



# Non-steroidal anti-inflammatory drugs (NSAIDs) block the late, prostanoid-dependent/ceramide-independent component of ovarian IL-1 action: implications for the ovulatory process

Motomu Ando <sup>a,1</sup>, Shahar Kol <sup>a,2</sup>, Minoru Irahara <sup>a,3</sup>, Jean Sirois <sup>b</sup>, Eli Y. Adashi <sup>a,\*</sup>

<sup>a</sup> Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, University of Maryland School of Medicine, Baltimore, MD 21201, USA

<sup>b</sup> Centre de Recherche en Reproduction Animale, University of Montreal, Montreal, Québec, Canada

Received 9 July 1999; accepted 27 July 1999

## Abstract

The therapeutic efficacy and antiovolatory properties of non-steroidal anti-inflammatory drugs (NSAIDs) is attributed to their ability to suppress prostaglandin endoperoxide synthase (PGS) activity. Given the likely role of interleukin (IL)-1 in the inflammatory (and probably the ovulatory) process, we set out to evaluate whether the antiovolatory property of NSAIDs is attributable, in part, to the inhibition of ovarian IL-1 action. Whole ovarian dispersates from immature rats were cultured under serum-free conditions in the absence or presence of the indicated agents. At the conclusion of the culture period, total RNA was extracted and probed for transcripts corresponding to PGS-1, PGS-2, IL-1 $\beta$ , IL-1 receptor antagonist (IL-1RA) or type I IL-1 receptor (IL-1R) by a solution hybridization/ribonuclease protection assay. Treatment with indomethacin was without significant effect on the early (1 h) response to IL-1 $\beta$ ; however, it led to complete and highly significant dose-dependent blockade of the late (48 h) response to IL-1 $\beta$  as assessed in terms of PGS-2 transcripts, proteins and activity. The addition of PGE<sub>2</sub> to cells augmented the ability of IL-1 $\beta$  to upregulate PGS-2 transcripts. Moreover, the addition of PGE<sub>2</sub> to indomethacin-treated cells all but reversed the ability of indomethacin to suppress the IL-1 $\beta$  effect at both the PGS-2 transcript and protein levels. The upregulation by IL-1 of IL-1 $\beta$ , IL-1R and IL-1RA transcripts was similarly inhibited by indomethacin. Taken together, these observations suggest that the anti-ovulatory property of NSAIDs may be due, in part, to blockade of the late, prostanoid-dependent component of ovarian IL-1 action. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Non-steroidal anti-inflammatory drugs; Prostaglandin biosynthesis; Interleukin-1; Ovary; Sphingomyelin–ceramide pathway

## 1. Introduction

A growing body of direct and indirect evidence supports the notion that intraovarian interleukin-1 $\beta$  (IL-

1 $\beta$ ) may constitute an intermediary in the ovulatory process (Brännström et al., 1993a; Peterson et al., 1993; Simón et al., 1994; Takehara et al., 1994). A separate set of experimental data documents the indispensability of prostaglandin biosynthesis to the rupture of the ovarian follicle (Diaz-Infante et al., 1974; Hamada et al., 1977; Downey and Ainsworth, 1980; Espey et al., 1986). In part, this compilation of information relies on pharmacologic inhibition of ovarian prostaglandin biosynthesis. Indeed, the therapeutic and anti-ovulatory efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) is attributed to their ability to suppress the enzymatic activity of prostaglandin endoperoxide synthase, also known as PGS (Roth et al., 1975; Kulmacz and Lands, 1985; Laneuville et al., 1994; Mitchell et al., 1994). The indispensability of prostaglandin pro-

\* Corresponding author. Present address: Division of Reproductive Sciences, Department of Obstetrics and Gynecology, University of Utah Health Sciences Center, ARUP II, Suite 1100, Room 109, 546 Chipeta Way, Salt Lake City, UT 84108, USA. Tel.: +1-801-585-9250; fax: +1-801-585-9256.

E-mail address: eadashi@hsc.utah.edu (E.Y. Adashi)

<sup>1</sup> Present address: Department of Obstetrics and Gynecology, Kyorin University School of Medicine, Tokyo 181, Japan.

<sup>2</sup> Present address: Department of Obstetrics and Gynecology, Rambam Medical Center, Haifa, Israel.

<sup>3</sup> Present address: Department of Obstetrics and Gynecology, University of Tokushima School of Medicine, Tokushima City, Japan.

duction to the ovulatory process was recently reaffirmed in that the knockout of the inducible PGS-2 isozyme resulted in infertility and anovulation (Dinchuk et al., 1995). Still, the precise role of prostaglandins in follicular rupture remains uncertain.

Given the likely role of IL-1 in the ovulatory process and its documented ability to upregulate ovarian prostaglandin biosynthesis (Kokia et al., 1992; Brännström et al., 1993b), we set out to examine the possibility that the antiovarian activity of NSAIDs may be due to suppression of ovarian IL-1 action consequent to the blockade of PGS activity. Assuming IL-1 plays a key role in ovulation (Brännström et al., 1993a; Peterson et al., 1993; Simón et al., 1994; Takehara et al., 1994), such hypothesis may explain the indispensable role of prostaglandins in this context (Tsafiriri et al., 1973; Diaz-Infante et al., 1974; Hamada et al., 1977; Downey and Ainsworth, 1980; Espey et al., 1986; Dinchuk et al., 1995). The above hypothesis is supported by the observed ability of NSAIDs to suppress and of prostaglandins to enhance IL-1 action in a number of extraovarian sites (Wu et al., 1991; Dinarello, 1992; Tetsuka et al., 1994; Matsumoto et al., 1995).

## 2. Materials and methods

### 2.1. Animals

Immature Sprague–Dawley female rats from Zivic-Miller Laboratories (Zelienople, PA) were sacrificed by CO<sub>2</sub> asphyxiation on day 25 of life. The project was approved by the Institutional Animal Care and Use Committee.

### 2.2. Hormones and reagents

Recombinant human IL-1 $\beta$  ( $2 \times 10^7$  U/mg) was generously provided by Drs Errol B. De Souza and C.E. Newton, DuPont-Merck (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, Upjohn (Kalamazoo, MI). Sphingomyelinase (SMase), D-sphingosin, indomethacin (Indo), Diclofenac, Flurbiprofen and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) were from Sigma (St Louis, MO).

McCoy's 5a medium (serum-free), penicillin–streptomycin solution, L-glutamine, trypan blue stain and bovine serum albumin (BSA) were from Gibco BRL Life Technologies (Grand Island, NY). Collagenase (Clostridium Histolyticum; CLS type I; 144 U/mg) was from Worthington Biochemical (Freehold, NJ). DNase (bovine pancreas), and RNase A were from Sigma (St Louis, MO). RNA Century Marker Template set was

from Ambion (Austin, TX). T7 and SP6 RNA polymerases, pGEM7Zf+ and other molecular biology grade reagents were from Promega (Madison, WI). Nitrocellulose filters (0.45  $\mu$ m) were from Schleicher & Schuell (Keene, NH), LC rainbow molecular weight markers from Amersham (Arlington Heights, IL), [<sup>125</sup>I]protein A from ICN Biochemicals (Costa Mesa, CA), and [<sup>32</sup>P]UTP from New England Nuclear (Boston, MA).

