Nuclear progesterone receptor A and B isoforms in mouse fallopian tube and uterus: implications for expression, regulation, and cellular function

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Am J Physiol Endocrinol Metab 291: E59–E72, 2006. First published January 31, 2006; doi:10.1152/ajpendo.00582.2005.—Progesterone and its interaction with nuclear progesterone receptors (PR) PR-A and PR-B play a critical role in the regulation of female reproductive function in all mammals. However, our knowledge of the regulation and possible cellular function of PR protein isoforms in the fallopian tube and uterus in vivo is still very limited. In the present study, we revealed that equine chorionic gonadotropin (eCG) treatment resulted in a time-dependent increase in expression of both isoforms, reaching a maximal level at 48 h in the fallopian tube. Regulation of PR-A protein expression paralleled that of PR-B protein expression. However, in the uterus PR-B protein levels increased and peaked earlier than PR-A protein levels after eCG treatment. With prolonged exposure to eCG, PR-B protein levels decreased, whereas PR-A protein levels continued to increase. Furthermore, subsequent treatment with human (h)CG decreased the levels of PR protein isoforms in both tissues in parallel with increased endogenous serum progesterone levels. To further elucidate whether progesterone regulates PR protein isoforms, we demonstrated that a time-dependent treatment with progesterone (P₄) decreased the expression of PR protein isoforms in both tissues, whereas decreases in p27, cyclin D₂, and p53, cyclin D₂, and proliferating cell nuclear antigen protein levels were observed only in the uterus. To define the potential PR-mediated effects on apoptosis, we demonstrated that the PR antagonist treatment increased the levels of PR protein isoforms, induced mitochondrial-associated apoptosis, and decreased in epidermal growth factor (EGF) and EGF receptor protein expression in both tissues. Interestingly, immunohistochemistry indicated that the induction of apoptosis by PR antagonists was predominant in the epithelium, whereas increase in PR protein expression was observed in stromal cells of both tissues. Taken together, these observations suggest that 1) the tissue-specific and hormonal regulation of PR isoform expression in mouse fallopian tube and uterus, where they are potentially involved in regulation of mitochondrial-mediated apoptosis depending on the cellular compartment; and 2) a possible interaction between functional PR protein and growth factor signaling may have a coordinated role for regulating apoptotic process in both tissues in vivo.

progesterone receptor protein isoforms

UNDER PHYSIOLOGICAL CONDITIONS, successful female reproduction depends on the coordinated function of the ovary, fallopian tube (2), and uterus. This coordination is, at least partly, mediated by the steroid hormone progesterone (20). The diverse biological actions of progesterone (P₄) are believed to be conveyed mostly through interaction with transcription-regulating nuclear progesterone receptors (PR), which are members of the steroid/nuclear receptor superfamily (3, 35, 72). PR binds its ligands, leading to dimerization of receptors, DNA-binding, activation or repression of specific hormone response elements on target genes, and further regulation of transcription of various functional downstream genes (72). The human and rodent nuclear PR consists of two distinct isoforms, PR-A and PR-B (20, 28), derived from a single gene independently regulated by separate A and B promoters (25). In mice, PR-A is an 83-kDa protein, whereas PR-B is a 115-kDa protein that contains an additional 164 amino acids at the NH₂ terminus that are missing in PR-A (60, 61). Although PR-A and PR-B have common ligand-binding specificities and DNA-binding domain and further activate transcription through binding to identical progestin response elements, these two isoforms possess different functional properties (3, 35). In vitro evidence indicates that PR-A functions as a transcriptional inhibitor of PR-B as well as of other steroid receptors, including estrogen, glucocorticoid, and mineralocorticoid receptors, where PR-A and PR-B are coexpressed (8). In contrast, PR-B acts as a stronger transcriptional activator than PR-A (35). These observations suggest that PR isoforms and their activities may change in opposite directions depending on the target cells, gene promoters, and levels of coregulators. Studies of gene mutant mice lacking various PR isoforms have provided important insight into the physiological function of PR in regulation of female fertility (8, 9, 44). For example, ablation of PR-A/B or just PR-A resulted in blockage or decrease of ovulation and infertility, and only a subset of progesterone action can be attributed to PR-B, whereas mice lacking PR-B did not affect biological responses of the ovary and uterus to progesterone, suggesting that the relative contribution of PR isoforms may have differential responses in cellular events.

PR-A and PR-B are coexpressed in most target tissues. However, the regulation of PR proteins has been found to be tissue and cell type specific in female reproductive tissues in vivo (20). Our laboratory has demonstrated that treatment with human chorionic gonadotropin (hCG), mimicking endogenous preovulatory luteinizing hormone surge, induces the levels of...
PR protein isoforms in periovulatory granulosa cells in mouse ovary (62). Similar observations have been made in ovaries from intact adult mice during the estrous cycle (19) and in humans (22). In addition, the developmental time course of PR mRNA and protein expression has been extensively studied in the uterus by use of either ovariec-tomized animals (a background devoid of endogenous steroid hormones) treated with selected steroid hormones (27, 30, 31, 70) or intact adult rats during the estrous cycle (46). Furthermore, it has previously been reported that the PR protein isoforms are expressed and regulated in human endometrium during the menstrual cycle (39, 43). Relative to ovary and uterus, earlier studies have demonstrated that specific binding sites for PR can be observed using the ligand-binding characteristic assay in human and rodent fallopian tubes, (37, 45, 52, 53, 73). Later reports have demonstrated that PR mRNA and protein expression can be detected by RT-PCR (6, 50, 66), Western blot analysis (6, 66), and immunohistochemical localization (1, 6, 18, 19, 50, 66). Although PR proteins display cyclic changes in human fallopian tubes during the menstrual cycle (1, 73), PR protein isoforms expressed in the fallopian tubes, their regulation, and their role in tubular function are much less well understood.

