Developmental and hormonal regulation of progesterone receptor A-form expression in female mouse lung *in vivo*: interaction with glucocorticoid receptors

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Abstract

Progesterone (P₄) regulates many aspects of physiological functions via two nuclear P₄ receptors (PR), PRA and PRB, which are members of a structurally related nuclear hormone receptor superfamily that includes glucocorticoid receptors (GR). The regulation and cellular distribution of PR protein isoforms have been extensively studied in reproductive tissues, but this is not the case in the lung. In the present study, reverse transcriptase (RT)-PCR, Western blotting, and immunolocalization supported the presence of PRA in the lung of female mice, with PRA protein levels significantly increased between postnatal day 7 and 12, declined at postnatal day 26, and minimal in adults when compared to postnatal day 2. The peak was temporally related to postnatal lung maturation in rodents. Immunoreactivity for PR was detected in the alveolar and bronchial epithelia. We then extended this study to examine, for the first time, the regulation of PRA protein expression in female mouse lung *in vivo*. Neither the increase in endogenous P₄ nor treatment with exogenous P₄ regulated PRA protein expression in female mouse lung. However,

treatment of mice with the GR/PR antagonist RU 486, but not Org 31710 (a specific PR antagonist), significantly increased PRA protein expression in parallel to a decrease in GR protein expression. In addition, treatment with the synthetic glucocorticoid dexamethasone led to a decrease in PRA protein expression independent of endogenous P₄ levels. Furthermore, immunoprecipitation followed by Western blot analysis revealed that, under *in vivo* conditions, PRA physically interacted with GR in mouse lung. Confocal laser microscopy revealed that PRA and GR co-localized in the nuclei of alveolar epithelia cells, whereas nuclear PR and cytoplasmic GR were detected in bronchial epithelium. Taken together, our observations suggest that PRA may be an important physiological factor involved in postnatal lung development and that the regulation of PRA protein expression is not dependent on P₄, but rather on functional glucocorticoid/GR signaling mediated by protein–protein interaction in the mouse lung.

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Introduction

Progesterone (P₄), an ovarian steroid hormone, exerts diverse functions in several physiological processes ranging from female reproduction to brain and bone cell metabolism (Graham & Clarke 1997). In many cases, the cellular actions of this hormone are mediated primarily by the nuclear P₄ receptors (PR), including two well-studied isoforms, PRA and PRB (Kastner *et al.* 1990a). Both isoforms function as hormone-activated transcription factors and are involved in the regulation of gene expression during development, growth, and differentiation in target organs (Graham & Clarke 1997, Shao *et al.* 2003, 2006). In addition, the human placenta and myometrial cells express high levels of

non-nuclear PR isoform, truncated PRC (Condon *et al.* 2006, Taylor *et al.* 2006). The roles of specific PRC in the steroid hormonal regulation and the importance of PRC gene expression are not clear. In the absence of P₄, the PR isoforms are maintained as a part of an inactive multiprotein complex that consists of the receptor and regulatory heat shock protein in the nuclei of target cells (Smith *et al.* 1990). When P₄ binds to PR, the receptors dissociate from heat shock proteins, undergo a change in conformation, dimerize, and are hyperphosphorylated, leading to the initiation of transcriptional regulation (Mohamed *et al.* 1994, McDonnell 1995). PRA lacks 164 amino acids at the N-terminus compared to the full-length PRB. The two isoforms arise from either transcription from alternative promoters (Kastner *et al.* 1990a, 1990b), or translation

from alternative sites in the same mRNA (Conneely *et al.* 1987, Giangrande & McDonnell 1999). The relative expression of the two PR isoforms is conserved in rodents and humans (Schneider *et al.* 1991, Kraus *et al.* 1993, Giangrande & McDonnell 1999), but varies dramatically between different tissues, cell types, physiological, and disease states, and is under developmental control (Ilenchuk & Walters 1987, Schneider *et al.* 1991, Graham & Clarke 1997, Bethea & Widmann 1998). Although both isoforms have similar hormone- and DNA-binding affinities, and form homo- and/or hetero-dimers (Lessey *et al.* 1983, Kastner *et al.* 1990a), several *in vitro* studies have shown that PRA and PRB display different transcriptional effects on progestin-responsive promoters in a cell- and promoter context-dependent manner (Vegeto *et al.* 1993, Wen *et al.* 1994, Giangrande & McDonnell 1999). For instance, PRA is often transcriptionally inactive and acts as a potent dominant repressor of PRB-mediated transcription (Tung *et al.* 1993). Furthermore, PRA has been found to diminish the activities of other nuclear receptors such as the glucocorticoid receptor (GR) under some conditions (Vegeto *et al.* 1993), whereas PRB is shown to activate transcription in several target tissues (McDonnell & Goldman 1994, McDonnell *et al.* 1994, Wen *et al.* 1994). Recently, studies in PR knockout mice confirmed that the two isoforms are not functionally equivalent and even imply tissue-selective isoform activity *in vivo* (Curtis & Korach 2000, Mulac-Jericevic *et al.* 2000). Thus, it is conceivable that the differential expression of PR protein isoforms in certain target tissues may influence the action of P₄. Several studies have demonstrated the potential for differential steroid receptor interactions at the level of transcription in numerous cell lines and tissue types *in vitro* (Uht *et al.* 1997, Kinyamu & Archer 2003). However, much less work has been done on the interaction between PR and other steroid receptors *in vivo*.

