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Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and Prolactin Signaling: $PGF_{2\alpha}$-Mediated Inhibition of Prolactin Receptor Expression in the Corpus Luteum

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It is well established that prolactin (PRL) sustains, whereas prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) curtails, progesterone production by the rodent corpus luteum (CL). We have previously shown that $PGF_{2\alpha}$ inhibits the expression of several luteal genes stimulated by PRL, whereas it stimulates other genes inhibited by this hormone. We have also found that $PGF_{2\alpha}$ stimulation of 20α-hydroxysteroid dehydrogenase (20αHSD), an enzyme that catabolizes progesterone, at the end of pregnancy is accompanied by a dramatic decrease in PRL receptor (PRL-R) expression. These findings, and the fact that the factors that inhibit PRL-R are not known, led us to examine *in vivo* whether the decline in PRL-R at the end of pregnancy is due to $PGF_{2\alpha}$ and to also find out whether $PGF_{2\alpha}$ opposes PRL action by inhibiting PRL-R expression. Using the $PGF_{2\alpha}$ receptor ($PGF_{2\alpha}$-R) knockout, we examined whether the absence of the $PGF_{2\alpha}$-R prevents the decline in the expression of both the short and long forms of the PRL-R in the CL. We found that, in sharp contrast to the wild-type mice, in which both forms of the PRL-R decline to low levels between d 18–20 of pregnancy, expression of these receptors remained elevated in the $PGF_{2\alpha}$-R null mice. Furthermore, administration of $PGF_{2\alpha}$ to pregnant rats inhibited PRL-R expression. Time-course analysis revealed that $PGF_{2\alpha}$ treatment decreases both isoforms of PRL-R within 1 h of treatment *in vivo*, whereas its stimulatory effect on 20αHSD expression was further delayed. Similar results were obtained with luteinized granulosa cells in culture. To examine whether the decline in PRL-R is involved/necessary for $PGF_{2\alpha}$ action, cells were transfected with a constitutively active PRL-R. The expression of this receptor did not prevent $PGF_{2\alpha}$ effect on PRL-R or 20αHSD expression. Taken together, these results demonstrate that $PGF_{2\alpha}$ inhibits the expression of the PRL-R and that the decline in both forms of the PRL-R that occurs at the end of pregnancy in the CL is due to $PGF_{2\alpha}$. The results further suggest that $PGF_{2\alpha}$-mediated stimulation of 20αHSD is independent from $PGF_{2\alpha}$ inhibition of PRL signaling in luteal cell. (*Endocrinology* 144: 3301–3305, 2003)

THE PROLACTIN (PRL) RECEPTOR (PRL-R) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor ($PGF_{2\alpha}$-R) knockout mice have clearly established the importance of PRL and $PGF_{2\alpha}$ in the normal evolution of pregnancy. The main cause of infertility in the PRL-R null mice is a defect in the corpus luteum (CL) formation and absence of progesterone support for implantation and placental development (1). On the other hand, mice rendered deficient for the $PGF_{2\alpha}$-R gene do not show the normal prepartum drop in progesterone and consequently do not give birth (2). Luteal expression of 20α-hydroxysteroid dehydrogenase (20αHSD), an enzyme that catabolizes progesterone, is the best example of the contrasting effects of these two hormones on the regulation of luteal function. Whereas PRL completely inhibits 20αHSD expression (3), $PGF_{2\alpha}$ massively increases it (4). Several other luteal genes are regulated in an opposite manner by PRL and $PGF_{2\alpha}$ (5). We have demonstrated that the CL of $PGF_{2\alpha}$-R knockout mice fails to express 20αHSD at the end of pregnancy, resulting in sustained level of progesterone in the circulation (4). We have also found that the physiological increase in 20αHSD expression at the end of pregnancy is accompanied by a dramatic decrease in PRL-R expression (6). These findings, and the fact that the factors that inhibit PRL-R expression are not known, led us to examine whether the decline in PRL-R at the end of pregnancy is due to $PGF_{2\alpha}$ and to find out whether $PGF_{2\alpha}$ opposes PRL action by inhibiting PRL-R expression.

