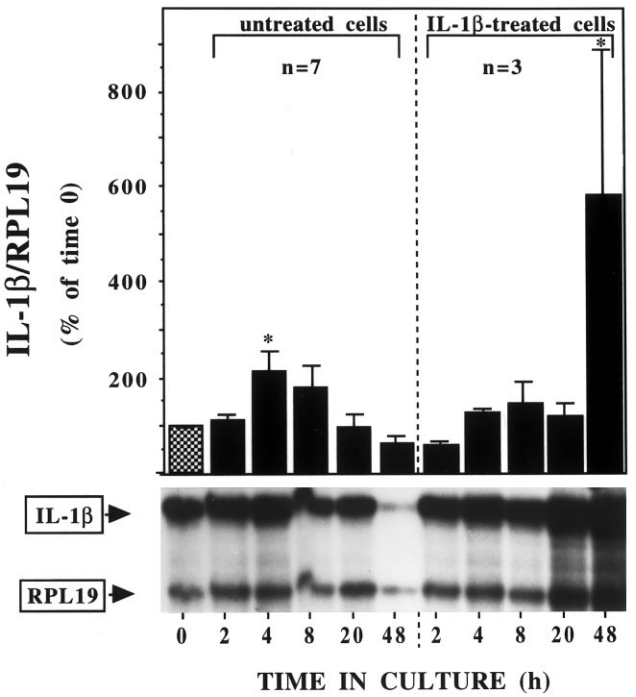

Fig. 4. IL-1β gene expression by cultured whole ovarian dispersates: time and IL-1-dependence. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for the duration indicated (up to 48 h) in the absence or presence of IL-1β. Total cellular RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1β and RPL19. The intensity of the signals was quantified as described. The left and right upper panels depict in bar graph form the mean ± SE of seven and three experiments, respectively. In each individual experiment, data were normalized relative to the time 0 value. A representative autoradiograph is shown below the bar graphs. Protected fragments are depicted in bold-faced letters. $*P < 0.05$ vs time 0.

a spontaneous (IL-1β-independent) early burst in ovarian IL-1β gene expression, as well as a delayed IL-1β-dependent upregulation of the same. Subject to the limitations of the time course, these data suggest that the delayed action of IL-1β took effect during the time window between 20 and 48 h.

3.4. Type I IL-1 receptor gene expression by cultured whole ovarian dispersates: time- and IL-1-dependence

To establish the basal expression pattern of ovarian type I IL-1R in vitro, 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, total RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat type I IL-1R and RPL19. As shown (Fig. 5, left panel), type I IL-1R transcripts, first noted at the time of cell plating (time 0), displayed a relatively rapid spontaneous increase (1.9-fold increase by 2 h, $P < 0.05$), continuing to increase to a 48 h high (3.1-fold increase over time 0, $P < 0.05$). Treatment with IL-1β was without apparent effect on the basal pattern of type I IL-1R expression during the first 20 h of culture (Fig. 5, right panel). However, thereafter, note was made of a further increase in type I IL-1R expression to a 48 h high (a 5.0-fold increase as compared with time 0, $P < 0.05$). These data document a spontaneous, continuous in vitro increase in type I IL-1R expression, one which is further augmented by IL-1β.

3.5. IL-1β and type I IL-1 receptor gene expression by cultured whole ovarian dispersates: IL-1β dose-dependence

To establish the dose requirements of IL-1β as it relates to the upregulation of IL-1β and type I IL-1R transcripts, whole ovarian dispersates were cultured for 48 h in the absence or presence of increasing concentrations of IL-1β. As shown (Fig. 6), provision of increasing concentrations of IL-1β produced dose-dependent increments in the expression of IL-1β and type I IL-1R (6.7- and 2.0-fold increments at the 50 ng/ml dose level, respectively). A statistically significant ($P < 0.05$) increase over untreated cells was noted at the 5 and 50 ng/ml doses for IL-1β and type I IL-1R transcripts, respectively. Overall, the IL-1β effect was characterized by approximate ED_{50s} of 6 and 10 ng/ml for IL-1β and type I IL-1R transcripts, respectively.