Although previous data suggest that P₄ might be important in lung physiology and diseases (e.g. asthma and cancer) (Milne 1979, Haggerty *et al.* 2003, Ishibashi *et al.* 2005), the regulation and cellular distribution of PR protein isoforms in lung remain largely unknown. Previous studies have shown that PR mRNAs and proteins are found in rabbit (Giannopoulos *et al.* 1982, Bullock *et al.* 1987, Nielsen *et al.* 1987, Camacho-Arroyo *et al.* 1994, 1998), rodent (Moser & Daxenbichler 1982, Beyer *et al.* 2003), and chicken (Pasanen *et al.* 1997) lungs. In the present study, we sought to initially establish the presence of PR isoforms in mouse lung using reverse transcriptase (RT)-PCR and Western blotting analyses and on finding that PRA mRNA and protein were specifically expressed in mouse lung, we examined the possibility of regulation of PRA protein expression in mice during postnatal growth and development. It is generally accepted that P₄'s actions on target tissues are mediated primarily by binding to its cognate nuclear receptors. Therefore, we examined the steroid hormone regulation of PRA protein expression in female mouse lung by different experimental approaches, including treatment with gonadotropins, P₄, dexamethasone, and PR and/or GR antagonists. Furthermore, we investigated whether under *in vivo*

conditions, PRA physically interacted with GR in mouse lung by using immunoprecipitation followed by Western blot and double-immunofluorescent assays.

Materials and Methods

Hormones and chemicals

Equine gonadotropin (eCG), dexamethasone, monoclonal anti- β -actin, and alkaline phosphatase-conjugated goat-anti-mouse immunoglobulin were obtained from Sigma. Human chorionic gonadotropin (hCG) and the specific PR-antagonist Org 31710 (Kloosterboer *et al.* 1994) were provided by N V Organon (Oss, Holland). The PR/GR antagonist RU486 (Cadepond *et al.* 1997) was provided by Exelgyn (Paris, France). Rabbit polyclonal anti-PR (c-19 and c-20) and their respective blocking peptides (c-19p and c-20p), as well as mouse monoclonal anti-GR were obtained from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). Mouse monoclonal anti-PRB (Joensuu *et al.* 1990) was obtained from Affinity BioReagents, Inc. (ABR, Golden, CO, USA). Mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) was obtained from Novocastra Laboratories Ltd (Newcastle upon Tyne, UK). Normal rabbit serum and normal goat serum were obtained from DAKO Corp. (Carpinteria, CA, USA). Alkaline phosphatase conjugated goat-anti-rabbit immunoglobulin was obtained from Tropix (Bedford, MA, USA). Biotin-SP-conjugated donkey anti-rabbit IgG, fluorescein (DTAF)-conjugated streptavidin, and Cy 3-conjugated donkey anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). All other chemicals were purchased from Merck, unless otherwise specified.

Animals and experimental design

Timed pregnant female mice (C57BL/6) were purchased from Taconic M&B, Copenhagen, Denmark. They were monitored daily between 0900 and 0930 h for delivery of pups and the day of birth was defined as postnatal day (PND) 0. Pups were sexed according to ano-genital distance and male pups were removed. All animals were housed and acclimatized to controlled temperature ($21 \pm 2^\circ\text{C}$) and with a ratio of 12 h light:12 h darkness cycles, with the standard rodent chow and tap water *ad libitum*. The animal studies were reviewed and approved by the ethics committee at Göteborg University, Sweden. At least five animals in each group were used for every experimental approach unless otherwise specified.

Experiment 1 This experiment was designed to determine the presence of the two PR isoforms (PRA and PRB) in mouse lung. Mice were killed following deep anesthetization with pentobarbitone sodium and selected whole tissues (lung, liver, fallopian tube, and uterus) were carefully removed and

lung tissue was frozen in liquid nitrogen for subsequent Western blot or RT-PCR analysis.

Experiment 2 This experiment was designed to examine the regulation and cellular localization of PRA protein expression in female mouse lung during postnatal growth and development. Female mice were deeply anesthetized with pentobarbitone sodium at various time intervals (PND 2, 7, 12, 26, and 90) between 0800 and 1000 h, whole lung tissue isolated from three mice at each time point were quickly dissected and either frozen in liquid nitrogen or stored at -135°C , or fixed in 4% formaldehyde in neutral buffer (Sigma) for 24 h at 4°C and embedded in paraffin wax for either Western blot or immunohistochemical analysis.

Experiment 3 This experiment was designed to examine the effects of endogenous or exogenous P_4 on regulation of PRA protein expression in female mouse lung. In our experiments, sequential administration of eCG and hCG is substituted for effects of follicle-stimulating hormone and luteinizing hormones *in vivo*. This *in vivo* model has been used for induction of ovarian P_4 production and further elevates serum P_4 levels in female mice *in vivo* (Shao *et al.* 2003, 2006). Female mice (PND 26) were given a single injection of eCG (5 IU, i.p.) followed by treatment with hCG (5 IU i.p.) after 48 h. Six hours after hCG treatment, mice were killed by approved methods and tissues were dissected. To determine the specific effect of P_4 on regulation of PR protein expression, female mice (PND 26) were randomly selected and received a single i.p. injection of P_4 (4 mg/kg per body weight in 100 μl sesame oil). A single pharmacological dose of P_4 was chosen on the basis of previous studies (Russo *et al.* 2001, Shao *et al.* 2006). Lung tissue was obtained at different time intervals (6, 24, 36, 48, and 72 h) for Western blot analysis.