Abbreviations: CL, Corpus luteum; 20αHSD, 20α-hydroxysteroid dehydrogenase; LGC, luteinized granulosa cells; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; $PGF_{2\alpha}$-R, $PGF_{2\alpha}$ receptor; PRL, prolactin; PRLR, PRL receptor; PRL-R_{CA}, constitutively active PRL-R; Stat, signal transducers and activators of transcription.

Materials and Methods

Animals and cell culture

Pregnant Sprague Dawley rats, purchased from Sasco Animal Labs (Madison, WI), were housed at 24 C on a 14-h light, 10-h dark cycle and allowed free access to Purina rat chow and water. $PGF_{2\alpha}$-R knockout mice with a mixed genetic background of 129/Ola and C57BL/6 strains were used (2). Wild-type and $PGF_{2\alpha}$-R knockout mice were maintained at 23 C under a 12-h light cycle. Virgin females (9–12 wk of age) housed overnight with males were checked the following morning for vaginal plug. The day the plug was found was counted as d 1 of pregnancy. Animal care and handling conformed to the National Institutes of Health (NIH) guidelines for animal research. The experimental protocol was approved by the Institutional Animal Care and Use Committee. Luteinized granulosa cells (LGC) were isolated and cultured as previously described (7). Briefly, LGC from pregnant mare serum gonadotropin/human chorionic gonadotropin superovulated immature rat were culture in DMEM/F12 medium plus 1% fetal bovine serum. After 3 d of culture, fetal bovine serum in the medium was reduced to 0.1%, and the cells were treated or transfected as depicted in figure legends.

RNA isolation and RT-PCR analysis

Total RNA from rat CL or mouse ovary was isolated by using Tri-Reagent (Sigma, St. Louis, MO) following the manufacturer's instructions. For mRNA analysis by RT-PCR, 1 μg of total RNA was reverse-transcribed at 42 C by using Advantage RT-for-PCR kit (Promega, Madison, WI). The PCR mixture containing specific oligonucleotide primers (20 pmol), deoxynucleotide triphosphate (150 μM), and *ExTaq* DNA polymerase (0.8 U) was added to each tube containing 5 μl of reveres transcription product. Each PCR included primer for rat or mouse ribosomal protein L19 mRNA, used as internal control. Before proceeding with the semiquantitative PCR, the conditions were established such that the amplification of the products was in the exponential phase, and the assay was linear with respect to the amount of input RNA. After electrophoresis in agarose gel, data were analyzed using MDS 120 software (Kodak, Rochester, NY).

For the rat samples, PRL-R message was amplified by using a common sense primer targeted to the conserved extracellular domain present both PRL-R isoforms: ATACTGGAGTAGATGGAGCCAGGAGAGTTC. For the long form of PRL-R, the antisense primer used was CTTCCGTGAACAGAGTCACTGTCGGGATCT; and for the PRL-R short form, the primer used was CTATTTGAGTCTGCAGCTTCAGTAGTCA (8). The same approach was used for mouse PRL-R determination: common sense primer, ATACTGGAGTAGATGGGGCCAGGAGAAATC; the antisense long-form primer, CTTCCATGACCAGAGTCACTGTCAGGATCT; or the antisense short-form primer, ATATTTGAGTCTGCTGCTTCAGTAGTCAAG. When a coamplifica-