Apoptosis is an active mode of cell death that is triggered by a variety of physiological and pathological stimuli (23). Inappropriate activation or inhibition of apoptosis may cause or contribute to a variety of diseases (69). Within the complex network of proteins regulating apoptosis, proteins for caspase family are universal effectors in the apoptotic process in mammalian cells (15). Members within this wide family have been divided into initiator caspsases (e.g., caspase-9) and executioner caspsases (e.g., caspase-3). It is known that functional caspase-9 is the critical player of the apoptotic stimuli, acting through mitochondrial-mediated death pathway (29). When release of cytochrome c (a proapoptotic factor) from mitochondria into cytosol is enhanced in response to apoptotic stimuli, procaspase-9 interacts with cytochrome c, Apaf-1, and ATP to liberate active caspase-9 (34), which in turn generates activation of caspase-3 (21). One of the biochemical hallmarks of apoptosis is the degradation of DNA that is induced by the DNA fragmentation factor (DFF). Caspase-activated deoxyribonuclease (CAD), a subunit of DFF, has been shown to cleave genomic DNA into interneclease fragments activated mainly by caspase-3 in humans and mice (16, 59), resulting in characteristic changes associated with the apoptotic nuclear morphology, such as membrane blebbing, nuclear condensation, and DNA fragmentation (24). In addition to physiological evidence for nuclear PR actions by effect of PR in female reproduction (e.g., the establishment and maintenance of pregnancy (20)), several studies from our laboratory and others have previously demonstrated that nuclear PRs as intracellular regulators act in an autocrine manner to influence granulose cell survival in humans and rodents (40, 62). However, the intracellular mechanisms involved in the activation of apoptotic pathways by PR signaling through regulation of PR protein levels in the fallopian tube and uterus are largely unknown.

The present study was designed to investigate the time-dependent expression and regulation of PR protein isoforms in response to gonadotropins in mouse fallopian tube and uterus in vivo using quantitative Western blotting and immunohistochemistry. To understand the impact of PR on the fallopian tube and uterus functions, we have demonstrated the effect of exogenous progesterone on expression of PR isoforms in parallel with expression of cell cycle and proliferation proteins. Furthermore, it assesses the effect of the PR antagonists on the PR protein expression, apoptosis induction and regulation of epidermal growth factor (EGF), and EGF receptor protein expression in both tissues.

MATERIALS AND METHODS

Hormones and reagents. Equine chorionic gonadotropin (eCG), P₄, mouse monoclonal anti-β-actin, mouse monoclonal anti-α-tubulin, alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG), and alkaline phosphatase-conjugated mouse anti-goat/sheep IgG were obtained from Sigma Chemical (St. Louis, MO). Human (b)CG and the PR antagonist Org 31710 (26) were obtained from N.V. Organon (Oss, Holland). The PR antagonist RU-486 (4) was obtained from Organon (Oss, Holland). The PR antagonist RU-486 (4) was obtained from Endocrine Assays (Paris, France). The antibodies used to detect PR in this study were raised against human PRs and recognized the ligand-binding domain (PR c-19) or DNA-binding domain (PR c-20) of PR in rodent tissues by Western blotting and immunohistochemical analysis. Rabbit polyclonal anti-PR (PR c-19 and PR c-20) and their respective blocking peptides rabbit polyclonal anti-cyclin D2, rabbit polyclonal anti- p27Kip1 (p27), mouse monoclonal anti-apoptosis-inducing factor (AIF), rabbit polyclonal anti-caspase-3, rabbit polyclonal anti-caspase-9, rabbit polyclonal anti-CAD, goat polyclonal anti-EGF, rabbit polyclonal anti-EGF receptor, and normal mouse IgG were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) was obtained from Novocastra Laboratories (Newcastle upon Tyne, UK). Mouse monoclonal anti-cytochrome c and rabbit polyclonal anti-activated caspase-3 were obtained from BD PharMingen (San Diego, CA). Normal rabbit serum and goat serum were obtained from DAKO (Carpinteria, CA). Alkaline phosphatase-conjugated goat anti-rabbit IgG was obtained from Tropix (Bedford, MA). Cy3-conjugated donkey anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Other reagents not mentioned in the text were purchased from Sigma or Merck (Darmstadt, Germany) and were of the highest purity grade available.

Experimental animals. The animal studies were reviewed and approved by the ethics committee at Göteborg University, Sweden. All murine analyses were performed in female C57BL/6 mice. Mice (21 days old) were purchased from Taconic M&B, Ejby, Denmark. Mice were initially allowed to acclimate to the facility for 5 days before the beginning of any study and were provided standard chow and tap water ad libitum. All animals were individually housed under defined conditions with temperature control, constant humidity, and a regular light-dark cycle. Mice at 26 days of age with a body weight ranging from 13–15 g were used in this study.

Hormone treatment, tissue preparation, and blood collection. To determine the response to gonadotropins and induction of ovulation (62), immature female mice (30 mice in one experiment) were given a single injection of 5 IU eCG or saline (hormone vehicle). eCG was administered intraperitoneally for 24 or 48 h. In addition, some of the animals (40 mice in one experiment) were injected intraperitoneally with 5 IU eCG followed 48 h later with a single injection of 5 IU hCG (ip) for the indicated time (6, 12, 24, and 48 h). The fallopian tubes and uteri were grossly dissected free of contaminating tissue (e.g., adipose tissue) after mice were killed by decapitation. One-half of the fallopian tube and uterine horn in each animal was either immediately frozen in liquid nitrogen and stored at −70°C for subsequent Western blot analysis or fixed in 4% formaldehyde neutral buffered solution for 24 h at 4°C and embedded in paraffin for histochemical analysis.

Protein was harvested from a matched portion of the organ in all cases to ensure that results did not differ because of organ composition. In all cases, trunk blood was obtained from heart...
puncture before tissue collection. Sera were prepared by clotting, centrifugation, and then collection of supernatants. Sera were stored at −20°C for subsequent analysis.