3.6. Type II IL-1 receptor gene expression by cultured whole ovarian dispersates: time-, IL-1- and hCG-dependence

Molecular probing of whole ovarian material and in situ hybridization of ovaries from untreated immature rats failed to detect type II IL-1R transcripts (see above). To explore the possibility of type II IL-1R gene expression under in vitro circumstances, whole ovarian dispersates were cultured for the duration indicated (up to 48 h) in the absence or presence of IL-1β, IL-1RA or hCG. At the conclusion of the culture period, total RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat type II IL-1R and RPL19. As shown (Fig. 7), type II IL-1R transcripts were noted at the time of cell plating (time 0). In the absence of treatment (left upper panel), note was made of a relatively rapid spontaneous increase to a 4 h peak (a 5.8-fold increase as compared with time 0, $P < 0.05$) followed by a gradual decline to a 48 h minimum. Treatment with IL-1β (Fig. 7; right upper panel) was without significant effect on the basal pattern of type II IL-1R expression, the 4 h peak representing a 4.1-fold increase over time 0 ($P < 0.05$). To explore the possibility that the 4 h peak observed for type II IL-1R transcripts is mediated by endogenously produced IL-1β, use was made of IL-1RA in an

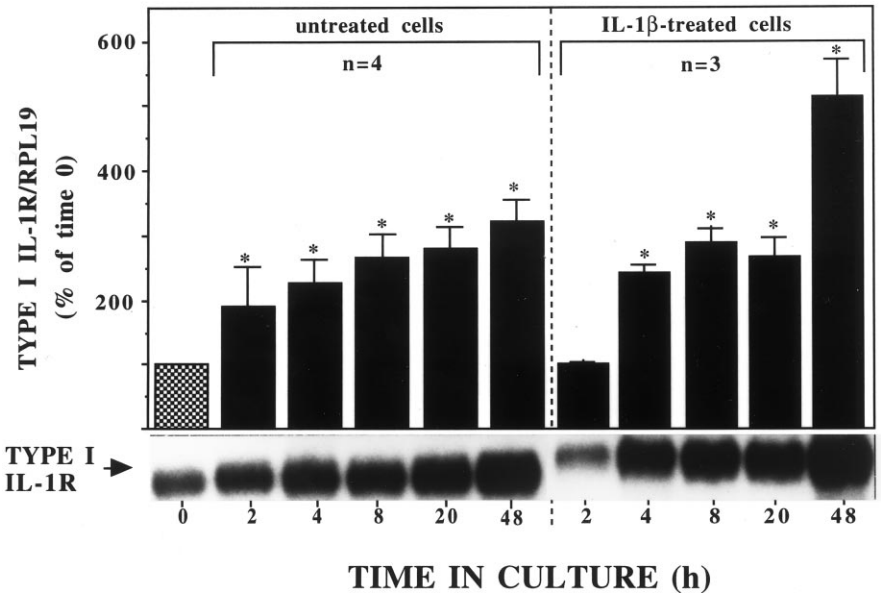

Fig. 5. Type I IL-1R gene expression by cultured whole ovarian dispersates: time- and IL-1-dependence. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for the duration indicated (up to 48 h) in the absence (left panel) or presence (right panel) of IL-1β. Total cellular RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat type I IL-1R and RPL19. The intensity of the signals was quantified as described. The left and right panels depict in bar graph form the mean ± SE of four and three experiments, respectively. In each individual experiment, data were normalized relative to the time 0 value. A representative autoradiograph is shown below the bar graphs. Protected type I IL-1R fragments are marked in bold-faced letters. $^*P < 0.05$ versus time 0.

effort to create an 'IL-1 vacuum' (Kokia and Adashi, 1994). As shown (Fig. 7; right lower panel), treatment with IL-1RA failed to affect the basal pattern of type II IL-1R expression, the 4 h peak constituting a 3.2-fold increase over untreated controls ($P < 0.05$). Finally, use was also made of hCG in recognition of the central role of luteotropic input to the ovulatory process. As shown (Fig. 7; left lower panel), treatment with hCG was without significant effect on the basal pattern of type II IL-1R expression, the 2 h peak constituting a 6-fold increase over untreated controls ($P < 0.05$). Taken together, these observations suggest that the ovary is a site of type II IL-1R gene expression the in vitro regulation of which is IL-1- and hCG-independent.

3.7. IL-1β and type I IL-1 receptor gene expression by cultured whole ovarian dispersates: an IL-1 receptor-mediated effect

To explore the possibility that the ability of IL-1β to upregulate IL-1β and type I IL-1R transcripts constitutes a receptor-mediated event, whole ovarian dispersates were cultured for 48 h in the absence or presence of IL-1β, with or without IL-1RA (Kokia and Adashi, 1994). At the conclusion of the culture period, media were removed, total RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1β, type I IL-1R and RPL19. As shown (Fig. 8), treatment with IL-1β produced significant increases in the relative expression of IL-1β (6.5-fold increase; $P < 0.01$) and type I IL-1R (2-fold increase; $P < 0.05$) transcripts over untreated controls. Importantly, both IL-1 effects were reversed by IL-1RA. These observations support the view that the ovarian effects of IL-1β are IL-1 receptor-mediated.