### 2.3. Tissue culture procedures

Whole ovarian dispersates were prepared and cultured as previously described (Hurwitz et al., 1991). Briefly, whole ovarian dispersates ( $1.5 \times 10^6$  viable cells) were inoculated onto 60-mm (diameter) tissue culture dishes (Falcon Plastic, Oxnard, CA) containing 3 ml McCoy's 5a medium supplemented with L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin sulfate (100  $\mu$ g/ml). Cell cultures were maintained for 48 h at 37°C under a water-saturated atmosphere of 5% CO<sub>2</sub> and 95% air with or without various experimental agents.

To examine the early response to various ovarian agonists, whole ovarian dispersates ( $1.5 \times 10^6$  viable cell/dish) were initially cultured under serum-free conditions for 24 h in the absence of treatment. Thereafter, cells were cultured for 1 h in the absence or presence of the indicated agents.

### 2.4. PGE<sub>2</sub> RIA

The radioimmunoassay for PGE<sub>2</sub> was carried out as previously described (Kokia et al., 1992).

### 2.5. Nucleic acid probes

The rat PGS-1 and PGS-2 cDNAs (Feng et al., 1993) were generously provided in Bluescript vectors by Drs Daniel Hwang and Shuenn S. Liou of Pennington Biochemical Research Center, Louisiana State University (Baton Rouge, LA). A 354 ClaI–EcoRI fragment of the original PGS-1 cDNA was sub-cloned into a pGEM7Zf+ vector. SP6-driven transcription of the EcoRI-linearized construct yielded a 411 nt riboprobe which upon hybridization was projected to generate a 354 nt protected fragment as well as a 200 nt protected fragment spanning a putative splicing variant previously reported for the human gene (Diaz et al., 1992). A 385 XbaI–EcoRI fragment of the original PGS-2 cDNA was sub-cloned into a pGEM7Zf+ vector. T7-driven transcription of the HindIII-linearized construct yielded a 328 nt riboprobe which upon hybridization was projected to generate a 297 nt protected fragment.

The rat IL-1 $\beta$  cDNA was provided in PUC8 by Dr A. Shaw of Glaxo (Geneva, Switzerland). A 222-bp

PstI fragment of the original cDNA was sub-cloned into pGEM2. T7-driven transcription of the EcoRI-linearized plasmid yielded a 272 nt riboprobe which upon hybridization was projected to generate a 222 nt protected fragment (Hurwitz et al., 1992).

Rat type I IL-1 receptor (type I IL-1R) 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.

The rat interleukin-1 receptor antagonist (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 published sequences (Eisenberg et al., 1991) using Primer Designer software (Scientific and Educational Software, State Line, PA). The sense strand primer was 5'-AGCGAATTCCTTGACA-CAAGACAGGCACA-3' and the antisense strand primer was 5'-ATAGTCGACCAGTCACCTAATCTCTCTCC-3'. These primers span the putative alternative splice acceptor site in the icIL-1RA isoform (Haskill et al., 1991; Cominelli et al., 1994). The resultant 286-bp PCR product was cloned into the EcoRI and BamHI 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 (Eisenberg et al., 1991). After digestion with HindIII and transcription with T7 RNA polymerase, a 300 nt full-length riboprobe was generated, which was projected to protect 267 and 224 nt segments of the secretory and intracellular IL-1RA mRNA, respectively.

The ribosomal protein large 19 (RPL19) probe was generated by reverse transcription of 1 µg of total RNA, with 0.25 OD260 units of pdN6 as primer and 200 U Moloney Murine Leukemia Virus reverse transcriptase in the recommended buffer. The product was amplified with 0.32 µg of primers 5'-CTGAAGGTCAAAGGGAATGTG-3' and 5'-GGACAGAGTCTTGATGATCTC-3' which were designed to amplify the rat gene (Chan et al., 1987), and 1.25 U AmpliTaq polymerase in the recommended reaction mixture. The mixture was heated to 95°C before the addition of polymerase. Thermal cycles, consisting of 95°C for 1.5 min, 55°C for 1.5 min, and 72°C for 3 min, were

performed in a DNA Thermal Cycler (Perkin Elmer, Norwalk, CT) for 20 cycles. The PCR product was then cloned into the pCR1000 vector, verified by DNA sequencing, digested with EcoRI or FokI and transcribed with T7 RNA polymerase to generate 283 or 234 nt probes capable of protecting a 194 or 153 nt segment, respectively. [ $\alpha$ -<sup>32</sup>P]UTP-labeled RNA size markers were generated by using the marker set kit as a DNA template.

## 2.6. RNA extraction

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

## 2.7. RNase protection assay

Linearized DNA templates were transcribed with the appropriate RNA polymerase to specific activities of 800 Ci/mmol [ $\alpha$ -<sup>32</sup>P]UTP (PGS-1, PGS-2, IL-1 $\beta$ , type I IL-1R, and IL-1RA) or 160 Ci/mmol [ $\alpha$ -<sup>32</sup>P]UTP (RPL19). The riboprobes were gel-purified as described (Kol et al., 1996) in an effort to eliminate transcribed products shorter than the full length probes. The assay was performed as previously described (Lowe et al., 1987). 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 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.

## 2.8. Immune western blot analysis

Methodology conformed to that previously described (Hedin et al., 1987; Wong and Richards 1991; Sirois and Richards 1992). Filters were incubated with affinity-purified antibody #9181 which recognizes both PGS-1 and PGS-2 (Wong and Richards, 1991; Sirois and Richards, 1992; Sirois, 1994).

## 2.9. Data analysis

Except as noted, each experiment was replicated a minimum of three times. Data points are presented as the mean  $\pm$  S.E. Statistical significance was determined by ANOVA (Fisher's Protected Least Significance Difference) or Student's *t*-test using Statview 512+ for MacIntosh (Brain Power, Calabasas, CA).

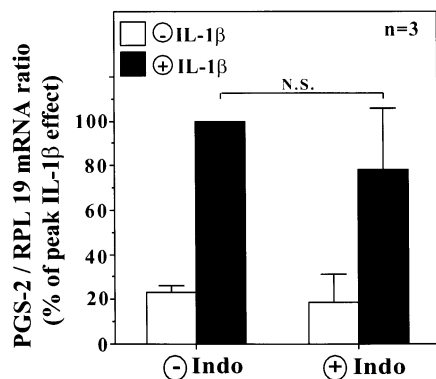


Fig. 1. Ovarian PGS-2 gene expression: effect of treatment with indomethacin on the early response to IL-1. Whole ovarian dispersates ( $1.5 \times 10^6$  viable cells/dish) were initially cultured under serum-free conditions for 24 h in the absence of treatment. Thereafter, cells were cultured for 1 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), with or without indomethacin (Indo: 10  $\mu$ g/ml). At the conclusion of the culture period, total RNA was extracted and probed for PGS-2 and RPL19 transcripts. Throughout, use was made of solution hybridization/ribonuclease protection assays. Ribosomal protein L19 transcripts were used as an internal control. To generate quantitative data, gels were also exposed to phosphor screen and the resultant digitized data analyzed with a phosphorimager. The data displayed in bar graph form depict the mean  $\pm$  S.E. of three experiments. In each individual experiment, data were normalized relative to the peak IL-1 $\beta$  effect.