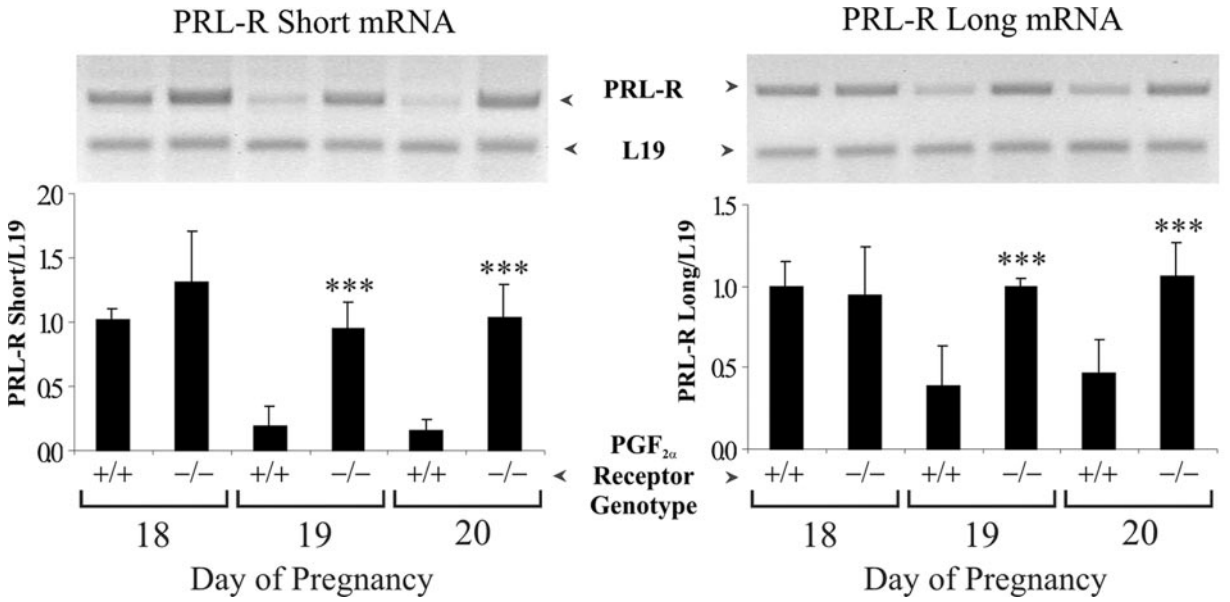

FIG. 1. PRL-R ovarian expression at the end of pregnancy in wild-type (+/+) and $PGF_{2\alpha}$-R knockout (−/−) mice. Total RNA was subjected to RT-PCR analysis using specific primers for mouse PRL-R and L19 as internal control. Values are expressed as the mean ± SEM (n = 3 animals). ***, $P < 0.001$ *vs.* +/+.