3.8. IL-1β and type I IL-1 receptor gene expression by cultured whole ovarian dispersates: nitric oxide-dependence

To determine whether the ability of IL-1β to induce IL-1β and type I IL-1R gene expression is contingent upon endogenously-produced nitric oxide (Ellman et al., 1993; Ben-Shlomo et al., 1994a,b; Bonello et al., 1995), whole ovarian dispersates were cultured for 48 h in the absence or presence of IL-1β, with or without AG, an established inhibitor of the inducible variety of ovarian nitric oxide synthase activity (Ellman et al., 1993). As shown (Fig. 9), treatment with AG by itself resulted in a modest (1.6-fold) albeit significant ($P < 0.05$) increase in IL-1β transcripts over untreated control. Similarly, treatment with AG produced a 1.2-fold increase in the relative expression of type I IL-1R ($P < 0.05$). However, co-treatment with AG was without effect on IL-1β action. These observations suggest that nitric oxide plays a limited role in the basal (but not IL-1-induced) expression pattern of IL-1β and type I IL-1R.

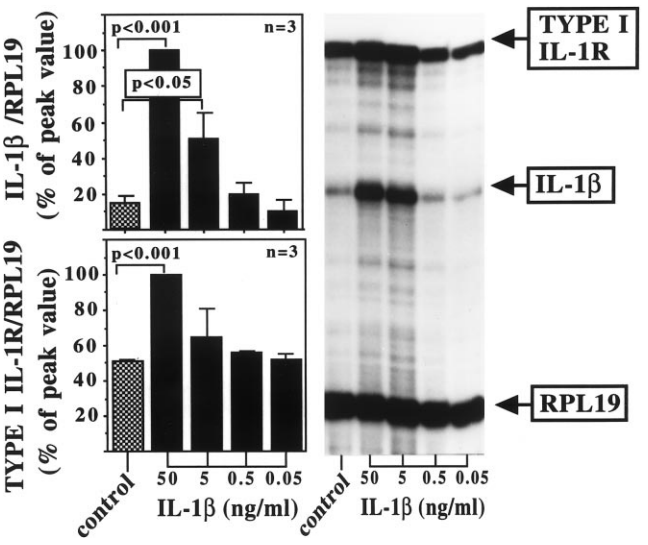

Fig. 6. IL-1β and Type I IL-1R gene expression by cultured whole ovarian dispersates: IL-1β dose-dependence. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for 48 h in the absence or presence of increasing concentrations of IL-1β. Total cellular RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1β, type I IL-1R and RPL19. The intensity of the signals was quantificd as described. The left panels depict in bar graph form the mean ± SE of three experiments. In each individual experiment data were normalized relative to the peak value. The right panel depicts a representative autoradiograph. Protected fragments are labeled in bold-faced letters.

3.9. IL-1β and type I IL-1 receptor gene expression by cultured whole ovarian dispersates: protein synthesis-dependence

To determine if the ability of IL-1β to induce IL-1β and type I IL-1R gene expression is contingent upon intact cellular protein synthesis, whole ovarian dispersates were cultured for 48 h in the absence or presence of IL-1β, with or without cycloheximide (CHX). As shown (Fig. 10), treatment with CHX completely abolished the ability of IL-1β to upregulate IL-1β and type I IL-1R gene expression. Importantly, RPL19 transcripts remained unaffected thereby arguing against an overt general toxic effect of CHX on the cellular transcription system. These observations suggest that the ability of IL-1β to upregulate IL-1β and type I IL-1R transcripts requires de novo protein biosynthesis.

3.10. IL-1β and type I IL-1 receptor gene expression by cultured whole ovarian dispersates: specificity studies

To determine the effect of ovarian agonists (other than IL-1β), whole ovarian dispersates were cultured for 48 h in the absence or presence of TNFα, IGF-I, activin-A, TGFα, or ET-1. As shown (Fig. 11), none of the above-mentioned agonists (with the exception of IGF-I and IL-1β) affected IL-1β or type I IL-1R gene expression as compared with untreated cells. Treatment with IGF-I resulted in a decrease in type I IL-1R transcripts by a modest but significant 23% ($P < 0.05$). These observations suggest that the upregulatory in vitro effect of IL-1β on IL-1β and type I IL-1R transcripts is not shared with the other agents used.