Validation of eCG and/or hCG mouse model. Ovarian weight of immature female mice remained relatively constant between 26 and 30 days of age. An increase in ovarian weight was noted in mice treated with eCG (day 26). They were increased further within 6 h after subsequent treatment with hCG, remained unchanged through 12 h, and then continued to decrease at 48 h (Fig. 1A). Treatment of mice with eCG for 48 h mimicked ovaries at the preovulatory phase of a reproductive cycle, such as increase in follicular size, cumulus expansion, and oocyte maturation as well as significant changes at each step-section of one ovary (data not shown). Subsequent hCG treatment (from 6 to 48 h) encompassed the interval preceding ovulation (Table 1) until the luteal phase of the ovarian cycle. Ovarian morphology (47, 48) and numbers of different stage of follicles (5) were assessed in mice with a regimen of 5 IU eCG and/or 5 IU hCG by examining histological step-sections of one ovary (data not shown). Serum estradiol concentration increased by 24 h and peaked at 48 h after eCG injection (Fig. 1B), whereas serum P4 concentration showed a tendency to significantly increase after post-hCG treatment and peaked at 48 h (Fig. 1B). The production of estradiol and progesterone in immature mice treated with eCG and/or hCG in this study is similar to that in adult cycling mice (74). This in vivo model has been used to stimulate follicular development, induce ovulation, and cause significant changes in the expression of PR protein isoforms in mouse ovarian granulose cells (62).

**Fig. 1.** Effect of gonadotropins on ovary weights (A) as well as serum estradiol and progesterone (B) concentrations in immature mice. The number of mice per group is indicated. eCG and hCG, equine and human chorionic gonadotropins, respectively. Trunk blood was obtained from heart puncture. Sera were collected after clotting and centrifugation. Serum estradiol and progesterone concentrations were measured in duplicate aliquots of each sample. Values represent means ± SE. ** and ***Significantly different from untreated animals (time, 0 h), P < 0.01 and P < 0.001, respectively.

<table>
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<th>Treatment</th>
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<th>Ova per Ovary (mean ± SE)</th>
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<tr>
<td>hCG</td>
<td>15</td>
<td>15</td>
<td>25±2</td>
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<tr>
<td>+RU-486</td>
<td>15</td>
<td>2</td>
<td>12±1*</td>
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<tr>
<td>+Org 31710</td>
<td>5</td>
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<td>+Dex</td>
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<td>+(RU-486/Dex)</td>
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hCG and eCG, human and equine chorionic gonadotropin, respectively. Dex, dexamethasone. Significance values for individual groups are indicated. *P < 0.001 vs. hCG treatment.

Treatment with P4 and PR antagonists and pharmacological procedures in vivo. To determine the specific effects of P4 on the regulation of PR isoform expression, 26-day-old mice randomly received a single injection of P4 (4 mg/kg ip in 100 μl of sesame oil). A single pharmacological dose of P4 was chosen on the basis of a previous study (58). Each time point consisted of five mice. The fallopian tubes and uteri were obtained at different time intervals (6, 24, 36, 48, and 72 h) for Western blot analysis. In addition, corresponding controls for test groups (3 mice each) consisted of administration of an equal volume of sesame oil (hormone vehicle) for 6 and 48 h, demonstrating that no changes in expression of proteins (PR isoforms, PCNA, and p27) were stress related (data not shown). No significant changes of serum estradiol concentration were apparent, whereas a rapid but transient increase in serum progesterone concentration was detected at 6 h followed by a decline to physiological levels (5–500 nM) within 24 h after P4 treatment (data not shown).

To test the effects of PR antagonists (RU-486 and Org 31710) on the regulation of PR isoform expression and ovulation in response to gonadotropin stimulation, animals were randomly given either 1 mg of RU-486 or 2 mg of Org 31710 in combination with 5 IU of hCG after treatment with 48-h eCG (5 IU ip). Both RU-486 and Org 31710 were dissolved in 100% ethanol and resuspended in sesame oil. All intraperitoneal injections were in a volume of 100 μl. An equal volume of 100% ethanol and sesame oil was injected to control mice. After 24 h of cotreatment with hCG and/or PR antagonist, either RU-486 or Org 31710, the fallopian tubes, and uteri were collected in the different groups for either Western blot or immunohistochemical analysis. In this study, the doses of RU-486 and Org 31710 were chosen on the basis of previous studies (36, 56). Additional treatment with dexamethasone [600 μg/kg dissolved in saline and resuspended in 100 μl of sesame oil (58)] was used for PR antagonist specific effect on ovulation. All drug solutions were freshly prepared before each experiment. In addition, the number of ovulations was determined by flushing the fallopian tubes and counting the number of oocytes retrieved under microscope. Mice exhibiting ovulation after PR antagonist treatments were discarded from the study (Table 1).

Protein extraction and Western blot analysis. Whole cell protein extract and Western blot were done essentially as described previously (64). Tissue extracts were homogenized at 4°C and incubated on ice for 30 min for a high-salt lysis buffer that effectively extracts all DNA-binding proteins. Subsequently, insoluble material was removed by centrifugation at 10,000 g for 30 min at 4°C. The protein content of the extracts was determined using a bichinonic acid protein assay (Pierce, Rockford, IL) with BSA as the standard. Equal amounts of protein per sample were heated at 95°C for 5 min with reducing agents and loaded onto 4–12% sodium dodecyl sulfate (SDS)-polyacrylamide gels (Novex, San Diego, CA) using a MOPS-SDS running system. The nonspecific protein-binding site on the polyvinylidene difluoride membranes (Amersham International, Buckinghamshire, UK) was blocked by incubation of the membrane with 5% nonfat milk in a Tris-buffered saline (TBS)-Tween 20 buffer (10 mM Tris, 150
mM NaCl, and 0.1% Tween 20, pH 8.0) for 4 h. The membranes were incubated with the indicated primary antibodies in blocking buffer overnight at 4°C and the appropriate alkaline phosphatase-linked secondary antibodies with 1:40,000 or 1:80,000 dilutions for 2 h. Preparation of anti-PR (c-19 + c-20, 1:250 for each), anti-PCNA (1:1,000), and anti-activated caspase-3 (1:500) has been described previously (62). The primary antibodies anti-cyclin D2 (1:1,000), anti-p27Kip1 (p27; 1:1,000), anti-AIF (1:250), anti-caspase-9 (1:250), anti-CAD (1:1,000), anti-EGF (1:250), and anti-EGF receptor (1:250) were used. The immunosignal-CDP-Star substrate for alkaline phosphatase system (Tropix, Bedford, MA) was used to visualize protein bands. To reprobe the blot with another antibody, the blot was rehydrated in methanol, rinsed, and incubated with stripping buffer (65 mM Tris·HCl, 2% SDS, and 100 mM β-mercaptoethanol, pH 6.8) at 50°C for 30 min. Blots were then washed for 2 h with several changes of TBS-Tween 20 buffer at room temperature. ECL films of the Western blots were captured with an image scanner (EPSON perfection 2450 photo). The intensities of the immunoreactive bands were converted into digitized signals by use of a computerized densitometry system and quantified by a gel documentation program (ImageQuant version 5.0; Molecular Dynamics, Sunnyvale, CA). Signal intensities of the mouse PR and caspase-3 proteins were normalized to those of mouse β-actin protein as ratios to produce arbitrary densitometric units (ADU) of relative abundance. Each Western blot was quantified using a standard curve consisting of serial dilutions of one of the samples included in the assay. All samples for a particular experiment were run on the same gel, or set of gels run at the same time, under the same conditions. Because exposure times, transfer efficiency, and other factors may vary between runs, ADUs for relative protein levels were only directly compared within a set of gels run at the same time. All steps were carried out at room temperature unless otherwise stated.