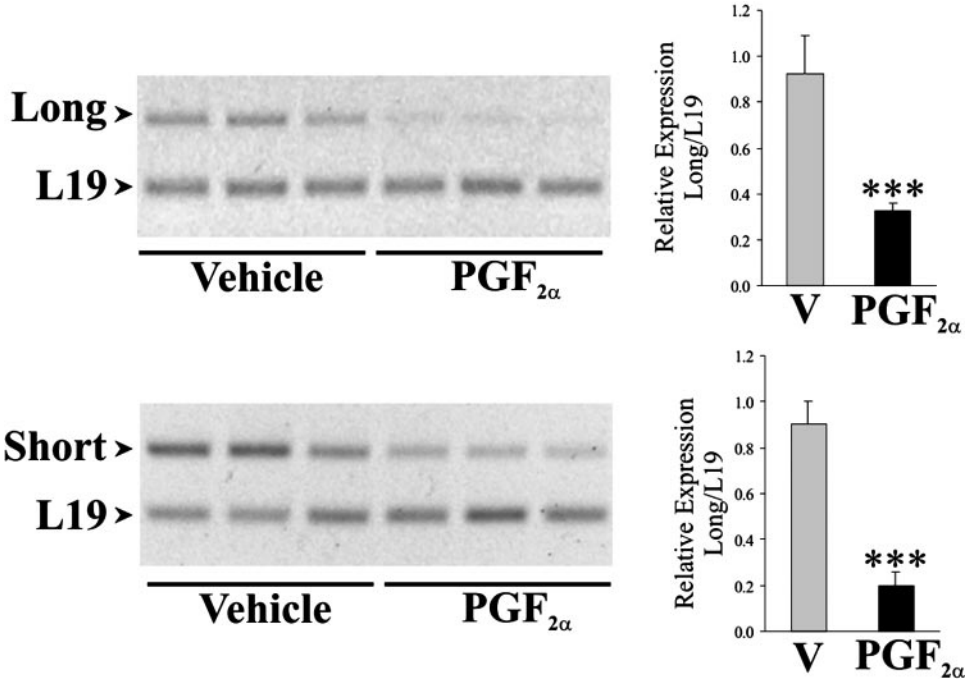

FIG. 2. Effect of $PGF_{2\alpha}$ administration on luteal expression of the long and short form of the PRL-R. Rats were injected with either 400 μg of $PGF_{2\alpha}$ ip or vehicle (V) on d 19 of pregnancy; luteal PRL-R long- and short-form mRNA levels were determined 24 h after. *Bars* represent the mean ± SEM (n = 3 animals). ***, $P < 0.001$ *vs.* vehicle (V).

tion of PRL-R short and long form and 20αHSD messages was performed, the following primers for 20αHSD were used, following a protocol previously described (4): TGTATCTCTGAGTTCCCAGG and ACTCTTCTAGGGAAGAGCAG. For the rat ribosomal protein L19, the primers used were GGACAGAGTCCAAGGGTCCGCTGCAGTC and TCCAAGGGTCCGTGCAGTC, which were included in the reaction to coamplify the message for PRL-Rs and the internal standard L19. For mouse ribosomal protein L19, the primers used were AGCGCCTCCAGGCCAAGAAGG and CCAGGCCGC TATGTACAGACACGA (4). The expected size of for each RT-PCR product was obtained. For all reactions, primers were used at a 0.6-μM concentration in a 50-μl final volume of reaction.

Statistical analysis

One-way ANOVA (ANOVA I) followed by the Tukey test was used for the statistical analysis of relative mRNA expression by using Prism software (GraphPad Software, Inc., San Diego, CA). Values were considered statistically significant at $P < 0.05$.

Results

PRL-R expression in $PGF_{2\alpha}$-R knockout mice at the end of pregnancy

To examine whether $PGF_{2\alpha}$ is responsible for the physiological drop in luteal PRL-R expression observed at the end of pregnancy in rodents, we examined the expression of this gene on d 18, 19, and 20 of pregnancy in wild-type and $PGF_{2\alpha}$-R knockout mice. PRL-R long and short mRNAs (Fig. 1) were equally expressed on d 18 in both CL of wild-type and $PGF_{2\alpha}$-R knockout mice. Similarly to the rat, in which the expression of both forms of the PRL-R drops 2 d before parturition (6), a dramatic decrease in these receptors was observed in wild-type mice on d 19 and 20. This decrease did not take place in CL of $PGF_{2\alpha}$-R-deficient mice, clearly establishing the participation of $PGF_{2\alpha}$ in the down-regulation of luteal PRL-R expression at the end of pregnancy.

In vivo administration of $PGF_{2\alpha}$ inhibits PRL-R expression in the CL

To further examine whether $PGF_{2\alpha}$ inhibits PRL-R expression, either $PGF_{2\alpha}$ (400 ug/rat, ip) or vehicle (saline solution) was administered to rats on d 19 of pregnancy, 2 d before the physiological decrease in the luteal expression of this gene (6). Twenty-four hours later (d 20 of pregnancy), luteal RNA was isolated and used to determine PRL-R mRNA expression level by semiquantitative RT-PCR. As shown in Fig. 2, $PGF_{2\alpha}$ caused a marked inhibition of both PRL-R long and short forms.

Time-dependent effect of $PGF_{2\alpha}$ on luteal PRL-R and 20αHSD expression

To examine the time course of $PGF_{2\alpha}$-mediated inhibition of PRL-R expression, d 19 pregnant rats were injected with $PGF_{2\alpha}$ and killed 0–10 h thereafter. Total RNA was isolated and subjected to RT-PCR with primers that allow coamplification of both forms of the rat PRL-R. Because we have previously studied in detail the stimulatory effect of $PGF_{2\alpha}$