Experiment 4 This experiment was designed to examine the effects of PR/GR antagonist RU 486 and PR antagonist Org 31710 on the regulation of PRA and GR protein expression in female mouse lung. Thirty female mice (PND 26), chosen at random, received eCG (5 IU, i.p.). The animals were then randomly divided into six groups of five mice for each group. Mice in the first two groups were given either 2 mg of RU 486 or 1 mg Org 31710 in 100 μl sesame oil by i.p. injection after treatment with eCG for 44 h. Mice in the remaining groups were injected with either 100 μl sesame oil (two groups, as controls), 2 mg RU 486 or 1 mg Org 31710 dissolved in 100 μl sesame oil after treatment with eCG for 48 h. All mice in the three groups received hCG (5 IU, i.p.) at 48 h after eCG treatment. Six hours after hCG treatment, the mice were killed by approved methods. Lung tissue was frozen in liquid nitrogen for subsequent Western blot analysis.

Experiment 5 This experiment was designed to study the effect of glucocorticoids on the regulation of PRA and GR expression in female mouse lung. Female mice (PND 26) were treated with gonadotropins and/or dexamethasone,

as described in Experiments 3 and 4. Animals were given a single i.p. injection of dexamethasone (600 $\mu\text{g}/\text{kg}$ per body weight) dissolved in saline, chosen on the basis of a previous study (Russo *et al.* 2001). Mice were killed by approved methods, lung tissues removed and frozen in liquid nitrogen for Western blot analysis.

RT-PCR

Total RNA was isolated from individual tissues using Trizol Reagent (Life Technologies) according to the manufacturer's instructions. The quantity of extracted RNA was determined by spectrophotometry. Any contaminating genomic DNA was digested by RNase-free DNase. cDNA was reversely transcribed from 5 μg total RNA using 0.25 μg random hexamer (Life Technologies) in 20 μl of a 1 $\times$ AMV-RT buffer containing 9 U AMV-RT (Invitrogen), 39 U ribonuclease inhibitor (RNasin) (Promega), and 4 μl 5 mM deoxyNTP (dNTP) mixtures (Amersham). Reactions were incubated for 1 h at 42°C , heated to 95°C for 5 min and then chilled on ice. An aliquot (5 μl) of RT product was used for PCR amplification in a total volume of 50 μl PCR-buffer presented with 2.5 U Taq-DNA polymerase (Promega), 2 μl 5 mM dNTP mixtures and the appropriate primer pairs as mentioned below. The PCR consisted of a denaturing step at 94°C for 5 min. The thermal cycle profile used in this study was (1) denaturing at 94°C for 30 s, (2) annealing the primers at 55°C for 30 s, (3) extending the primers at 72°C for 2 min. After the final amplification, the reaction was elongated for an additional 7 min at 72°C . PRA/B and PRB were amplified after 30 or 39 cycles respectively. The amount of cDNA and the number of PCR cycles needed to detect PR were determined in a preliminary experiment. A portion (10 μl) of the PCR mixture was electrophoresed in 1.2% agarose gel along with a 100 bp DNA ladder (Life Technologies) and visualized by ethidium bromide staining. Specificity of amplified products was confirmed by including RT control reactions. Ribosomal protein L19 was selected as an internal control to confirm that cDNA had been synthesized and to check for genomic DNA contamination (Chan *et al.* 1987). PCR was performed using primers specific for mouse PRA/B (sense 5'-GGCAAATCCCACAGGAGTTTG-3', antisense 5'-AGACATCATTTCCGGAAATTC-3'; expected PCR-amplified product size, 327 bp) (Beyer *et al.* 2002); mouse PRB (sense 5'-ATGACTGAGCTGCAGGCAAAG-3', antisense 5'-CTTCTACCCCAGAGAAAGCGC-3'; expected PCR-amplified product size, 277 bp) (Beyer *et al.* 2002); ribosomal protein L19 (sense 5'-GAAATCGCCAATGCCAACTC-3', antisense 5'-ACCTTCAGGTACAGGCTGTG-3', 290 bp) (Robker *et al.* 2000).

Western blot and co-immunoprecipitation analyses

Protein extraction and Western blotting were performed according to standard methods as described previously in Shao *et al.* (2003). Equal amounts of protein in the cell extracts were pretreated with 4 $\times$ SDS before loading and

resolved by 4–12% SDS-polyacrylamide gels (Novex, San Diego, CA, USA) under reducing conditions. The separated samples were electrotransferred to polyvinylidene difluoride membranes (Amersham) and treated with blocking buffer (0.2% I-Block, 0.2% BSA, 5 mM MgCl₂, 3 mM NaN₃, and 0.3% Tween 20 in PBS, pH 7.4) for 2 h. The antibodies used to detect PR in this study were raised against human PRs and recognized ligand-binding domain (PR c-19) or DNA-binding domain (PR c-20) of PR in rodent tissues by Western blotting and immunohistochemical analyses. The membranes were incubated with primary antibody for either anti-PRA/B (c-19 + c-20, 1:250), anti-PRB (1:50), anti-PCNA (1:1000), anti- β -actin (1:1000), or anti-GR (1:500) in blocking buffer overnight at 4 °C. After washing in blocking buffer for 2 h, the membranes were incubated with alkaline phosphatase-linked secondary antibody (polyclonal secondary antibody at 1:40 000 or monoclonal secondary antibody at 1:80 000 dilutions respectively) in blocking buffer for 2 h with gentle shaking and detected using CDP-Star as substrate (Tropix, Bedford, MA, USA). Immunoblotted signals were exposed, developed on ECL-film (Amersham) and subsequently scanned into a computer. Individual bands were quantified directly from membranes by densitometry using ImageQuant (version 5.0) software program (Molecular Dynamics, Inc., Sunnyvale, CA, USA). Equal protein loading was determined by the expression of β -actin, as well as confirmed by staining the gels with Coomassie blue after the transfer. As a negative control, the two PR antibodies were pre-absorbed with tenfold excess of neutralizing synthetic PR peptides for 4 h at room temperature before use to demonstrate antigen specificity.