**Histological and immunohistochemical analyses.** Tissue blocks were sectioned at 5 μm thickness onto poly-l-lysine-coated slides. They were dewaxed by two 5-min washes in xylene and rehydrated through graded alcohol by 5-min washes with a final wash in water. A standard citrate boiling antigen retrieval procedure was used (62, 64). Sections were blocked in 0.3% H2O2 in TBS and 10% normal goat serum. The primary antibody was diluted 1:250 (anti-PR c-19 or 1:50 (anti-activated caspase-3) in TBS containing 1% BSA, and the sections were incubated overnight at 4°C. The appropriate secondary antibody was added. After a wash with TBS, sections were stained using the avidin-biotinylated peroxidase complex detection system (ABC kit, Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Immunostaining was then visualized using 3,3-diaminobenzidine tetrahydrochloride (0.5 mg/ml in PBS + 0.01% H2O2, pH 7.6) for 10 min. Slides were viewed on a Nikon E-1000 microscope under bright-field optics and photomicrographed using Easy Image 1 (Bergström Instrument, Gothenburg, Sweden). Ovary sections from mice treated with eCG and hCG (62) and human breast carcinoma sections (41) were used as positive controls because nuclear PR is highly expressed in these tissues. Three negative-control procedures (exclusion of either primary antibody or secondary antibody in the tissue sections, application of either normal goat serum or normal rabbit serum, and preabsorption of the anti-PR antibody with a 100-fold excess of antigen) were run simultaneously to confirm the specificity of the immunostaining. Serial sections of the fallopian tubes and uteri were examined in a blinded manner under light microscopy.

For fluorescence immunohistochemistry of cytokerine c, sections were incubated with antibody against cytokerine c diluted 1:200 in PBS containing 1% BSA and 3% fat-free milk. Immunodetection was accomplished using Cy3-conjugated anti-mouse IgG antibody. Sections were washed and mounted with Fluorescent Mounting Media (DAKO, Carpinteria, CA). Tissue images were then acquired with an Axiosvert 200 microscope (Zeiss, Jena, Germany) equipped with a laser-scanning confocal imaging LSM 510 META system (Zeiss). The immunohistochemical findings illustrated are representative of those observed in numerous sections from multiple animals.

**Visualization of apoptosis.** Immunohistochemistry was used for detection of denatured DNA with a monoclonal antibody against single-strand DNA (ssDNA) to identify specific apoptotic cells, as described previously (17). All steps were processed according to the manufacturer’s instructions. Negative controls were processed in an identical manner in the same experiment except that anti-ssDNA primary antibody was omitted; incubation was performed with either normal mouse IgG (Santa Cruz Biotechnologies) or 100 U/ml S1 nuclease (Sigma-Aldrich) in acetate buffer after a rinse in ice-cold PBS or PBS with 1% nonfat dry milk; and the controls yielded no reaction product in parallel experiments (data not shown).

**Measurement of estradiol and progesterone.** Concentrations of total serum estradiol and P4 were quantified by a radioimmunoassay (RIA) (10, 63) using a Delfia assay kit (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) according to a protocol provided by the manufacturer. All samples were tested as duplicates together in one run. When different volumes of serum were assayed, concentrations were parallel to the standard curve. The sensitivity of the assay was typically better than 50 pmol/l, and the intra-assay coefficient of variation was 3.8–10% for measurement of estradiol, whereas the sensitivity of the assay was typically better than 0.8 pmol/l, and the intra-assay coefficient of variation was 3.3–7.3% for measurement of P4.

**Data analysis and statistics.** Data from Western blot analysis are presented in the figures as means ± SE. Five mice for each group were used in one experiment except for the RU-486 treatment, where 10 mice were used for each group. To confirm reproducibility of results, three independent experiments for time course study of PR protein isoform expression in mouse fallopian tubes and uteri were performed and analyzed. All data were analyzed using the Analyze-It program (Analyze-It Software, Leeds, UK). Multiple comparisons between groups were made using one-way ANOVA with Tukey’s post hoc test. Statistical significance was set at P < 0.05.

### RESULTS

**Effect of gonadotropins on expression and regulation of PR protein isoforms in fallopian tubes and uteri.** The Western blot analysis revealed the expression pattern of PR protein isoforms during gonadotropin treatment in the fallopian tubes comparable to that in the uterus. Treatment of immature mice with eCG resulted in the induction of proteins for total PR, PR-A, and PR-B in the fallopian tubes, commencing at 24 h and reaching maximum at 48 h. Furthermore, additional treatment with hCG reduced the total PR protein levels, reaching a minimum at 24 h (Fig. 2A). In the uterus, induction of total PR protein expression was seen, with a maximum at 48 h eCG treatment. However, the changes in protein levels of both PR isoforms in the uterus were different from those in the fallopian tubes. PR-B protein was induced earlier than PR-A protein after eCG treatment in the uterus. As with expression of PR proteins in the fallopian tubes, hCG treatment reduced the total PR, PR-A, and PR-B protein levels to baseline levels from 12 to 48 h (Fig. 3A). In all tissues, no induction of PR or its isoforms was seen at 48 h after injection of saline (data not shown).