4. Discussion

The present observations localize IL-1β and type I IL-1R in topographically-intact rat ovarian tissue through the use of in situ hybridization technology (Figs. 1 and 2). The latter localized IL-1β transcripts and type I IL-1R transcripts to the granulosa cell compartment, the innermost (Brannstrom et al., 1993a; Ben-Shlomo and Adashi, 1994) cell layers of the theca interna and to the oocyte. Consequently, one must assume that the detection of the relevant transcripts in whole ovarian material (Fig. 3) or in cultured whole ovarian dispersates (Figs. 4–11) of rat origin depends on the contribution of multiple cellular compartments inclusive of both somatic and germ cell elements. Comparable findings in the rat were previously reported for the type I IL-1 receptor in adult rats, although no signal was found in oocytes (Wang et al., 1997). Immunohistochemical studies in the mouse ovary demonstrated a shift in the expression of IL-1β and type I IL-1R from the theca-interstitial layer to the granulosa cell layer immediately after follicular rupture (Simon et al., 1994b). The functional significance of the apparent difference between the rat and mouse intraovarian IL-1 systems remains unknown.

Special note must be made of the apparent localization of IL-1β and type I IL-1R transcripts to the innermost (Brannstrom et al., 1993a; Ben-Shlomo and Adashi, 1994) cell layers of the theca interna (Figs. 1 and 2). It must be pointed out however that the latter designation need not necessarily imply that the cells in question are androgen-producing cells. Indeed, there remains significant doubt as to the precise identity of the cells in question. For one, it would appear that these cells are devoid of LH receptors, as has been shown for the swine (Meduri et al., 1992). Instead, these cells may well be amply endowed with 'differentiation antigen' (Fujiwara et al., 1994) or aminopeptidase-N (Fujiwara et al., 1992). Consequently, one cannot rule out the possibility that these cells constitute somatic (but perhaps non-steroidogenic) cell elements possibly representative of the endothelial or even myoepithelial series. Irrespective of the precise identity of the cells in question, our current observations place IL-1β and the type I IL-1R in an extrafollicular environment thereby suggesting potential novel roles not previously envisioned. Immunohistochemical studies of