For co-immunoprecipitation experiments, tissues were extracted with ice-cold lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1% SDS, 200 μ M sodium deoxycholate, 1 mM dithiothreitol (DTT), 5 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM *N*-ethylmaleimide, 10 mM iodoacetamide, and a cocktail of protease inhibitors (Roche). Either anti-PR or anti-GR antibodies were added to 500 μ g protein extracts and incubated for 4 h at room temperature. Immune complexes were obtained by the addition of 50 μ l Pansorbin cells (Calbiochem, San Diego, CA, USA). The resulting immobilized immune complexes were washed in RIRA buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 15 mM MgCl₂, 0.5% Nonidet P-40, 0.3% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 1 mM DTT, and a cocktail of protease inhibitors). The bound protein was eluted by boiling in 30 μ l SDS sample reducing loading buffer (Novex) for 5 min. Immunoprecipitated complexes for either GR or PRA/B were loaded in the same gels, which were run at the same time within the same electrophoresis unit and examined by immunoblotting as described above.

To re-probe the blot with another antibody, the blot was re-hydrated in methanol, rinsed and incubated with stripping buffer (62.5 mM Tris-HCl, 2% SDS and 100 mM β -mercaptoethanol, pH 6.8) at 50 °C for 30 min.

Immunohistochemistry and microscopy

PR immunolabeling was undertaken essentially as described previously (Shao *et al.* 2003, 2006). Paraffin-embedded lung sections were deparaffinized in xylene and rehydrated through graded series of ethanol and water. Antigens were retrieved by 10 mM sodium citrate buffer. After three washes with Tris-buffered saline (TBS 50 mM Tris, 0.9% NaCl, pH 7.5), endogenous peroxidase activity was abolished by 3% hydrogen peroxide in TBS and non-specific binding was blocked by incubating sections in 10% normal goat serum. Sections were incubated with primary antibodies (anti-PRA/B (c-19 + c-20) at 1:100, and anti-PRB at 1:50 dilutions respectively) in TBS containing 1% BSA overnight at 4 °C in a humidified chamber. After a series of washing with TBS, sections were then stained using the avidin-biotinylated-peroxidase complex detection system (ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's instructions. Immunostaining was then visualized using 3, 3'-diaminobenzidine tetrahydrochloride (0.5 mg/ml in PBS and 0.01% H₂O₂, pH 7.6) for 10 min. Slides were viewed on Nikon E-1000 microscope (Bergström Instruments AB, Stockholm, Sweden) under brightfield optics, and photomicrographed using Easy Image 1 (Bergström Instrument AB, Göteborg, Sweden). To determine the specificity for detection of PR, the primary antibodies (c-19 + c-20, 1:250 each) were pre-incubated with tenfold excess of neutralizing synthetic PR peptides (c-19 p + c-20 p) for 4 h at room temperature. These pre-absorbed antibodies were substituted for primary antibodies, while adjacent sections were incubated with PR primary antibodies alone. Serial sections of lung were examined blind under light microscopy.

Double-immunofluorescent staining of PR and GR was performed according to a previously described procedure (Shao *et al.* 2004). Sections were incubated with a mixture of antibodies against PR and GR diluted to 1:100 and 1:150 respectively, in PBS containing 1% BSA and 5% fat-free milk. Each primary antibody was serially diluted to optimize the concentration used to achieve maximum sensitivity and specificity. Immunodetection was accomplished using the species-specific secondary antibodies, either a biotin-conjugated anti-rabbit antibody together with streptavidin conjugated with DTA-FITC or Cy 3-conjugated anti-mouse antibody. Sections were washed and mounted with Fluorescent Mounting Media (DAKO). Slides were viewed on an Axiocvert 200 microscope (Zeiss, Jena, Germany) equipped with a laser-scanning confocal imaging LSM 510 META system (Carl Zeiss, Jena, Germany) and photomicrographed. The resulting stain was evaluated blind by three observers. The immunohistochemical figures illustrated are representative of those observed in numerous sections from multiple animals.

Serum P₄ assay

Serum P₄ concentration was measured by RIA according to a protocol provided by the manufacturer (Perkin-Elmer Life

and Analytical Sciences, Wallac Oy, Turku, Finland) (Shao *et al.* 2006). Trunk blood was obtained from cardiac puncture in immature mice treated with or without gonadotropins, PR antagonists, and dexamethasone. All samples were tested as duplicates together in one run. The sensitivity of the assay was typically better than 0.8 nmol/l, and the intra-assay coefficient of variation was 3.3–7.3%.

Statistical analysis

Results are presented as the mean $\pm$ S.E.M. Statistical analysis was performed by one-way ANOVA using Analyse-It program (Analyse-It Software, Ltd, Leeds, UK). Significant differences between the treatment groups were determined by the *post hoc* Tukey's test. Effects were considered to be significant if *P* value was less than 0.05.