### 3. Results

#### 3.1. Ovarian PGS-2 gene expression: effect of treatment with indomethacin on the early response to IL-1

To assess the ability of indomethacin to modulate

the early response to IL-1, whole ovarian dispersates were initially cultured under serum-free conditions without treatment for 24 h. Thereafter, cells were treated for 1 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), with or without indomethacin (10  $\mu$ g/ml; Indo). As shown in bar graph form (Fig. 1), treatment with IL-1 produced a 4.4-fold increase in the steady state levels of PGS-2 transcripts ( $P < 0.001$ ). Treatment with indomethacin, an established prototypic inhibitor of PGS-2 activity by itself, was without significant effect on the basal content of PGS-2 transcripts. Similarly, co-treatment with indomethacin was without significant effect on the early, 1 h response to IL-1 $\beta$  as assessed by the induction of PGS-2 transcripts.

#### 3.2. Ovarian PGS-2 gene expression: effect of treatment with indomethacin on the early response to sphingomyelinase or sphingosine

To further validate the apparent inability of indomethacin to block the early response to IL-1, whole ovarian dispersates were initially cultured for 24 h under serum-free conditions in the absence of treatment. Thereafter, cells were treated for 1 h in the absence or presence of sphingomyelinase (0.3 U/ml; SMase), sphingosine (10  $\mu$ M), or IL-1 $\beta$  (10 ng/ml), with or without indomethacin (10  $\mu$ g/ml; Indo). As shown (Fig. 2), treatment with SMase produced 4.8-fold increments in the steady state level of ovarian PGS-2 transcripts ( $P < 0.001$ ). A 2.6-fold increment was noted for sphingosine ( $P < 0.01$ ). Treatment with IL-1 $\beta$  resulted in a 6.1-fold increase in ovarian PGS-2 gene expression

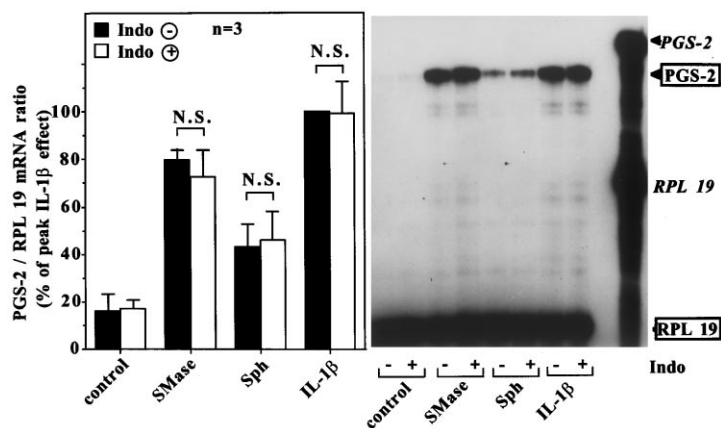


Fig. 2. Ovarian PGS-2 gene expression: effect of treatment with indomethacin on the early response to sphingomyelinase or sphingosine. Whole ovarian dispersates ( $1.5 \times 10^6$  viable cells/dish) from immature rats were initially cultured under serum-free conditions for 24 h in the absence of treatment. Thereafter, cells were cultured for 1 h in the absence or presence of Sphingomyelinase (Smase: 0.3 U/ml), Sphingosine (10  $\mu$ M), or IL-1 $\beta$  (10 ng/ml), with or without indomethacin (Indo: 10  $\mu$ g/ml). At the conclusion of the culture period, total RNA was extracted and subjected to solution hybridization/ribonuclease protection assays using antisense riboprobes corresponding to rat PGS-2 and RPL19. The intensity of the signals was quantified as described. The data displayed in bar graph form (left panel) depict the mean  $\pm$  S.E. of three experiments. In each individual experiment, data were normalized relative to the peak IL-1 $\beta$  effect. Right panel depicts a representative autoradiograph. Protected fragments are depicted in bold-faced letters. Full-length riboprobes are depicted in italics.

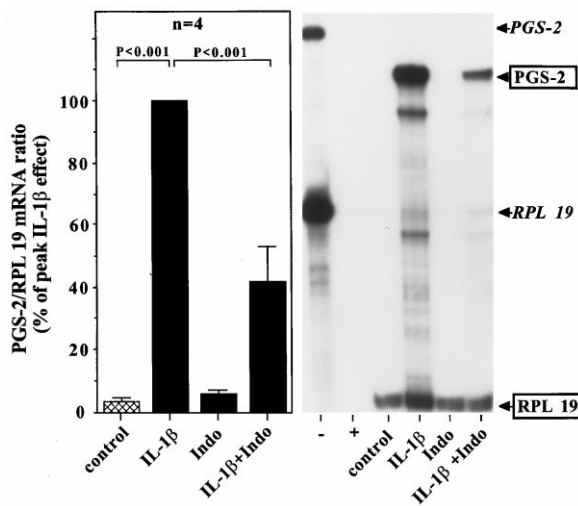


Fig. 3. Ovarian PGS-2 transcript: effect of treatment with indomethacin on the late response to IL-1. Whole ovarian dispersates ( $1.5 \times 10^6$  viable cells/dish) from immature rats were cultured under serum-free conditions for 48 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), with or without indomethacin (Indo: 10  $\mu$ g/ml). Total cellular RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat PGS-2 and RPL19. The intensity of the signals was quantified as described. The data displayed in bar graph form (left panel) depict the mean  $\pm$  S.E. of four experiments. In each individual experiment, data were normalized relative to the peak IL-1 $\beta$  effect. Right panel depicts a representative autoradiograph. Plus and minus designate riboprobe lanes treated with or without RNase. Protected fragments are depicted in bold-faced letters. Full-length riboprobes are depicted in italics.

( $P < 0.001$ ). Co-treatment with indomethacin proved ineffective in suppressing the early response to SMase or sphingosine, both congeners of the sphingomyelin–ceramide transduction pathway and capable of generating ceramide, an early messenger of IL-1.