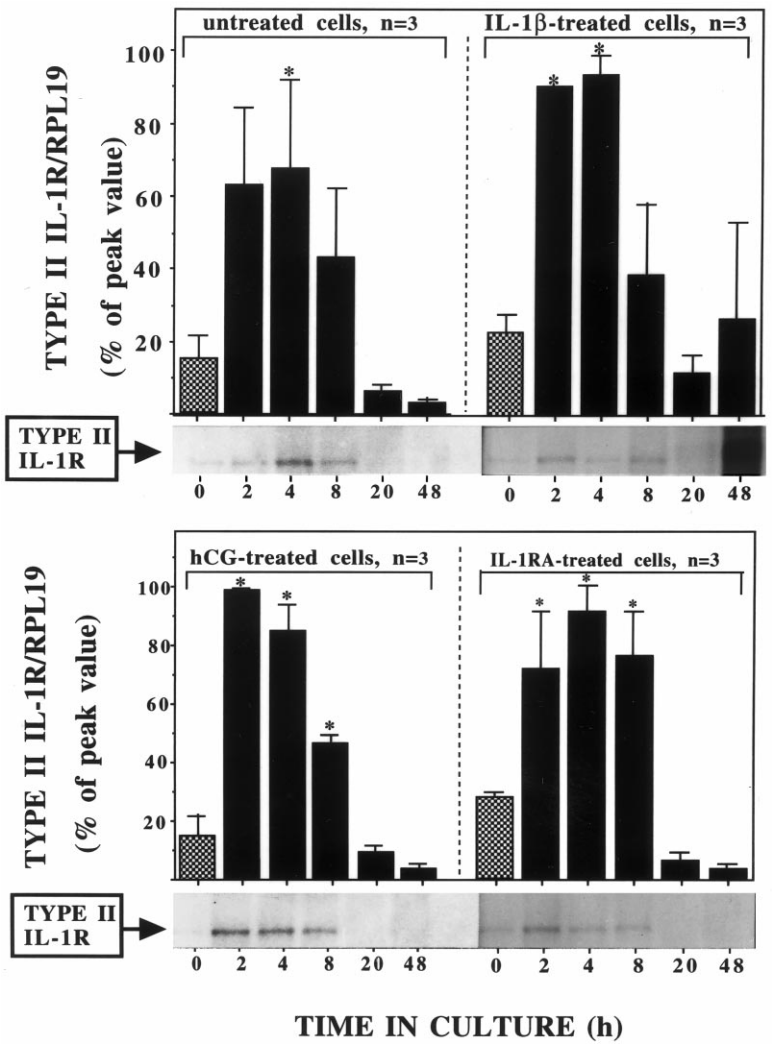

Fig. 7. Type II lL-lR gene expression by cultured whole ovarian dispersates: time-, IL-1- and hCG-dependence. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for the duration indicated (up to 48 h) in the absence or presence of IL-1β, IL-1RA or hCG. Total cellular RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat type II IL-1R and RPL19. The intensity of the signals was quantified as described. The data depict in bar graph form the mean ± SE of three experiments. In each individual experiment data were normalized relative to the peak value. Corresponding representative autoradiographs are shown below the bar graphs. Protected fragments for type II IL-1RA are marked in bold-faced letters. $^*P < 0.05$ vs time 0.

the mouse ovary revealed IL-1β and type I IL-1R staining to be confined to the theca-interstitial layer of growing follicles (Simon et al., 1994b).

For both IL-1β and type I IL-1R transcripts note was made of an in vivo peak approximately 12 h after hCG administration, i.e. at the time of projected ovulation (Fig. 3). Accordingly, temporal considerations alone would suggest that both IL-1β and the type I IL-1R may play a role during the periovulatory period and by extension, in the process of follicular rupture. Increased periovulatory expression of both the ligand (IL-1β) and its receptor (type I IL-1R) clearly serves the explosive characteristics of ovulation as a phenomenon which commits the dominant follicle to rupture and to the release of a fertilizable ovum.

Although the dependence of IL-1β (Zoja et al., 1992) and type I IL-1R transcripts on IL-1β has been demonstrated in several extraovarian sites (Akahoshi et al., 1988; Grenfell et al., 1992; Takii et al., 1992; Ye et al., 1992; Takii et al., 1994), the ability of IL-1β to modulate the expression of these genes in the context of the ovary has been the subject of limited investigation (Hurwitz et al., 1991a, 1992). Our present studies document the ability of IL-1β to produce time- (Figs. 4 and 5) and dose (Fig. 6)-dependent increments in IL-1β and type I IL-1R transcripts, as assessed in cultured whole ovarian dispersates from immature rats. Importantly, this IL-1 effect appears to be receptor-mediated in that IL-1β action is completely abrogated in the presence of IL-1RA (Fig. 8). Qualitatively comparable but quantitatively reduced results were obtained for isolated granulosa cells (not shown). The present observations establish the ability of IL-1β to exert a positive upregu-latory effect on its own transcripts, as well as on those of its receptor.