Results

PRA is the predominant PR in mouse lung

PRA/B mRNA was detected in female mouse lung at PND 7 and 26, whereas PRB transcripts in the same lung tissues

were absent (Fig. 1A). In parallel with PCR analysis, we used the same selected tissues for Western blot analysis to demonstrate PR protein isoform expression, with only PRA protein expression found in mouse lung, at PND 7 and 26 (Fig. 1B). Male mice at PND 26 also displayed similar levels of PRA protein expression in lung (data not shown). Furthermore, we used a specific PRB antibody to confirm this result under PRA/B antibodies, and no PRB immunoreactive band was detected in all lung tissues examined, while expression of PRB was observed in mouse fallopian tube and uterus (Fig. 1C) (Shao *et al.* 2006).

Developmental regulation of PRA protein expression in female mouse lung

To determine whether PRA protein levels are regulated during postnatal development in female mouse lung, we examined PRA protein expression by Western blot analysis. PRA immunoreactive protein was detected in all lung tissue extracts isolated from female mice of different ages. Levels of PRA protein increased with age, peaking at PND 7, although significantly increased levels of PRA protein were also

Figure 1 Expression of PR isoforms in mouse lung. (A) Total RNA from the indicated female mouse lungs was reversely transcribed and amplified by PCR with primers specific for mouse PRA and PRB as well as L-19 (as described in Materials and Methods). Amplified products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. A mouse uterus-positive control and a mouse liver-negative control were also included. Similar analysis with two different RNA preparations from different animal tissues ($n=3$ each). This figure is representative of two separate experiments in which same results were obtained. (B) and (C) Protein samples were isolated from mouse lungs and selected tissues ($n=3$ each). The equal protein (50 μ g/lane) underwent Western blot analysis with either anti-PRA/B (B) or anti-PRB antibody as described in Materials and Methods. The immunoblots are representative of duplicate blots of two independent experiments from both female and male mice. The molecular mass markers (kDa) are indicated on the left. X denotes protein band that cross-reacted with neutralizing synthetic PR peptide-PR antibodies. PND, postnatal days.

detected at PND 12. PRA protein levels declined by PND 26 and were barely detectable in adults (PND 90) compared to PND 2 (Fig. 2A). There is rapid cell proliferation during early- and mid-gestational lung organogenesis (Burri 1997,

Kaplan 2000). At PNDs 2–4, the number of proliferating cells substantially increases to support postnatal growth in mouse lung (Thaete *et al.* 1989). The PCNA synthesis correlates with the proliferative state of the cell and it represents a key

Figure 2 Regulation and cellular localization of PRA protein expression in female mouse lung during postnatal development. (A) Lung tissues from different age female mice were subjected to quantitative Western blot analysis as described in Materials and Methods. Total protein was prepared and analyzed for PR protein isoform and PCNA expression together with the negative control using mouse liver and the positive controls using mouse uterus and fallopian tube. The expression of β-actin was used as an internal standard to verify equal loading of proteins in all experiments. Five sets of densitometric values obtained from three independent experiments with separate mice for each experiment (five mice in each time point) were normalized by β-actin and presented as percent of mean ± S.E.M. relative to PRA protein levels in PND 2 animals. **P* < 0.05; †*P* < 0.01. PND, postnatal days. (B) Standard curve showing a linear relationship between the immunoreactive band intensity and the amount of whole-lung extract protein loaded for mice at PND 7. The equation of the line of best fit was generated by least squares linear regression analysis. (C) Lung tissue sections from 7-day-old female mice stained with PRA/B antibody (I and II) as described in Materials and Methods. No immunostaining of the PRA/B was detected in an adjacent lung section (III) when the synthetic PR blocking peptide was pre-absorbed with excess antigen, confirming the specificity of the PR antibody. Random sections from the lung of three animals provided the same results. Alveolar and bronchial epitheliums are indicated by arrowhead and arrow respectively. All original magnifications: ×100.

gene necessary for the transition of cells from quiescence to S phase (Tsurimoto 1999). PCNA was used previously as a reliable marker protein for cell proliferation in mouse lung (Thaete *et al.* 1989). We demonstrated that the expression pattern of PCNA protein was similar to that of PRA protein in female mice in a parallel experiment. Next, we determined the cellular localization of PR in a 7-day-old mouse lung by immunohistochemical analysis using a mixture of two different polyclonal PRA/B antibodies (c-19+c-20). Positive immunostaining for PR was observed in the alveolar and bronchial epitheliums (Fig. 2C I–II). Comparison of immunostaining between adjacent sections was used to confirm immunoreactive specificity. In adjacent sections of lung stained with either PRA/B antibody and blocking peptides (Fig. 2C III), or PRB antibody (data not shown), no positive signals were detected. Sections from human breast carcinoma as positive controls (data not shown), were run in parallel to reduce discrepancies related to inter-assay variability in immunostaining intensity.

Neither endogenous nor exogenous P₄ regulates PRA protein expression in female mouse lung

Sequential treatment with eCG/hCG to 26-day-old female mice results in induction of ovarian P₄ production and further elevates serum P₄ levels *in vivo* (Shao *et al.* 2003, 2006). To determine whether PRA protein expression is regulated by P₄, 26-day-old peripubertal female mice were injected with either eCG/hCG or P₄. Western blot analysis demonstrated that there was no concomitant change of PRA protein expression in lung (Fig. 3A) when endogenous serum P₄ levels were increased (Shao *et al.* 2006). After treatment with exogenous P₄, no significant changes in serum estradiol concentration were apparent (data not shown), whereas a rapid but transient increase in serum P₄ concentration was detected at 6 h followed by a decrease to basal level within 24 h after P₄ treatment (Fig. 3C). Moreover, time-course studies showed that in 26-day-old female mice, treatment with P₄ did not affect PRA protein expression in lung (Fig. 3B). Furthermore, time-course studies also showed that treatment with exogenous diethylstilbestrol (20 µg/kg per body weight, a non-steroidal synthetic estrogen) (Savu *et al.* 1979) or dihydrotestosterone (1 mg/kg per body weight, a more biologically active androgen) (Gray *et al.* 1995) did not change PRA protein expression in female mouse lung (data not shown).