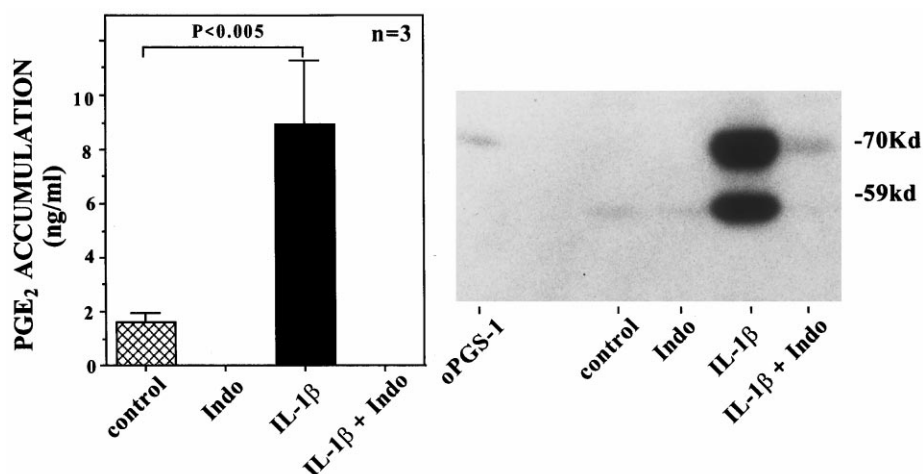


Fig. 4. Ovarian PGS-2 protein: effect of treatment with indomethacin on the late response to IL-1. Whole ovarian dispersates ( $1.5 \times 10^6$  viable cells/dish) were cultured under serum-free conditions for 48 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), with or without indomethacin (Indo: 10  $\mu$ g/ml). Medium PGE<sub>2</sub> content was measured by RIA. The immunoreactive content of PGS-2 (antibody 9181) was determined by immune Western blot analysis (50  $\mu$ g protein/lane). In addition to the 72-kDa holoenzyme, note was made of a 59-kDa proteolytic fragment in keeping with previous observations (Wong and Richards, 1991; Sirois and Richards, 1992). The data displayed in bar graph form (left panel) depict the mean  $\pm$  S.E. of three experiments. Right panel depicts a representative autoradiograph. oPGS<sub>1</sub> = ovine PGS-1 standard (12.5 ng/lane).

### 3.3. Ovarian PGS-2 transcripts and proteins: effect of treatment with indomethacin on the late response to IL-1

To examine the ability of indomethacin to modulate the late response to IL-1, whole ovarian dispersates were cultured for 48 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), with or without indomethacin (10  $\mu$ g/ml; Indo). As shown (Fig. 3), treatment with IL-1 $\beta$  produced a 34.4-fold increment in ovarian PGS-2 transcripts ( $P < 0.001$ ). In contrast, treatment with indomethacin by itself was without significant effect on the basal content of ovarian PGS-2 transcripts. However, co-treatment with indomethacin led to a significant decrease in the late, 48-h response to IL-1 $\beta$  as assessed by the upregulation of PGS-2 transcripts. Co-treatment with indomethacin also led to complete blockade of the late, 48-h response to IL-1 $\beta$  as assessed at the level of the PGS-2 protein (Fig. 4; right panel). In the representative Western immune blot (right panel), the 70-kDa band represents the holoenzyme whereas the 59-kDa band constitutes a proteolytic fragment thereof (Wong and Richards 1991; Sirois and Richards 1992). In addition, co-treatment with indomethacin resulted in complete abolition of IL-1-induced PGS activity ( $P < 0.01$ ) as assessed by the accumulation of PGE<sub>2</sub> in the corresponding conditioned media (left panel).

### 3.4. Indomethacin-mediated suppression of IL-1-induced ovarian PGS-1 transcripts, PGS-2 transcripts and PGS activity: dose-dependence

To assess the dose requirements of the indomethacin effect, whole ovarian dispersates were cultured to 48 h

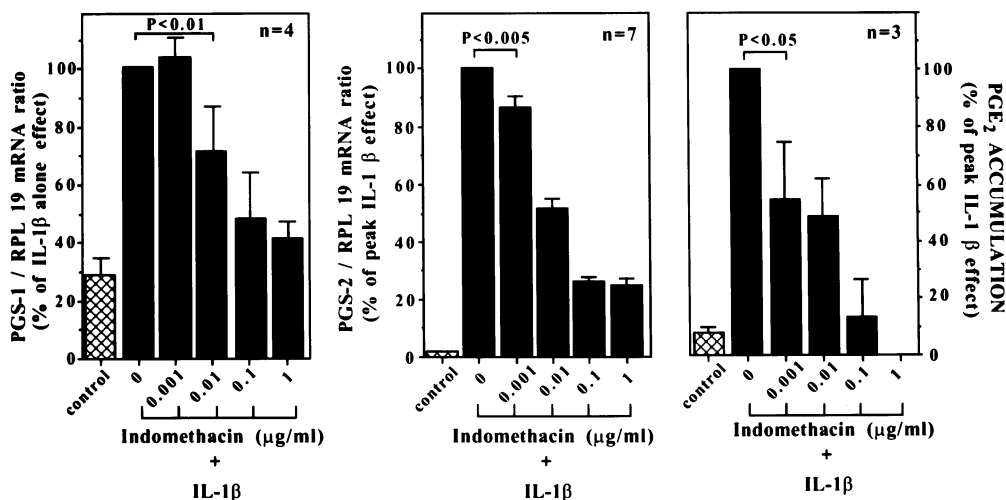


Fig. 5. Indomethacin-mediated suppression of IL-1-induced ovarian PGS-1 transcripts, PGS-2 transcripts and PGS activity: dose-dependence. Whole ovarian dispersates ( $1.5 \times 10^6$  viable cells/dish) were cultured under serum-free conditions for 48 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), with or without increasing concentrations (0–1  $\mu$ g/ml) of indomethacin. Medium PGE<sub>2</sub> content was measured by RIA. Total cellular RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat PGS-1, PGS-2 and RPL19. The intensity of the signals was quantified as described. The data displayed in bar graph form depict the mean  $\pm$  S.E. of 3–7 experiments. In each individual experiment, data were normalized relative to the peak IL-1 $\beta$  effect.

in the absence or presence of IL-1 $\beta$  (10 ng/ml), with or without increasing concentrations (0–1  $\mu$ g/ml) of indomethacin. As shown (Fig. 5), treatment with IL-1 $\beta$  (10 ng/ml) produced 3.3-, 59.9- and 13.3-fold increments in ovarian PGS-1 transcripts, PGS-2 transcripts, and PGS activity, respectively. However, co-treatment with increasing concentrations of indomethacin resulted in dose-dependent inhibition of the IL-1 $\beta$  effect. A concentration as low as 1 ng/ml of indomethacin produced significant ( $P < 0.05$ ) inhibition of the IL-1 $\beta$ -mediated induction of PGS-2 transcripts. The corresponding minimal effective doses for the PGS-1 transcript and PGS activity endpoints were 10 and 100 ng/ml, respectively.