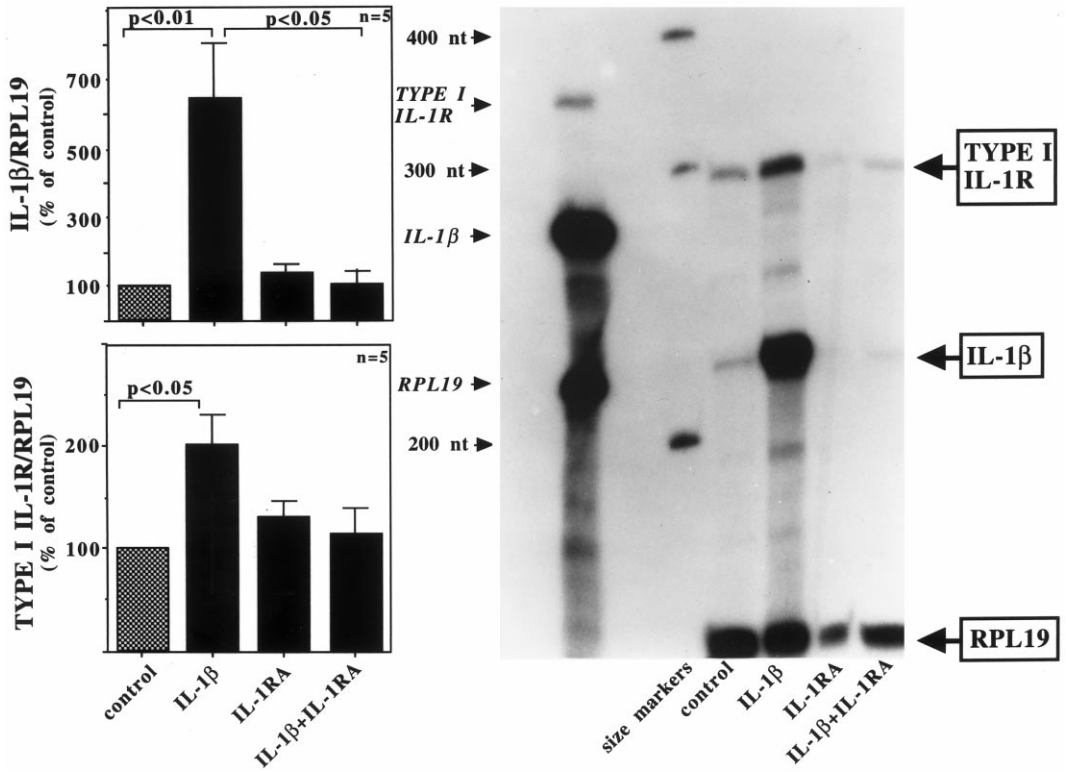

Fig. 8. IL-1β and type I IL-1R gene expression by cultured whole ovarian dispersates: an IL-1 receptor-mediated effect. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for 48 h in the absence or presence of IL-1β, with or without IL-1RA. The resultant RNA samples were subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-lβ, type I IL-1R and RPL19. The left panels depict in bar graph form the mean ± SE of five experiments. In each individual experiment, data were normalized relative to control. The right panel depicts a representative autoradiograph. Full length probes are marked in *italics* and protected fragments in bold-faced letters.

Studies at the transcript level suggest that the ability of IL-1β to upregulate IL-1β and type I IL-1R transcripts is characterized by ED_{50s} of 6 and 10 ng/ml, respectively. Such concentrations of IL-1β are higher then those required to upregulate secretory PLA_2 (0.3 ng/ml) transcripts, nitric oxide synthase activity (0.7 ng/ml), cytosolic PLA_2 transcripts (2 ng/ml), prostaglandin endoperoxide synthase-2 transcripts (2 ng/ml) or Glut1 and Glut3 transcripts (2 and 3 ng/ml) (Kol et al., unpublished observations). Thus, relative to other endpoints of IL-1, the induction of IL-1β and type I IL-1R transcripts constitutes a relatively insensitive endpoint. To the extent that IL-1 may play a role in the ovulatory cascade, these observations suggest that the induction of IL-1β (self-amplification) and type I IL-1R may constitute one of the later events in the sequence leading to follicular rupture.

The basal in vitro expression of IL-1β and its two receptors follows distinct temporal patterns. IL-1β (Fig. 4) and type II IL-1R (Fig. 7) transcripts peak early in the culture period (2–4 h) and decline thereafter. In contrast, the level of type I IL-1R appears to increase throughout the 48 h culture period (Fig. 5). Still, even the latter displays a marked and indeed significant ($P < 0.05$) increase during the first 2–4 h of culture. Importantly, these early 'bursts' in expression proved IL-1- and hCG-independent (Figs. 4, 5 and 7). In contrast, assessment of longer-term in vitro expression patterns disclose IL-1β and type I IL-1R transcripts to be IL-1-dependent (Figs. 4–6 and 8–10). Type II IL-1R transcripts in turn proved entirely autonomous by displaying IL-1- as well as hCG-independence (Fig. 7).

Given the documented ability of IL-1β to stimulate ovarian nitric oxide synthase activity (Ellman et al., 1993; Ben-Shlomo et al., 1994a,b; Bonello et al., 1996), we examined the possible role of nitric oxide in IL-1β action. To this end, use was made of aminoguanidine, an established inhibitor of the inducible variety of ovarian nitric oxide synthase (Ben-Shlomo et al., 1994a). As shown (Fig. 9), the generation of a nitric oxide 'vacuum' was without significant effect on the ability of IL-1 to upregulate IL-1β and type I IL-1R transcripts. Conceivably, the IL-1 effect may be mediated through activation of the prostanoid (Nothnick and Page, 1990; Kokia et al., 1992; Pitzel et al., 1993; Brannstrom et al., 1993b; Townson and Pate, 1994) or sphingomyelin (Hannun and Bell, 1989; Kolesnick and Golde, 1994; Zhang and Kolesnick, 1995) pathways. Studies are currently underway to evaluate these possibilities. Irrespective of the transduction pathway, the IL-1 effect is contingent upon an intact cellular protein synthesis (Fig. 10). The nature of the intermediary protein(s) remains unknown.