Effect of PR antagonist treatment on PRA and GR protein expression in female mouse lung

PR antagonists regulate expression of PR protein isoforms in female reproductive tissues by blocking the actions of P₄ *in vivo* (Donath *et al.* 2000, Shao *et al.* 2003, 2006). To determine the effects of PR antagonists on PRA and GR protein expression in mouse lung, 26-day-old female mice, under a gonadotropin regimen, were treated with either RU 486, a PR/GR antagonist, or Org 31710, a specific PR

Figure 3 Time-course effects of endogenous and exogenous progesterone (P₄) on PRA protein expression in female mouse lung. Fresh lungs from 26-day-old female mice treated with eCG and/or (A) hCG or (B) P₄ with the indicated times were collected. Protein samples were prepared and subjected to Western blot analysis for detection of PRA protein expression as described in Materials and Methods. The expression of β-actin was used as an internal standard to verify equal loading of proteins in all experiments (three mice in each experimental group). Corresponding serum P₄ levels in trunk blood are shown in (C). The number of mice per group is indicated. Serum P₄ concentration was measured in duplicate aliquots of each sample. Values represent the mean ± S.E.M. *significantly different from untreated animals (time, 0 h), $P < 0.001$.

antagonist (Kloosterboer *et al.* 1994). Western blot analysis showed that treatment with RU 486 increased PRA protein expression (Fig. 4A), in parallel with a decrease in GR protein expression (Fig. 4C), without affecting serum P₄ concentration (Fig. 4E). However, administration of Org 31710 did not affect either PRA or GR protein expression in the lung (Fig. 4B and D).

Effect of exogenous glucocorticoid dexamethasone treatment on PRA protein expression in female mouse lung

To determine the effects of glucocorticoids on PRA and GR protein expression in mouse lung, 26-day-old female mice were either vehicle treated or treated with dexamethasone. Western blot analysis indicated that GR protein expression was increased at 6 h after dexamethasone treatment (Fig. 5A), in association with a decrease in serum P₄ concentration (Fig. 5C). Furthermore, dexamethasone treatment in female mice, undergoing a gonadotropin regimen, decreased PRA

Figure 5 Effect of dexamethasone (Dex) on GR and PRA protein expression in female mouse lung. Twenty-six-day-old female mice treated with Dex for 6 h without gonadotropin treatment (A) or for 10 and 6 h, given either 4 h before hCG or at the same time combined with hCG after 48 h eCG treatment (B). After lung tissues were isolated, protein samples were prepared and subjected to Western blot analysis for detection of GR (A) and PRA (B) protein expression as described in Materials and Methods. The expression of β -actin was used as an internal standard to verify equal loading of proteins in all experiments. All densitometric values for quantitative analysis of PRA protein levels obtained from the individual female mouse lung (five mice in each experimental group) were normalized in relation to β -actin and presented as percent of mean $\pm$ S.E.M. relative to GR or PRA protein levels in mice treated with eCG/hCG only. * $P<0.01$. Corresponding serum P_4 levels in trunk blood are shown in (C). The number of mice per group is indicated. Serum P_4 concentration was measured in duplicate aliquots of each sample. Values represent the means $\pm$ S.E.M. $^\dagger P<0.001$.

Figure 4 Effects of RU 486 and Org 31710 on PRA and GR protein expression in female mouse lung. Twenty-six-day-old female mice treated with RU 486 (A) and (C) or Org 31710 (B) and (D) for 10 and 6 h, given either 4 h before hCG or at the same time combined with hCG after 48 h eCG treatment. After lung tissues were isolated, protein samples were prepared and subjected to Western blot analysis for detection of PRA (A) and (B) and GR (C) and (D) protein expression as described in Materials and Methods. Mouse uterus and liver were used for the positive and negative controls for PRA/B protein expression. The expression of β -actin was used as an internal standard to verify equal loading of proteins in all experiments. All densitometric values for quantitative analysis of PRA protein levels obtained from the individual female mouse lung (five mice in each experimental group) were normalized by β -actin and presented as percent of mean $\pm$ S.E.M. relative to PRA or GR protein levels in mice treated with eCG/hCG only. * $P<0.05$; $^\dagger P<0.01$; $^\ddagger P<0.001$. Corresponding serum P_4 levels in trunk blood are shown in (E). The number of mice per group is indicated. Serum P_4 concentration was measured in duplicate aliquots of each sample. Values represent means $\pm$ S.E.M. N.D., no significant difference.

protein expression (Fig. 5B), depending on injection time. However, dexamethasone treatment decreased serum P_4 concentration regardless of injection time (6 and 10 h) (Fig. 5C). Our findings demonstrate that dexamethasone-induced suppression of PRA protein expression is independent of circulating P_4 concentration.