### 3.5. Late response to IL-1: prostaglandin-dependence of the PGS-2 transcript and protein

To assess further the apparent dependence of the late response to IL-1 on prostaglandins, whole ovarian dispersates were cultured for 48 h under serum-free conditions in the absence or presence of IL-1 $\beta$  (10 ng/ml), PGE<sub>2</sub> (10  $\mu$ g/ml), indomethacin (10  $\mu$ g/ml; Indo), or combinations thereof. As shown (Fig. 6), the provision of PGE<sub>2</sub> by itself was without significant effect on ovarian PGS-2 transcripts. In contrast, the addition of PGE<sub>2</sub> to indomethacin-untreated cells modestly augmented the ability of IL-1 $\beta$  to upregulate PGS-2 transcripts. However, the addition of PGE<sub>2</sub> to indomethacin-treated cells all but reversed the ability of indomethacin to suppress the IL-1 $\beta$  effect at the PGS-2 transcript level. These phenomena were also noted at

the PGS-2 protein level as shown by the corresponding Western immune blot (Fig. 7). Note was made of the fact that the addition of indomethacin not only reduced

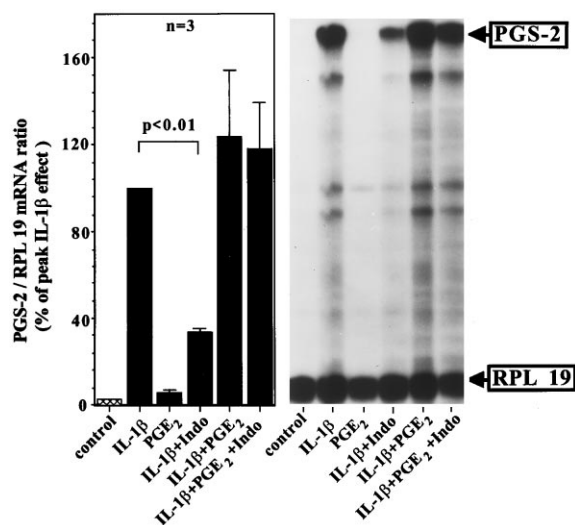


Fig. 6. Late response to IL-1: prostaglandin-dependence of the PGS-2 transcript. Whole ovarian dispersates ( $1.5 \times 10^6$  viable cells/dish) were cultured under serum-free conditions for 48 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), PGE<sub>2</sub> (10  $\mu$ g/ml), indomethacin (Indo: 10  $\mu$ g/ml), or combinations thereof. Total cellular RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat PGS-2 and RPL19. The intensity of the signals was quantified as described. The data displayed in bar graph form (left panel) depict the mean  $\pm$  S.E. of three experiments. In each individual experiment, data were normalized relative to the peak IL-1 $\beta$  effect. Right panel depicts a representative autoradiograph. Protected fragments are depicted in bold-faced letters.

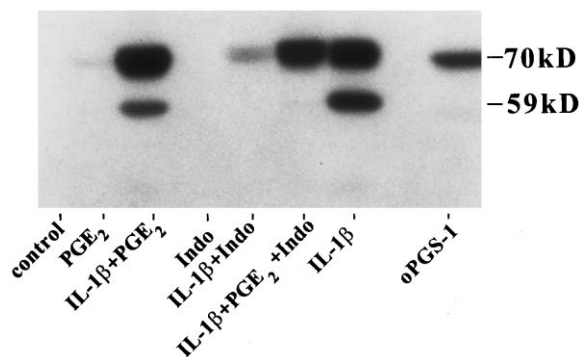


Fig. 7. Late response to IL-1: prostaglandin dependence of the PGS-2 protein. Whole ovarian dispersates ( $1.5 \times 10^6$  viable cells/dish) were cultured under serum-free conditions for 48 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), PGE<sub>2</sub> (10  $\mu$ g/ml), indomethacin (Indo: 10  $\mu$ g/ml), or combinations thereof. The immunoreactive content of PGS-2 was determined by immune Western blot analysis (antibody # 9181). In addition to the 70-kDa holoenzyme, note was made of a 59-kDa fragment in keeping with previous observations (Wong and Richards, 1991; Sirois and Richards, 1992). oPGS<sub>1</sub> = ovine PGS-1 standard (12.5 ng/lane).

the relative representation of the 70-kDa band but all but eliminated the 50-kDa band. Whether the removal of the latter represents the differential antiproteolytic effect of indomethacin remains to be established. It is also possible that reduction in PGS-2 biosynthesis overall may contribute to the diminished representation of its 59-kDa breakdown product.

To determine whether or not the blockade of the late response to IL-1 was indomethacin-selective, whole ovarian dispersates were cultured under serum-free conditions for 48 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), with or without increasing concentrations (0–10  $\mu$ g/ml) of Diclofenac. The inhibitory effect of indomethacin was reproduced by Diclofenac, a related NSAID, as evident by the dose-dependent suppression of IL-1 $\beta$ -induced PGS-2 transcripts and PGE<sub>2</sub> accumulation. Treatment with increasing concentrations (0–10  $\mu$ g/ml) of Flurbiprofen, a related NSAID, also resulted in dose-dependent inhibition of IL-1 $\beta$ -induced PGS-2 transcripts and PGE<sub>2</sub> accumulation (not shown).

### 3.6. Effect of treatment with indomethacin on IL-1-induced ovarian IL-1 $\beta$ , type I IL-1R, and IL-1RA gene expression

To determine whether the ability of indomethacin to block the late response to IL-1 is PGS-2 exclusive, whole ovarian dispersates were cultured under serum-free conditions for 48 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), with or without indomethacin (10  $\mu$ g/ml). As shown (Fig. 8), the inhibitory indomethacin effect was also noted for other IL-1 endpoints such as the IL-1-mediated induction of IL-1 $\beta$  ( $P < 0.05$ ) and of type I IL-1 receptor (IL-1R) ( $P < 0.005$ ) transcripts,

respectively. The IL-1-mediated induction of IL-1 receptor antagonist (IL-1RA) gene expression was likewise inhibited ( $P < 0.05$ ) by indomethacin (Fig. 9).

## 4. Discussion

The present manuscript deals with the inhibitory effects of indomethacin on IL-1 action in whole ovarian dispersates. It is recognized that the work described is constrained by its in vitro nature and the lack of unequivocal comparable in vivo information. Furthermore, it is recognized that the more recent availability of the specific inhibitors for PGS-2 and PGS-1 may require a modification of the conclusions reached. Work is currently underway to establish whether or not PGS-2 and PGS-1-specific inhibitors behave in a manner distinct from that displayed by indomethacin and related congeners.