In the fallopian tubes, weak immunoreactivity was present in the nuclear compartments of luminal epithelial, stromal, and smooth muscle cells in immature mice, and no differences in expression were noted between cell types. At 48 h after eCG treatment, there was an intense overall PR immunoreactivity in all cell types (Fig. 2C). Furthermore, the level of PR immunostaining was decreased when endogenous P4 levels were high.
after hCG treatment (Fig. 2C). Specific nuclear PR immuno-
histochemical staining was observed, with no difference in the
isthmus and ampullary regions in the fallopian tubes during
gonadotropin treatment. In the uterus, immunoreactivity was
weak in the nuclear compartments of endometrial stromal cells
and almost absent in the endometrial luminal epithelia and
myometrium in immature mice. After eCG treatment for 48 h,
intense immunoreactivity was detected in the endometrial stroma-
cells, whereas a low level of PR immunostaining was
detected in the endometrial luminal epithelia and myometrium.
In addition, there was intense immunostaining in the endo-
metrial luminal epithelia and less immunoreactivity in myome-
trium after 12 h of hCG treatment. In contrast, endometrial
stromal cell PR immunoreactivity was minimal (Fig. 3B).

**Fig. 2. Regulation of progesterone receptor (PR) protein isoforms A and B after gonad-

otropin treatment in immature mouse fallo-

pian tubes. A: fresh fallopian tubes from

26-day-old female mice treated with eCG

and/or hCG with the indicated times were

collected. Top: total protein was isolated and

used for Western blot analysis as described

in MATERIALS AND METHODS. Samples were

run on a SDS-polyacrylamide gel and immu-
noblotted with antibody against PR. Bottom:

densitometric analysis of levels of PR iso-

form protein expression in 33 independent

experiments (n = 5–10 mice/group in each

experiment). β-Actin was used as internal

control. Levels of PR isoform proteins were

expressed as ratio of either PR-A or PR-B
densitometric value to β-actin. Data are

expressed as arbitrary densitometric units

(ADU) and represent means ± SE of 3

independent experiments in the graph. **

and ***Significantly different from un-
treated animals (time, 0 h), P < 0.01 and

P < 0.001, respectively. B: standard curve

showing linear relationship between immu-

noreactive band intensity and amount of

whole tissue extract protein loaded for gona-
dotropin-treated mice. Equation of line of

best fit was generated by least squares linear

regression analysis. C: immunohistological

localization of PR in sections of mouse fal-
llopian tube from mice untreated (I-1 and

I-2), or treated with eCG for 48 h (I-1 and

I-2), or treated with eCG for 48 h (II-1 and

II-2) and additionally treated with hCG for

12 h (III-1 and III-2), as described in mate-

rials and methods. High specific immuno-

staining of PR was observed in nuclei of

luminal epithelial (le), stromal (str), and

smooth muscle cells (m) (48-h eCG treat-

ment); is, isthmic segment; as, ampullary

segment. IV: ovary from mouse treated

with eCG/hCG served as a positive control

tissue. Strong immunoreactivity was seen in

granulosa cells. V: the same concentration of

nonimmune IgG instead of the primary anti-

body was used as a negative control in ovar-
inian section. Original magnification (I, II, and

III)-1, ×10; (I, II, and III)-2, IV, and V, ×100. These experiments were repeated using 5 mice/group with similar results.
Fig. 3. Regulation of PR protein isoforms after gonadotropin treatment in immature mouse uteri. A: fresh uteri from 26-day-old female mice treated with eCG and/or hCG with the indicated times were collected. Top: total protein was isolated and used for Western blot analysis as described in MATERIALS AND METHODS. Samples were run on an SDS-polyacrylamide gel and immunoblotted with antibody against PR. Bottom: densitometric analysis of levels of PR isoform protein expression in 3 independent experiments (n = 5–10 mice/group in each experiment). β-Actin was used as internal control. Levels of PR isoform proteins were expressed as ratio of PR-A or PR-B densitometric value to β-actin. Data are expressed as ADU and represent means ± SE of 3 independent experiments in graph. ** and ***Significantly different from untreated animals (time, 0 h), P < 0.01 and P < 0.001, respectively. B: immunohistological localization of PR in sections of mouse uterus from mice untreated (I-1 to I-3), or treated with eCG for 48 h (II-1 to II-3) and additionally treated with hCG for 12 h (III-1 to III-3), as described in MATERIALS AND METHODS. High specific immunostaining of PR was observed in nuclei of endometrial stromal (str; 48-h eCG treatment) and endometrial luminal epithelial (le; additional 12-h hCG treatment) cells; myo, myometrium. Some stromal cells were positive for PR immunostaining in untreated mouse uterus. IV: strong immunoreactivity seen in human breast carcinoma served as a positive control tissue. V: the same concentration of nonimmune IgG instead of the primary antibody was used as negative control in human breast carcinoma section. All original magnification, ×100. These experiments were repeated using 5 mice/group with similar results.
Decreased PR protein isoform abundance after P₄ treatment in fallopian tubes and uteri. The data presented above indicate that the decrease in PR protein isoforms after additional hCG treatment presumably arises from changes in endogenous P₄ levels in vivo. Therefore, we demonstrated the effect of an exogenous P₄ exposure on the levels of PR protein isoforms in the fallopian tubes and uteri determined by Western blot analysis. A time-dependent reduction of PR-A and PR-B protein expression was seen in the fallopian tubes and uteri from immature mice treated with a single injection of P₄ (Fig. 4, A and B). To analyze the molecular mechanisms involved in the effects of P₄, we also compared the expression of the cell cycle proteins (cyclin D₂ and p27) and PCNA (as an indicator of proliferation-related gene expression). In the fallopian tubes, the levels of expression of cyclin D₂, PCNA, and p27 remained relatively constant throughout the various time points during P₄ manipulations (Fig. 4A). However, all of these proteins displayed a decrease in uteri following P₄ treatment (Fig. 4B), suggesting that the P₄ effect on the expression of cyclin D₂, PCNA, and p27 proteins appears to be tissue specific.