PRA physically interacts with GR in mouse lung

To investigate whether PRA could interact with GR, a co-immunoprecipitation analysis was performed in female mouse lung. Lung tissue extracts from 7- to 26-day-old female mice were subjected to immunoprecipitation with either: anti-GR, anti-PRA/B antibody, anti-PRA/B antibody with specific blocking peptides or anti-PRB antibody. Immunoprecipitates were separated by denaturing electrophoresis and analyzed by Western blot with either the anti-PRA/B (Fig. 6A, left) or anti-GR antibody (Fig. 6A, right). In both the anti-GR and anti-PRA/B precipitates of lung tissue proteins from female mice of different ages, immunoreactive bands for either PRA or GR were revealed. No GR-immunoreactive band was found in immunoprecipitates from protein samples with either anti-PRA/B antibody with specific blocking peptides or anti-PRB antibody (Fig. 6A, right). To further examine the interaction of PRA and GR *in vivo*, we performed a double-immunofluorescent analysis on lung tissue sections from 26-day-old mice. The majority of PR-positive nuclei co-localized with GR in alveolar epithelia cells, whereas nuclear PR and cytoplasmic GR were detected in bronchial epithelium in both female (Fig. 6B) and male (data not shown) mice.

Discussion

The molecular basis for the regulation of PR protein isoforms is of great importance for understanding their physiological functions in both reproductive (Shao *et al.* 2003, 2006) and non-reproductive tissues (Graham & Clarke 1997). Profiling PR isoform expression and determining their cellular localization in mouse lung would help us in delineating the role of P_4 in lung physiology. In this study, we present a detailed demonstration of developmental and hormonal regulation of PRA protein expression in female mouse lung *in vivo*. More specifically, our results strongly suggest that regulation of PRA protein expression is mediated by the actions of GR in response to glucocorticoids.

The presence of PR in lungs of different species (Giannopoulos *et al.* 1982, Moser & Daxenbichler 1982, Bullock *et al.* 1987, Nielsen *et al.* 1987, Camacho-Arroyo *et al.* 1994, 1998, Pasanen *et al.* 1997, Beyer *et al.* 2003), is not surprising. However, our experiments do not provide evidence for the existence of PRB in mouse lung. The results, rather, are consistent with the existence of PRA only. First, we obtained the same result when probing blots with different primary PR antibodies in different tissues. Secondly,

in order to determine sufficient extraction of receptor from tissue preparation for Western blot procedure, we have used different internal controls (PCNA, β -actin, and GR). Thirdly, using different molecular techniques, including PCR, Western blot, and co-immunoprecipitation analyses, we confirmed the lack of PRB in mouse lung under very stringent conditions. Uotinen *et al.* (1999) previously failed to detect PR protein expression by immunohistochemistry in the female adult mice lung. This difference may be explained by the use of different antibodies, experimental techniques, and observation times. Although a specific physiological role for PRA in lung remains to be defined, its identification has provided a potential explanation for the biological actions of P_4 in cells where no immunoreactive PRB could be detected.

Considerable evidence has accumulated to indicate that many transcription factors are involved in lung development (Korfhagen & Whitsett 1997). To further assess the role of PRA as a transcription factor involved in normal lung physiology, we are the first to show in a quantitative manner that the expression of PRA protein in lung is regulated in an age-dependent manner in female mice during postnatal growth and development. At present, the possible function of PRA in lung is not clear. In the first and second weeks of postnatal development, physiological changes take place in the mouse lung, i.e. termination of sac ends on PND 5, alveoli start to form on PND 5 and mature until PND 30 (Burri 1997, Kaplan 2000). Of note, the levels of PRA protein increase and peak at PND 7 when rapid formation of alveoli occurs, whereas decline of PRA protein expression is positively correlated to the postnatal termination of lung development. Moreover, glucocorticoids as a physiological regulator have been implicated in the regulation of postnatal alveolar formation in rodent lung (Burri 1997, Kaplan 2000). In female mice, endogenous serum corticosterone concentrations are very low on PND 5 until PND 15 and significantly increase on PND 25, remaining high during postnatal life compared to that on PND 5 (Morale *et al.* 1995). In line with previous data, the current study shows that expression of PRA protein is negatively correlated with endogenous serum corticosterone concentrations (Morale *et al.* 1995). We also show that treatment with dexamethasone, a synthetic glucocorticoid, decreases PRA protein expression in 26-day-old female mice lung, suggesting that glucocorticoids may be involved in the regulation of PRA in mouse lung. Taken together, these observations indicate that PRA may be a significant physiological factor involved in lung postnatal development. On the other hand, the biological effects of PRA expression in lung could be questioned, because it has been reported that mice, lacking either PRA/B or PRA are viable, although exhibiting abnormalities in reproductive systems and function (Curtis & Korach 2000, Mulac-Jericevic *et al.* 2000). However, this does not necessarily imply that the respiratory system of these mice is unaffected. Dimerization of the PR isoforms has a potential role as a regulatory step to determine the biochemical properties and biological activity of PRA and

Figure 6 Endogenous PRA physically interacted with GR in mouse lung. (A) Cellular extracts of lung tissues from 7- to 26-day-old female mice were immunoprecipitated with antibodies as indicated. Subsequently, immunoprecipitates were analyzed by Western blot analysis with either anti-PRA/B (left) or anti-GR (right) antibody as described in Materials and Methods. Arrows indicate the presence of PRA and GR protein expression. Running position of molecular weight markers is indicated in kDa on the left. IP, immunoprecipitation; PND, postnatal days; BP, neutralizing synthetic PR peptide. (B) Double-label fluorescence immunohistochemistry for PRA/B and GR in lung from 26-day-old female mice. On the left, PR-immunoreactive nuclei are shown in red (I and IV); in the center panels, GR immunoreactivity is shown in green (II and V) and double exposures are shown on the right (III and VI), demonstrating substantial double labeling of both nuclear PR and GR in alveolar epithelium (III), whereas nuclear PR and cytoplasmic GR in bronchial epithelium (VI). Immunostaining is representative of two different experiments with lung tissues obtained from 26-day-old female and male mice. All original magnifications: $\times 40$.