Although the dependence of the PGS-2 gene on IL-1 $\beta$  has been demonstrated in several extraovarian sites (Raz et al., 1988; Maier et al., 1990; Kawaguchi et al., 1994), the ability of IL-1 $\beta$  to modulate ovarian PGS gene expression has received limited attention. We previously documented the ability of IL-1 $\beta$  to produce dose- and time-dependent increments in PGS-2 gene expression as assessed in cultured whole ovarian dispersates from immature rats (Ando et al., 1998). Qualitatively comparable upregulation of the PGS-2 protein

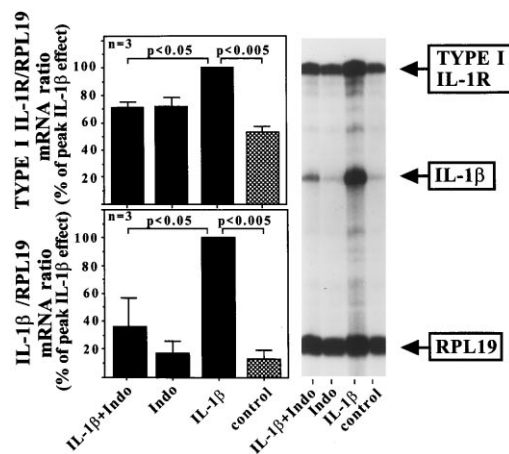


Fig. 8. Effect of treatment with indomethacin on IL-1-induced ovarian IL-1 $\beta$  and type I IL-1R gene expression. Whole ovarian dispersates ( $1.5 \times 10^6$  viable cells/dish) were cultured under serum-free conditions for 48 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), with or without indomethacin (Indo: 10  $\mu$ g/ml). Total cellular RNA was extracted and subjected to an RNase protection assay using antisense riboprobes corresponding to rat IL-1 $\beta$ , type I IL-1R and RPL19. The intensity of the signals was quantified as described. The data displayed in bar graph form (left panel) depicts the mean  $\pm$  S.E. of three experiments. In each individual experiment, data were normalized relative to the peak IL-1 $\beta$  effect. Right panel depicts a representative autoradiograph. Protected fragments are depicted in bold-faced letters.

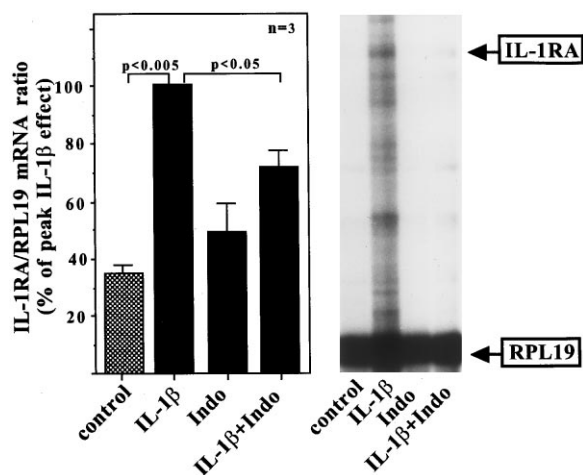


Fig. 9. Effect of treatment with Indomethacin on IL-1-induced ovarian IL-1RA gene expression. Whole ovarian dispersates ( $1.5 \times 10^6$  viable cells/dish) were cultured under serum-free conditions for 48 h in the absence or presence of IL-1 $\beta$  (10 ng/ml), with or without indomethacin (Indo: 10  $\mu$ g/ml). 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. The data displayed in bar graph form (left panel) depict the mean  $\pm$  S.E. of three experiments. In each individual experiment, data were normalized relative to the peak IL-1 $\beta$  effect. Right panel depicts a representative autoradiograph. Protected fragments are depicted in bold-faced letters.

and PGS activity was also noted. To the extent that IL-1 may play a role in the ovulatory cascade, we reported that the induction of PGS-2 may constitute one of the early and most sensitive events in the sequence leading to follicular rupture. It is highly likely the ovarian IL-1 effect is mediated via the type I IL-1 receptor, the role of which in signal transduction has been amply documented (Sims et al., 1993). Indeed, the type II IL-1 receptor may be an IL-1 binding protein or 'decoy' receptor (Colotta et al., 1993), the overall abundance of which in the rat ovary is substantially reduced (Scherzer et al., 1995).

The precise site(s) wherein indomethacin perturbs the ovarian IL-1 transduction signal was investigated. Indomethacin proved ineffective in suppressing the induction of PGS-2 by congeners of the sphingomyelin-ceramide cycle, the role of which in the transduction of the IL-1 signal and in the early IL-1 response is the subject of active investigation (Kuno and Matsushima 1994; Schütze et al., 1994). SMases have been implicated in a number of IL-1 responses, including stimulation of cell growth, differentiation, cytotoxicity and apoptosis (Kaipia et al., 1997; Witty et al., 1997). Ceramide, the active cleavage product of SMase, is a multifunctional lipid second messenger (Kuno and Matsushima, 1994). IL-1 rapidly induces sphingomyelin turnover in various types of cells, producing ceramides, which may act as a second messenger molecule in an intracellular signaling cascade (Kuno and Matsushima,

1994). Indeed, ceramide was shown to mediate granulosa cell apoptosis and follicle atresia as an important intraovarian regulator (Kaipia et al., 1997; Witty et al., 1997). In this study (Fig. 2), use was made of sphingomyelinase, a cell membrane-anchored enzyme and a putative proximal effector capable of degrading cell membrane sphingomyelin into ceramide. The activity of sphingosine, a metabolite of ceramide, was likewise examined. Taken together, these observations suggest that the ability of indomethacin to attenuate ovarian IL-1 action may be ceramide-independent.

Given the inability of indomethacin to affect an early ovarian response to IL-1, consideration was given to the possibility that NSAIDs may perturb the late ovarian response to the cytokine. As shown (Figs. 3–7), treatment with indomethacin produced dose-dependent inhibition of the late response to IL-1 as assessed at the level of PGS-2 transcripts, protein, and activity. Similarly, the addition of PGE<sub>2</sub> to indomethacin-untreated cells modestly augmented the ability of IL-1 $\beta$  to upregulate PGS-2 transcripts (Fig. 6). However, the addition of PGE<sub>2</sub> to indomethacin-treated cells all but erased the ability of indomethacin to suppress the IL-1 effect at the PGS-2 transcript level (Fig. 6). This phenomenon was also noted at the PGS-2 protein level (Fig. 7). It is recognized that the addition of PGE<sub>2</sub> may not substitute satisfactorily for all the missing products downstream of this metabolite. However, assuming comparable metabolic pathways, it is conceivable that the metabolism of PGE<sub>2</sub> may in fact yield partial replacement of downstream products. Indomethacin, a classic non-selective PGS inhibitor, binds deeply within the PGS active site (Kurumbail et al., 1996). Diclofenac is an equipotent inhibitor of PGS-1 and PGS-2. Flurbiprofen, a slowbinding competitive inhibitor of both PGS-1 and PGS-2, binds in the long hydrophobic channel and excludes substrate from the PGS active site. PGS inhibitors such as indomethacin, Flurbiprofen and Diclofenac inhibit this enzyme by an essentially irreversible time-dependent mechanism (Mancini et al., 1995).

Given that the preceding observations may be unique to PGS-2, we undertook to examine additional endpoints reflective of the late ovarian response to IL-1. In this context, our observations reveal indomethacin to inhibit the IL-1 mediated induction of ovarian IL-1 $\beta$ , IL-1R, and IL-1RA transcripts. As such, these observations suggest that the ability of NSAIDs to block the late ovarian response to IL-1 are not PGS-exclusive thereby suggesting a global effect as distinct from a discrete phenomenon.