Treatment with PR antagonists increases PR protein isoform expression in fallopian tubes and uteri. The expression of PR protein isoforms in both tissues from mice treated concurrently with hCG and RU-486 by 24 h after 48-h eCG treatment was examined by Western blot analysis. Exogenous administration of RU-486 showed an induction of PR protein isoforms in the fallopian tubes (Fig. 5A) and uteri (Fig. 5B). Meanwhile, serum estradiol concentration decreased, whereas serum P₄ concentration was not significantly changed in RU-486-treated mice relative to untreated mice (Fig. 5C), in agreement with a previous report (14). Although RU-486 is known mostly as a PR antagonist, it is also an antagonist of glucocorticoid receptor (GR) (4). It is therefore important to ascertain that treatment of mice with dexamethasone, a potent synthetic glucocorticoid, did not increase the expression of PR protein isoforms (data not shown) nor induce ovulation (Table 1). Furthermore, RU-486-induced PR protein isoforms were not neutralized by exogenous administration of dexamethasone (data not shown), suggesting that the effect of RU-486 on ovulation and regulation of PR isoform protein expression is unlikely mediated via the GR in the fallopian tubes and uteri. Because Org 31710 is a highly selective PR antagonist (26), we demonstrated the effect of Org 31710 on the PR expression in both tissues by immunohistochemistry analysis. In the fallopian tubes, intense immunoreactivity was detected in the nuclear compartments of luminal epithelia and stromal cells in mice treated with RU-486, whereas most, but not all, of the PR immunostaining appeared in both cytoplasm and nucleus of luminal epithelia in mice treated with Org 31710 (Fig. 5D). However, PR immunostaining was especially dark in the endometrial stromal cells after treatment with both PR antagonists, in particular Org 31710 (Fig. 5D).

Effect of PR antagonists on induction of apoptosis differs in fallopian tubes and uteri. The regulation of AIF, cytochrome c, caspase-3 and -9, and CAD protein expression and DNA fragmentation was investigated by either Western blot and/or immunohistochemical analyses with the same tissues from the same animals. Levels of activated caspase-9, activated caspase-3, and CAD protein expression were increased in parallel with the levels of PR protein isoforms (Fig. 5, A and B) in the fallopian tubes (Fig. 6A) and uteri (Fig. 6B) in RU-486-treated mice compared with controls. There was no change in any AIF protein expression after RU-486 treatment relative to controls (Fig. 6A and 7A). To confirm the data from Western blot analyses, we examined cytochrome c, caspase-3 activation, and DNA fragmentation microscopically by immunohistochemistry. We observed that intense immunoreactivities for both cytochrome c and activated caspase-3 and DNA fragmentation were consistently detected in the luminal epithelia of the fallopian tubes (Fig. 6B) as well as in the endometrial luminal and glandular epithelia of uterus (Fig. 7B) from mice treated with either RU-486 or Org 31710. There were, in general, no detectable intensities of immunoreactive cytochrome c, activated caspase-3, and DNA fragmentation in stromal cells in both tissues.

Effect of PR antagonists on inhibition of EGF and EGF receptor protein expression in fallopian tubes and uteri. To assess whether PR antagonist-induced apoptosis affects the expression of growth factors, we demonstrated regulation of EGF and its receptor expression in both tissues by means of Western blot analysis in a same-setting experiment. The stimulatory effect of hCG and the suppressive effect of RU-486 on expression of EGF and its receptor proteins compared with those in mice treated with 48-h eCG were clearly observed in fallopian tubes (Fig. 8A) and uteri (Fig. 8B).

DISCUSSION

Information about the tissue relative distribution of PR protein isoforms is necessary for understanding the specific functions of these two isoforms in female reproduction in all mammals. Indeed, unequal expression of PR isoforms exhibits...
different effects during the initiation of ovulation and successful intruterine implantation in mice (8, 44). Considering the importance of the nuclear PR isoforms in the ovary (62) and uterus (27, 46), little is known about the regulation of the PR protein isoforms in vivo and the possible cellular functions of PR in the fallopian tubes. The principal aim of this study was to investigate and compare the regulation and functional role of PR isoforms in mouse fallopian tubes and uteri. This was done by studying the gonadotropin regulation of PR protein isoforms by demonstrating effects of exogenous progesterone exposure and by examining effects of blocking PR signaling using PR antagonists. To better understand the biological role of nuclear PR in mouse fallopian tubes and uteri, we have focused here on how regulation of PR protein expression affects apoptosis and on molecules that regulate this process.

The time course of PR protein expression in response to gonadotropin stimulation in the fallopian tubes and uteri is different from that in ovary in mice. The current results showed that eCG treatment resulted in an increase in PR protein levels in the fallopian tubes and uteri that appears to be a response to the presence of high levels of serum and ovarian estradiol concentrations in vivo (Fig. 1B and Ljungström K, Weijdegård B, and Shaw R, unpublished data). There is evidence that estrogen peaked from ovulatory follicles induces PR mRNA and protein in rat uterus in vivo (27), and in most target cells in vitro (20) the present results suggest that increase in the expression of PR protein isoforms in the fallopian tubes and uteri may be important for the control of tubular and uterine responsiveness to estrogens in the early events of reproduction (e.g., cell proliferation for tissue development). This effect was attenuated by subsequent treatment with hCG, suggesting that the increase in endogenous P4 levels (Fig. 1B) inhibits PR protein expression in both tissues. This hypothesis is strongly supported by the finding that exogenous P4 treatment led to a time-dependent decrease in PR protein expression. After ligand binding, PR protein is rapidly and extensively downregulated in vitro (32). The mechanism for P4-dependent reduction of PR protein levels has been demonstrated to be due to an alteration in ubiquitination in chicken fallopian tubes in vivo (67). Our results for regulation of PR protein expression are consistent with these findings, demonstrating that PR protein expression is downregulated in both tissues after hCG treatment.