PRB (Mohamed *et al.* 1994, McDonnell 1995). Since PRA cannot only dimerize to PRB, but also homodimerize to modulate target cell responsiveness to hormones (Leonhardt *et al.* 1998) and RU 486 (Mohamed *et al.* 1994), it is possible that PRA plays a transient role in mouse lung development during the early postnatal period. Future studies will be needed to address the effects of PRA on cell differentiation and function in lung during postnatal development.

Previously, we have shown that PR protein isoform expression in female reproductive tissues including ovary, fallopian tube, and uterus is regulated by gonadotropin treatment that increase endogenous P_4 concentration, as well as acute treatment with P_4 in female mice (Shao *et al.* 2003, 2006). The current study demonstrates that neither endogenous nor exogenous P_4 regulate PRA protein expression in lung using the same *in vivo* experimental model. In addition, a time-course study of both RU 486 and dexamethasone treatment supports the idea that changes in PRA protein expression are independent of circulating P_4 concentration in female mouse lung. To our knowledge, this is the first report that demonstrates a functional dissimilarity of P_4 and glucocorticoids in the regulation of PRA protein expression in female mouse lung *in vivo*. Although previous studies have reported that the pure antagonist effect of RU 486 is mediated by the PRA signaling pathway within target cells *in vitro* (McDonnell & Goldman 1994, Hurd *et al.* 1999), RU 486 can interact with both PR and GR (Guettari *et al.* 1990, McDonnell & Goldman 1994, Cadepond *et al.* 1997). We therefore used Org 31710, a specific PR antagonist (Kloosterboer *et al.* 1994), to distinguish the effects of RU 486 on PRA from the effects on GR. There is no change in PRA protein expression after Org 31710 treatment, suggesting that RU 486 binding to GR regulates PRA protein expression in mouse lung. Furthermore, GR appears to overlap PR cellular distribution in lung (Camacho-Arroyo *et al.* 1994, Adcock *et al.* 1996), interacts with estrogen receptors (ER) (Kinyamu & Archer 2003), and PR has been shown to interact with ER (Uht *et al.* 1997), suggesting a possibility of interaction between PRA and GR *in vivo*. The amount of PRA/GR complex detected in PRA and GR immunoprecipitates in this study suggests that PRA and GR are able to crosstalk with each other in response to glucocorticoids through protein–protein interaction *in vivo*. Interestingly, cellular localization patterns of PR and GR are diverse. Unliganded PR is predominantly nuclear (Smith *et al.* 1990) whereas unliganded GR resides entirely in the cytoplasm (Hache *et al.* 1999). Lung is composed of multiple cell types (Amy *et al.* 1977, Burri 1997) and our immunohistochemistry results show that in the presence of low levels of endogenous corticosterone (Ferrandez & De la Fuente 1999), PRA is localized predominantly in the nucleus of both alveolar and bronchial epithelia, whereas GR is distributed in either the nucleus of alveolar epithelium or cytoplasm of bronchial epithelium, suggesting that interaction between PRA and GR may differ in a cell type-specific manner. Additional studies will determine whether the

interactions of PRA and GR are ligand-dependent using a cell fractionation procedure. Evaluating the significance of the interactions described here requires identification of targets of GR signal activated by glucocorticoids in mouse lung.

Clinically, corticosteroids are the most potent and effective therapy for treatment of chronic inflammatory diseases such as asthma, and they are generally believed to exert their therapeutic benefits by specifically binding to GR, which on activation translocates to the nucleus and either increases or decreases expression of responsive genes and also suppresses inflammation (Adcock *et al.* 1996, Umland *et al.* 2002). It is known that receptor regulation is an important mechanism for modulating target cell responsiveness to hormones. As previously mentioned, several *in vitro* studies have shown that PRA functions as a strong inhibitor of other steroid receptors including GR (Vegeto *et al.* 1993, McDonnell *et al.* 1994). Since the cellular sensitivity to glucocorticoid is directly proportional to GR concentration and the anti-inflammatory activity of GR is mediated by protein–protein interaction (Reichardt *et al.* 2000), our results suggest that suppression of PRA protein expression by glucocorticoids may affect the regulation and/or modulation of GR levels or activity to enhance lung responsiveness to circulating glucocorticoids, i.e. activation of GR signaling.

In summary, the results of this study document the presence of PRA mRNA and protein in the female mouse lung and demonstrate that PRA protein is expressed and regulated in female mice during postnatal lung development. The relationship between PRA, PCNA protein expression, and alveoli maturation suggests that PRA may play a role in postnatal lung development. However, there was no direct relationship between circulating P_4 concentrations and PRA protein expression, suggesting that it is unlikely that P_4 is responsible for the regulation of PRA expression in female mouse lung. Moreover, we have shown that exposure to dexamethasone and RU 486, but not Org 31710, *in vivo* significantly changes PRA protein expression independently of circulating P_4 levels. Furthermore, the interaction and co-localization of PRA and GR at physiologically relevant levels depends on the lung cell type, suggesting that further study of such interactions may be relevant to our understanding of the mechanism of action and interaction between PRA and GR signaling pathway in the lung.

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