Taken together, these observations suggest that the antiovarian properties of NSAIDs may be due, in part, to the suppression of the late, prostanoid-dependent component of ovarian IL-1 action. This line of reasoning suggests that the indispensable ovarian role



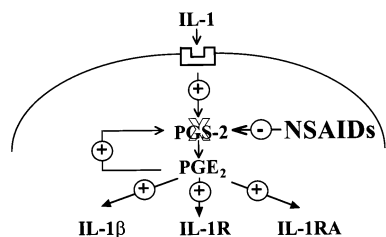


Fig. 10. Schematic depiction of the mechanism(s) underlying the ability of NSAIDs to suppress ovarian IL-1 action.

of prostaglandins may be as mediators of IL-1 action. As such, these observations provide additional indirect support to the notion that IL-1 may play a meaningful role as an intermediary in the ovulatory cascade and that prostaglandins constitute key signalling molecules on its behalf in this regard. In schematic terms (Fig. 10), these findings suggest an important role for cellular prostaglandins in the amplification of the IL-1 signal, an effect which is markedly attenuated with the application of NSAIDs.

### Acknowledgements

This work was supported in part by NIH Research Grant HD-30288 (E.Y. Adashi) and MRC of Canada Grant MT-13190 (J. Sirois). The authors wish to thank Linda Elder and Andrea Raposa for their assistance in the preparation of this manuscript.

### References

- Ando, M., Kokia, E., Ruutianen-Altman, K., Sirois, J., Rihan, R.M., Payne, D.W., Adashi, E.Y., 1998. Rat ovarian prostaglandin synthase-1 and -2: periovulatory expression of granulosa cell-basal interleukin-1-dependent enzymes. *Endocrinology* 139, 2501–2508.
- Brännström, M., Wang, L., Norman, R.J., 1993a. Ovulatory effect of interleukin-1 on the perfused rat ovary. *Endocrinology* 132, 399–404.
- Brännström, M., Wang, L., Norman, R.G., 1993b. Effects of cytokines on prostaglandin production and steroidogenesis of incubated preovulatory follicles of the rat. *Biol. Reprod.* 48, 165–171.
- Chan, Y.-L., Lin, A., McNally, J., Peleg, D., Meyuhas, O., Wool, I.G., 1987. The primary structure of rat ribosomal protein L19. *J. Biol. Chem.* 262, 1111–1115.
- Colotta, F., Re, F., Muzio, M., Bertini, R., Polenarutti, N., Sironi, M., Giri, J.G., Dower, S.K., Simms, J.E., Mantovani, A., 1993. Interleukin-1 type II receptor: A decoy target for IL-1 that is regulated by IL-1. *Science* 261, 472–475.
- Cominelli, F., Bortolami, M., Pizarro, T.T., Monsacchi, L., Ferretti, M., Brewer, M.T., Eisenberg, S.P., Ng, R.K., 1994. Rabbit interleukin-1 receptor antagonist cloning, expression, functional characterization and regulation during intestinal inflammation. *J. Biol. Chem.* 269, 6962–6971.
- Diaz, A., Reginato, A.M., Jimenez, S.A., 1992. Alternative splicing of human prostaglandin G/H synthase mRNA and evidence of differential regulation of the resulting transcripts by transforming growth factor  $\beta$ 1, interleukin-1b, and tumor necrosis factor  $\alpha$ . *J. Biol. Chem.* 267, 10816–10822.
- Diaz-Infante, A.J., Wright, K.H., Wallach, E.E., 1974. Effects of

- indomethacin and prostaglandin F<sub>2a</sub> on ovulation and ovarian contractility in the rabbit. *Prostaglandins* 5, 567–581.
- Dinarelo, C.A., 1992. Role of interleukin-1 in infectious disease. *Immunol. Rev.* 127, 119–146.
- Dinchuk, J.E., Car, B.D., Focht, R.J., Johnston, J.J., Jaffee, B.D., Covington, M.B., Contel, N.R., Eng, V.M., Collins, R.J., Czeraniak, P.M., Gorry, S.A., Trzaskos, J.M., 1995. Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. *Nature* 378, 406–409.
- Downey, B.R., Ainsworth, L., 1980. Reversal of indomethacin blockade of ovulation in gilts by prostaglandins. *Prostaglandins* 19, 17–22.
- Eisenberg, S.P., Brewer, M.T., Verderber, E., Heimdal, P., Brandhuber, B.J., Thompson, R.C., 1991. Interleukin 1 receptor antagonist is a member of the interleukin 1 gene family: Evolution of a cytokine control mechanism. *Proc. Natl. Acad. Sci. USA* 88, 5232–5236.
- Espey, L.L., Norris, C., Saphire, D., 1986. Effect of time and dose of Indomethacin on follicular prostaglandins and ovulation in the rabbit. *Endocrinology* 119, 746–754.
- Feng, L., Sun, W., Xia, Y., Tang, W.W., Chanmugam, P., Soyoola, E., Wilson, C.B., Hwang, D., 1993. Cloning two isoforms of rat cyclooxygenase: Differential regulation of their expression. *Arch. Biochem. Biophys.* 307, 361–368.
- Hamada, Y., Bronson, R.A., Wright, K.H., Wallach, E.E., 1977. Ovulation in the perfused rabbit ovary: The influences of prostaglandins and prostaglandin inhibitors. *Biol. Reprod.* 17, 58–63.
- Haskill, S., Martin, G., Van Morris, J.L., Peace, A., Bigler, C.F., Jaffe, G., Hammerberg, C., Sporn, S., Fong, S., Arend, W.P., Ralph, P., 1991. cDNA cloning of an intracellular form of the human interleukin 1 receptor antagonist associated with epithelium. *Proc. Natl. Acad. Sci. USA* 88, 3681–3685.
- Hedin, L., Gaddy-Kurten, D., Kurten, R., DeWitt, D.L., Smith, W.L., Richards, J.S., 1987. Prostaglandin endoperoxide synthase in rat ovarian follicles: Content, cellular distribution, and evidence for hormonal induction preceding ovulation. *Endocrinology* 121, 722–731.
- Hurwitz, A., Payne, D.W., Packman, J.N., Andreani, C.L., Resnick, C.E., Hernandez, E.R., Adashi, E.Y., 1991. Cytokine-mediated regulation of ovarian function: Interleukin-1 inhibits gonadotropin-induced androgen biosynthesis. *Endocrinology* 129, 1250–1256.
- Hurwitz, A., Ricciarelli, E., Botero, L., Rohan, R.M., Henandez, E.R., Adashi, E.Y., 1992. Endocrine- and autocrine-mediated regulation of rat ovarian (theca-interstitial) interleukin-1b gene expression: Gonadotropin-dependent preovulatory acquisition. *Endocrinology* 129, 3427–3429.
- Kaipia, A., Chun, S.-Y., Eisenhauer, K., Hsueh, A.J.W., 1997. Tumor necrosis factor- $\alpha$  and its second messenger, ceramide, stimulate apoptosis in cultured ovarian follicles. *Endocrinology* 137, 4864–4870.
- Kawaguchi, H., Raisz, L.G., Voznesensky, O.S., Alander, C.B., Hakeda, Y., Pilbeam, C.C., 1994. Regulation of the two prostaglandin G/H synthases by parathyroid hormone, interleukin-1, cortisol, and prostaglandin E<sub>2</sub> in cultured neonatal mouse calvariae. *Endocrinology* 135, 1157–1164.
- Kokia, E., Hurwitz, A., Ricciarelli, E., Tedeschi, C., Resnick, C.E., Mitchell, M.D., Adashi, E.Y., 1992. Interleukin-1 stimulates ovarian prostaglandin biosynthesis: evidence for heterologous contact-independent cell–cell interaction. *Endocrinology* 130, 3095–3097.
- Kol, S., Ben-Shlomo, I., Adashi, E.Y., Rohan, R.M., 1996. Simplified riboprobe purification using translucent straws as gel tubes. *Genet. Anal. Biomol. Eng.* 12, 129–132.