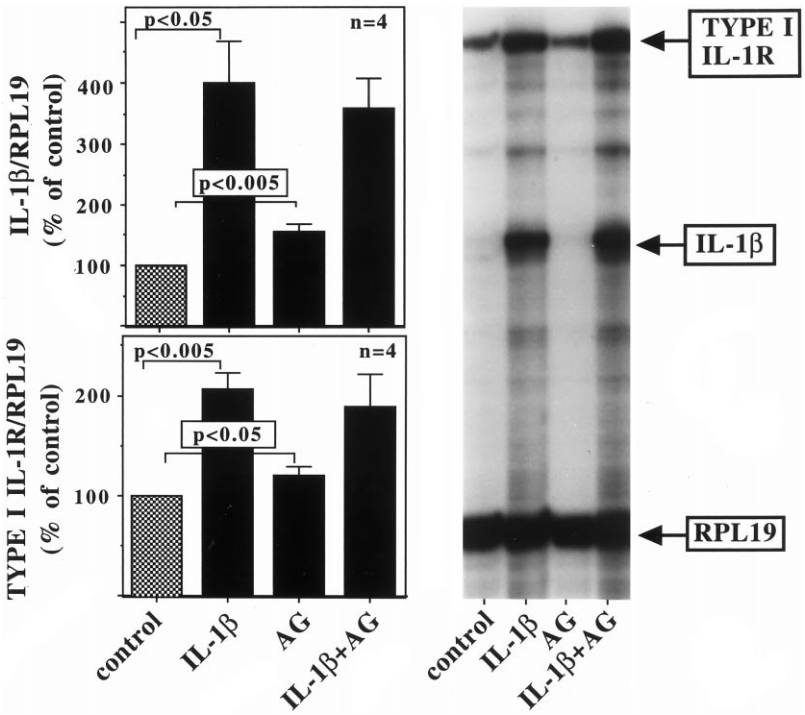

Fig. 9. IL-1β and type I IL-1R gene expression by cultured whole ovarian dispersates: nitric oxide-dependence. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for 48 h in the absence or presence of IL-1β, with or without AG. The resultant RNA samples were subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1β, type I IL-1R and RPL19. The intensity of the signals was quantified as described. The left panels depict in bar graph form the mean ± SE of four experiments. In each individual experiment data were normalized relative to control. The right panel depicts a representative autoradiograph. Protected fragments are marked in bold-faced letters.

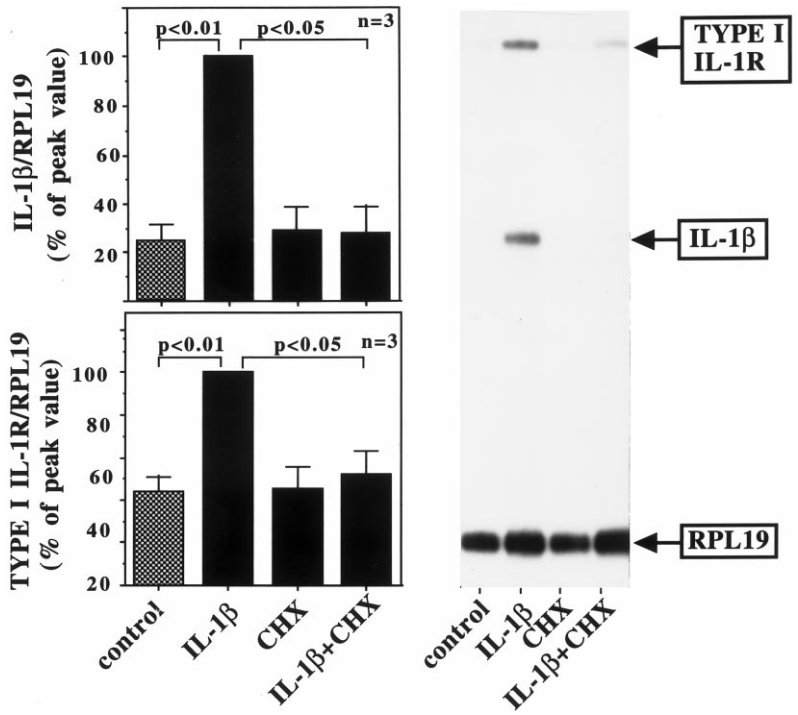

Fig. 10. IL-1β and type I IL-1R gene expression by cultured whole ovarian dispersates: protein synthesis dependence. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for 48 h in the absence or presence of IL-1β, with or without CHX (0.1 μg/ml). The resultant RNA samples were subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1β, type I IL-1R and RPL19. The intensity of the signals was quantified as described. The left panels depict in bar graph form the mean ± SE of three experiments. In each individual experiment, data were normalized relative to the peak value. The right panel depicts a representative autoradiograph. Protected fragments are marked in bold-faced letters.