**Fig. 5. Regulation of PR protein isoforms after PR antagonist treatment in eCG/hCG-primed mouse fallopian tubes and uteri. Top:** results from Western blot analysis performed on whole tissue protein preparation as described in MATERIALS AND METHODS. Fallopian tubes (A) and uterus (B) were obtained from immature mice treated with hCG for 24 h only (lanes 1–3) or combined with RU-486 (lanes 4–6) for 24 h followed by eCG for 48 h. Lanes 1–6 represented the same tissue from different mice. Samples were run on an SDS-polyacrylamide gel and immunoblotted with antibody against PR. β-Actin was used as control to monitor loading of protein. **Bottom:** relative expression levels of PR, PR-A, and PR-B proteins after RU-486 treatment. Densitometric values were normalized to that of β-actin protein expression. Data are from 3 mice/group of 3 independent performed experiments and are presented as means ± SE. ***P < 0.001, ****P < 0.0001 compared with mice not treated with RU-486. NC, no change. **C:** effect of RU-486 on serum estradiol and serum P4 in female mice. No. of mice per group is indicated. Trunk blood was obtained from heart puncture. Sera were subsequently collected after clotting and centrifugation. Serum estradiol and P4 concentrations were measured in duplicate aliquots of each sample. Values represent means ± SE. ***Significantly different from animals treated with eCG for 48 h, P < 0.001.

**Fig. 5. Regulation of PR protein isoforms after PR antagonist treatment in eCG/hCG-primed mouse fallopian tubes and uteri.**

**A.** Fallopian tube

<table>
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<th>Sample no.</th>
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<td>PR-B</td>
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<td>PR-A</td>
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**B.** Uterus

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<td>PR-B</td>
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**C.** Estradiol and progesterone expression in serum of female mice treated with eCG and hCG. No. of mice per group is indicated. Trunk blood was obtained from heart puncture. Sera were subsequently collected after clotting and centrifugation. Serum estradiol and P4 concentrations were measured in duplicate aliquots of each sample. Values represent means ± SE. ***Significantly different from animals treated with eCG for 48 h, P < 0.001.

**D.** Immunohistological localization of PR in sections of mouse fallopian tube and uterus from eCG- and hCG-primed mice treated with oil, RU-486, and Org 31710, as described in MATERIALS AND METHODS. All original magnification, ×100. These experiments were repeated using 5 mice/group with similar results.
with other reports, which demonstrate cyclic changes in PR proteins in human (52, 53) and rat (18, 50) fallopian tubes as well as human (39, 43, 75) and rat (27) uteri. Together, our animal experiments suggest that the effect of ovarian steroid hormones on regulation of PR protein expression in the fallopian tubes is similar to that in the uteri. It should be pointed out that we observed differences in pattern and expression levels of PR protein isoforms in mouse fallopian tube compared with uterus after gonadotropin stimulus. Previously, work in this laboratory indicated that both PR protein isoforms are regulated by additional treatment with hCG in ovarian granulosa cells (62). The data presented herein demonstrated that regulation of PR-A protein expression always paralleled that of PR-B protein expression in the fallopian tube (Fig. 2A) as well as in ovarian granulosa cells (62). However, PR-B protein levels in uterus were increased and peaked earlier than PR-A protein levels after eCG treatment. Moreover, although the levels of PR-B protein were decreased, PR-A protein expression further increased in the uterus (Fig. 3A). This may reflect that PR-B is the principal ligand-dependent transcriptional activator of P4-responsive genes, whereas PR-A acts as a dominant repressor mediated by PR-B when the two isoforms are coexpressed in vitro (8, 35). Together, our observations indicate that regulation of relative expression of PR isoforms seems to differ in a stage- and tissue-specific way in female reproductive tissues. The fallopian tube provides an appropriate environment for fertilization and early embryo development (2). P4 has been reported to decrease epithelial cilia beat frequency in human fallopian tubes (38). We showed that blocking P4 action by the PR antagonist RU-486 increased the expression of PR protein isoforms in mouse fallopian tubes, in parallel with increased epithelial cilia beat frequency and tubal contractions (38) and accelerated tubal oocyte transport in rodents (65). Although P4 via nuclear PR functions for remodeling of the uterus during the estrous cycle and pregnancy in mammals (20), our findings imply that nuclear PR protein isoforms may be involved in P4-mediated physiological processes such as transportation of the oocyte, sperm, and embryo through the fallopian tubes.

Reproductive tissues exist in coordination and balance between the processes of proliferation, differentiation, cell cycle inhibition, and apoptosis (7, 42). In previous studies, P4 inhibited apoptosis in rat endometrial epithelial cells, which expressed functional nuclear PR in vitro (51), whereas RU-486 induced apoptosis in rabbit uterine epithelium in vivo (57). Our laboratory has also demonstrated that PR antagonists induce apoptosis in mouse ovarian granulosa cells that were expressing nuclear PR in vivo (62) and in vitro (63). These demonstrations suggest that suppression of apoptosis is likely mediated by existence of nuclear PR in P4 target tissues. In mouse fallopian tube and uterus, the present study has detailed the molecular events involved in the apoptotic process affected by PR antagonists by using the same experimental models (62) for in vivo study and establish several new points. First, a considerable amount of experimental evidence has conclusively demonstrated a unique role for PR in the regulation of
apoptosis by orchestrating mitochondria-mediated activation of caspase-9 and caspase-3, resulting in DNA fragmentation. Little evidence for regulation of AIF protein expression after RU-486 treatment relative to controls was observed, suggesting that AIF may not be involved in PR antagonist-induced caspase-3-dependent apoptosis in both tissues, in agreement with a previous observation showing apoptotic effect on regulation of AIF protein expression in uterus (68). Treatment with either RU-486 (4) or Org 31710, which is devoid of any action on glucocorticoid receptors (26), yielded similar results, indicating that a generalized mechanism for induction of apoptosis is mediated by blockade of PR protein function. Therefore, our results suggest a direct link between PR protein expression and alterations in the mitochondrial-mediated apop-
Understanding the molecular components of the PR-mediated apoptotic program in female reproductive tissues will be an important step toward the development of novel therapeutic regimens to control cell proliferation and apoptosis during abnormal pregnancy as well as more targeted approaches to female contraception. To the best of our knowledge, this is the first study that shows the molecular details of a linkage between the regulation of PR expression and development of apoptosis in the fallopian tube and uterus of any species. Second, both fallopian tube and uterus are relatively complex tissues composed of many different cell types. For the interpretation of the consequences of an increase in the levels of PR protein isoforms on induction of apoptosis by PR antagonists, the exact localization of apoptotic cells is of great importance. We showed that the induction of apoptosis (i.e., extensive cytochrome c release, caspase-3 activation, and increase in DNA fragmentation) by PR antagonists was predominant in the epithelium, whereas an increase