- Kulmacz, R.J., Lands, W.E.M., 1985. Stoichiometry and kinetics of the interaction of prostaglandin H synthase with anti-inflammatory agents. *J. Biol. Chem.* 260, 12572–12578.
- Kuno, K., Matsushima, K., 1994. The IL-1 receptor signal pathway. *J. Leukoc. Biol.* 56, 542–547.
- Kurumbail, R.G., Stevens, A.M., Gierse, J.K., McDonald, J.J., Stegeman, R.M., Pak, J.J., Gildehaus, D., Miyashiro, J.M., Penning, T.D., Seibert, K., Isakson, P.C., Stallings, W.C., 1996. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature* 384, 644–648.
- Laneuville, O., Breuer, D.K., Dewitt, D.L., Hla, T., Funk, C.D., Smith, W.L., 1994. Differential inhibition of human prostaglandin endoperoxide H synthases-1 and -2 by nonsteroidal anti-inflammatory drugs. *J. Pharmacol. Exp. Ther.* 271, 927–934.
- Lowe, J.W.L., Roberts, J.C.T., Lasky, S.R., LeRoith, D., 1987. Differential expression of alternative 5' untranslated regions in mRNA encoding rat insulin-like growth factor 1. *Proc. Natl. Acad. Sci. USA* 84, 8946–8950.
- Maier, J.A., Hla, T., Maciag, T., 1990. Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. *J. Biol. Chem.* 265, 10805–10808.
- Mancini, J.A., Riendeau, D., Falgouty, J.-P., Vickers, P.J., O'Neil, G.P., 1995. Arginine 120 of prostaglandin G/H synthase-1 is required for the inhibition by nonsteroidal anti-inflammatory drugs containing a carboxylic acid moiety. *J. Biol. Chem.* 270, 29372–29377.
- Matsumoto, K., Okazaki, H., Nakamura, T., 1995. Novel function of prostaglandins as inducers of gene expression of HGF and putative mediators of tissue regeneration. *J. Biochem.* 117, 458–464.
- Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J., Vane, J.R., 1994. Selectivity of nonsteroidal anti-inflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc. Natl. Acad. Sci. USA* 90, 11693–11697.
- Peterson, C.M., Hales, H.A., Hatasaka, H.H., Mitchell, M.D., Rittenhouse, L., Jones, K.P., 1993. Interleukin-1b (IL-1b) modulates prostaglandin production and the natural IL-1 receptor antagonist inhibits ovulation in the optimally stimulated rat ovarian perfusion model. *Endocrinology* 133, 2301–2306.
- Raz, A., Wyche, A., Siegel, N., Needleman, P., 1988. Regulation of fibroblast cyclooxygenase synthesis by interleukin-1. *J. Biol. Chem.* 263, 3022–3028.
- Roth, G.J., Stanford, N., Majerus, P.W., 1975. Acetylation of prostaglandin synthase by aspirin. *Proc. Natl. Acad. Sci. USA* 72, 3073–3076.
- Scherzer, W.J., Ruutiainen-Altman, K.S.L., Putowski, L.T., Kol, S., Adashi, E.Y., Rohan, R.M., 1995. Detection and in vivo hormonal regulation of rat ovarian type I and type II interleukin-1 receptor mRNAs: Increased expression during the periovulatory period. *J. Soc. Gynecol. Invest.* 3, 131–140.
- Schütze, S., Machleidt, T., Krönke, M., 1994. The role of diacylglycerol and ceramide in tumor necrosis factor and interleukin-1 signal transduction. *J. Leukoc. Biol.* 56, 533–541.
- Simón, C., Tsafiriri, A., Chun, S.-Y., Piquette, G.N., Dang, W., Polan, M.L., 1994. Interleukin-1 receptor antagonist suppresses hCG-induced ovulation in the rat. *Biol. Reprod.* 51, 662–667.
- Sims, J.E., Gayle, M.A., Slack, J.L., Alderson, M.R., Bird, T.A., Giri, J.G., Colotta, R.R.F., Mantovani, A., Shanebeck, K., Grabstein, K.H., Dowe, S.K., 1993. Interleukin-1 signaling occurs exclusively via the type I receptor. *Proc. Natl. Acad. Sci. USA* 90, 6155–6159.
- Sirois, J., 1994. Induction of prostaglandin endoperoxide synthase 2 by human chorionic gonadotropin in bovine preovulatory follicles in vivo. *Endocrinology* 135, 841–848.
- Sirois, J., Richards, J.S., 1992. Purification and characterization of a novel, distinct isoform of prostaglandin endoperoxide synthase induced by hCG in granulosa cells of rat periovulatory follicles. *J. Biol. Chem.* 267, 6382–6388.
- Takehara, Y., Dharmarajan, A.M., Kaufman, G., Wallach, E.E., 1994. Effects of interleukin-1b on ovulation in the in vitro perfused rabbit ovary. *Endocrinology* 134, 1788–1793.
- Tetsuka, T., Daphna-Iken, D., Srivastava, S.K., Baier, L.D., Du-Maine, J., Morrison, A.R., 1994. Cross-talk between cyclooxygenase and nitric oxide pathways: prostaglandin E2 negatively modulates induction of nitric oxide synthase by interleukin 1. *Proc. Natl. Acad. Sci. USA* 91, 12168–12172.
- Tsafiriri, A., Koch, Y., Lindner, H.R., 1973. Ovulation rate and serum LH levels in rats treated with Indomethacinmethacin or prostaglandin E2. *Prostaglandins* 3, 461–467.
- Witty, J.P., Bridgman, J.T., Johnson, A.L., 1997. Induction of apoptosis cell death in hen granulosa cells by ceramide 137. *Endocrinology* 137, 5269–5277.
- Wong, W.Y.L., Richards, J.S., 1991. Evidence for two antigenically distinct molecular weight variants of prostaglandin H synthase in the rat ovary. *Mol. Endocrinol.* 5, 1269–1279.
- Wu, K.K., Sanduja, R., Tsai, A.L., Ferhanoglu, B., Loose-Mitchell, D.S., 1991. Aspirin inhibits interleukin 1-induced prostaglandin H synthase expression in cultured endothelial cells. *Proc. Natl. Acad. Sci. USA* 88, 2384–2387.