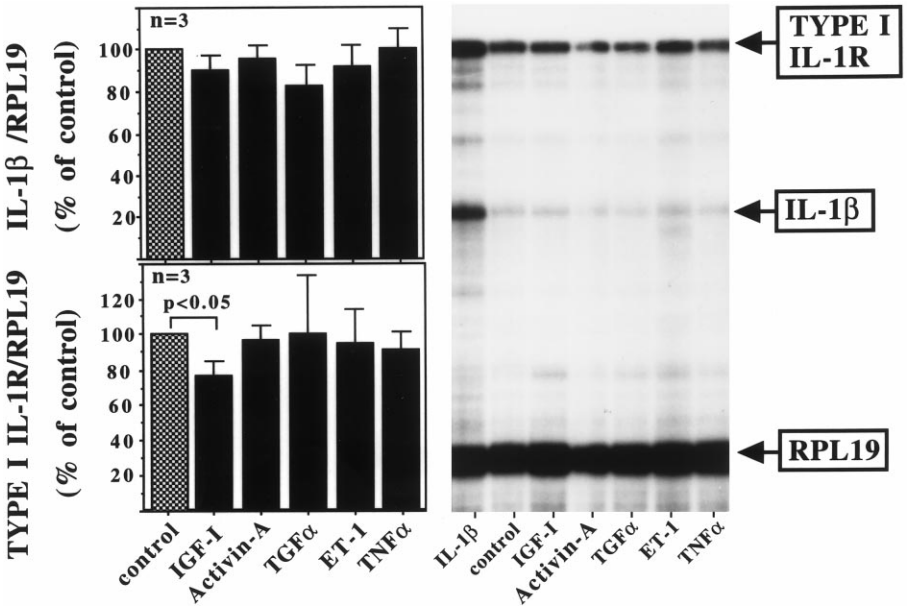

Fig. 11. IL-1β and type I IL-1R gene expression by cultured whole ovarian dispersates: specificity studies. Whole ovarian dispersates (1.5×10^6 cells per dish) were cultured for 48 h in the absence or presence of TNFα, IGF-I, activin-A, TGFα or ET. The resultant RNA samples were subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1β, type I IL-1R and RPL19. The intensity of the signals was quantified as described. The left panels depict in bar graph form the mean ± SE of three experiments. In each individual experiment data were normalized relative to control. The right panel depicts a representative autoradiograph. Protected fragments are marked in bold-faced letters. A lane with RNA from IL-1β-treated cells is shown as a positive control.

We have previously observed the expression of type II IL-1R transcript in whole ovarian material prior to ovulation (Scherzer et al., 1995). However, type II IL-1R mRNA was at the detection limit of the RNase protection assay (25). It was estimated that the ovarian content of type I IL-1R transcripts is 70-fold higher than that of its type II IL-1R counterpart. Our present findings document the IL-1- and hCG-independent expression of type II IL-1R in cultured whole ovarian dispersates (Fig. 7). In this respect, type II IL-1R transcripts differ from their type I IL-1R counterparts, the dependence of which on IL-1 proved substantial (Figs. 4–6). Taken together, these findings suggest a more limited role for the ovarian type II IL-1R in a manner compatible with its seemingly constitutive expression pattern and its relatively low abundance (Scherzer et al., 1995). Such observations are in keeping with the finding that the transduction of IL-1 action occurs solely via the type I receptor (Sims et al., 1993) and that the type II IL-1 receptor may be an IL-1 binding protein or 'decoy' receptor (Colotta et al., 1993).

Taken together, the present observations provide additional indirect support to the hypothesis that the intraovarian IL-1 system plays a key role in the ovulatory process. In part, this conclusion is supported by the periovulatory expression pattern of both IL-1β and its receptor. Indeed, temporal considerations alone would suggest that both IL-1β and the type I IL-1R may play a role in the process of follicular rupture. The ability of IL-1 to amplify its own production and that of its receptor further argues for a self-amplifying exponential process in keeping with the nature of the ovulatory cascade. The finding of type I IL-1R in the oocyte suggests that IL-1 may be involved in periovulatory events relevant not only to the somatic ovarian cell but also to germ cell components.

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