Fig. 7. Effect of PR antagonists on apoptosis in eCG/hCG-primed mouse uteri. A: Western blot analysis was performed on whole tissue protein preparation as described in MATERIALS AND METHODS. Uteri were obtained from immature mice treated with hCG for 24 h only (lanes 1–3) or combined with RU-486 for 24 h (lanes 4–6) followed by eCG for 48 h. Lanes 1–6 represent the same tissue from different mice. Samples were run on an SDS-polyacrylamide gel and immunoblotted with antibody against AIF, caspase-3, caspase-9, or CAD. β-Ac
tin was used as control to monitor loading of protein. B: immunohistological localization of cytochrome c (a–c), activated caspase-3 (d–f), and DNA fragmentation (g–i) in sections of mouse uteri from eCG- and hCG-primed mice treated with control oil (a, d, g, and j), RU-486 (b, e, h, and k), and Org 31710 (c, f, i, and l) as described in MATERIALS AND METHODS. Original magnification: a–c, ×63; d–f, ×10; g–l, ×100. ge, Endometrial glandular epithelia. These experiments were repeated using 5 mice/group with similar results.
in PR protein expression was observed in stromal cells of both tissues, suggesting that different cell types within a P₄ target tissue can have different cellular fates in response to PR antagonist treatment. Moreover, the inhibition of stromal cells undergoing apoptosis by increase in stromal PR could indicate negative regulation at one or more points within a mitochondrial-dependent apoptotic process. P₄-dependent inhibition of uterine cell proliferation has been demonstrated previously in intact (71) and PR knockout mice (8, 9, 44). Therefore, it is likely that both antiapoptotic (in this study) and antiproliferative effects (7, 42) are mediated by the functional PR protein expression in the fallopian tube and uterus during their development as a result of changing serum steroid levels. However, whether one isoform is more important than the other requires further study. Third, various growth factors, including EGF, play an essential role in regulating cell proliferation and differentiation and in suppressing the cell apoptotic pathway (13). A previous report has shown that treatment with EGF induces PR mRNA expression in adult mouse uterus (11), suggesting that PR-mediated inhibition of apoptosis may be mediated, at least in part, through growth factors. Therefore, we demonstrated that an increase in endogenous P₄ by hCG treatment resulted in an increase in EGF and EGF receptor protein expression, pointing to the pivotal link between P₄-PR and EGF signaling pathways in both tissues. Moreover, knowledge about the specific effects of PR on EGF and its receptor protein expression was supported by treatment with PR antagonists, in accord with previous reports that RU-486 reduces EGF receptor expression in rat uterine stromal cells in vivo (12, 49) and in human endometrial cells in vitro (76). Given the similarity of PR-mediated regulation of EGF and EGF receptor expression in mouse fallopian tube and uterus in the current study, we propose the existence of a possible interaction between PR and growth factor signaling in the regulation of cell proliferation and apoptosis in both tissues. On the other hand, we found that the protective effects of PRs were partial, as epithelial apoptosis still occurred despite an increase in PR protein expression after PR antagonist treatment in the fallopian tubes, suggesting the existence of an additional cell death mechanism that functions against the protective effect of PR. One interpretation of our result is that changes in EGF protein levels could be a main alternative for determination of epithelial cells committed to die in the fallopian tubes.

Our study also showed that in the fallopian tube the effect of P₄ treatment on reduction of PR protein isoforms could be dissociated from changes in p27, cyclin D₂, and PCNA protein expression, whereas in uterus this effect was accompanied by decreased levels of p27, cyclin D₂, and PCNA proteins, suggesting that PR may be one of the intracellular molecules

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**Fig. 8.** Effect of PR antagonists on expression of epidermal growth factor (EGF) and EGF receptor (EGFr) proteins in eCG/hCG-primed mouse fallopian tubes and uteri. *Top:* Western blot analysis performed on whole tissue protein preparation as described in MATERIALS AND METHODS. Fallopian tubes (A) and uteri (B) were obtained from immature mice treated with eCG for 48 h (lanes 1–3), additional hCG for 24 h (lanes 4–6), or combined hCG with RU-486 for 24 h (lanes 6–9) followed by 48-h eCG. Lanes 1–9 represented the same tissue from different mice. Samples were run on an SDS-polyacrylamide gel and immunoblotted with antibody against EGF or EGFr. β-Actin was used as control to monitor loading of protein. *Bottom:* relative expression levels of EGF and EGFr proteins after RU-486 treatment. Densitometric values were normalized to that of β-actin protein expression. Data are from 3 mice/group and are presented as means ± SE. **P < 0.01, ***P < 0.001 compared with 48-h eCG.
involved in cell cycle progression in the uterus (71) and ovary (55) but not in the fallopian tube.

In summary, the present study highlights the developmental regulation of PR protein isoforms by gonadotropins in mouse fallopian tube and uterus in vivo. Our investigation suggests that both isoforms are mediated by estradiol stimulation and P₄ inhibition. Aside from the physiological functions of PR and its isoforms in the female reproductive tract, cellular events leading to cell death or survival are mediated by modulation of PR expression in different cell types in mouse fallopian tube and uterus. Moreover, the decision for cells to undergo apoptosis or survival are mediated by modulation of PR inhibition. Aside from the physiological functions of PR and its isoforms, both isoforms are mediated by estradiol stimulation and P₄ requirement for caspase 9 in apoptotic pathways in vivo. Proc Natl Acad Sci USA 93: 1603–1614, 1996.

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