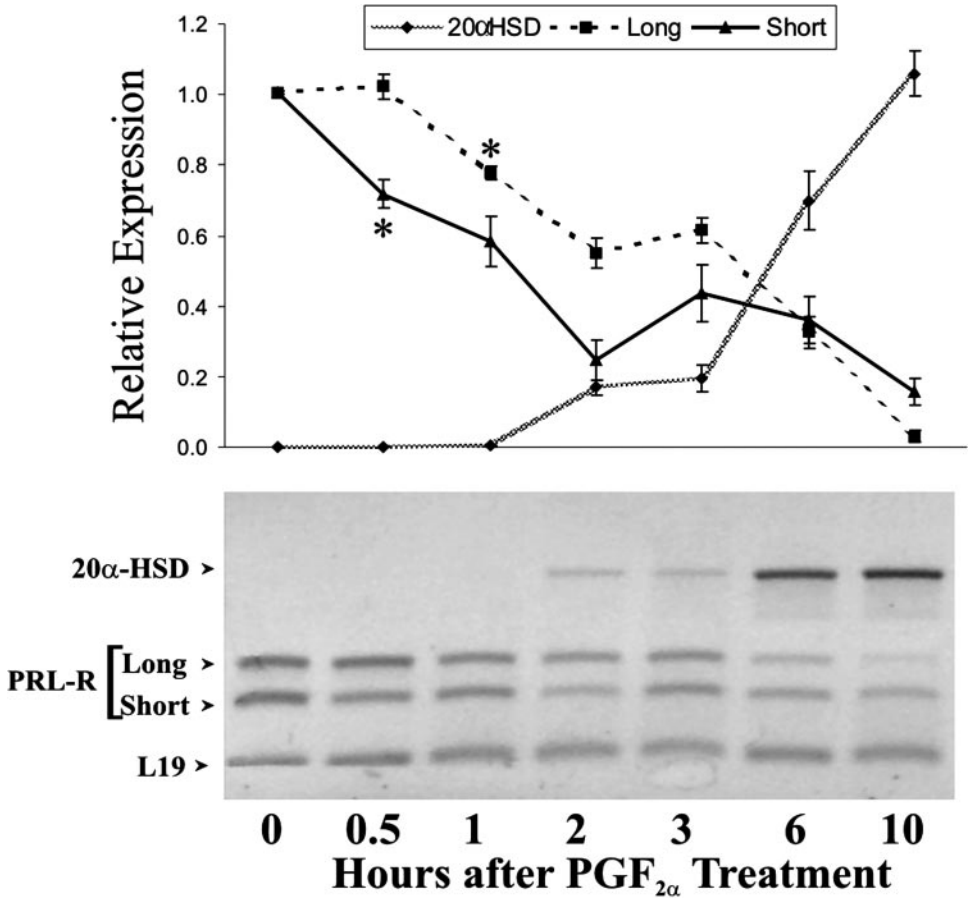

FIG. 3. Time-course inhibition of PRL-R expression by $PGF_{2\alpha}$ in the rat CL. Rats on d 19 of pregnancy were treated with 400 μg $PGF_{2\alpha}$ ip and killed 0–10 h thereafter. RT-PCR analysis was performed using primers that allow the coamplification of both forms of the PRL-R, 20αHSD, and L19 messages. Each *point* represents means ± SEM, n = 3. *, $P < 0.05$ *vs.* 0 h; thereafter, all *points* are significantly different.

on 20αHSD expression (4), we sought to examine whether the induction of 20αHSD by $PGF_{2\alpha}$ may be preceded by a decrease in PRL-R expression. To accomplish this, we co-amplified 20αHSD mRNA along with that of PRL-R in luteal RNA of rats treated with $PGF_{2\alpha}$ for different periods of time. The results shown in Fig. 3 reveal that $PGF_{2\alpha}$ treatment causes a time-related decrease in the expression of both forms of PRL-R. A significant inhibition in the expression of the short and long forms occurred within 30 and 60 min, respectively, whereas 20αHSD expression was induced by $PGF_{2\alpha}$ only 2 h after treatment. 20αHSD mRNA levels increased progressively thereafter (Fig. 3).

Effect of $PGF_{2\alpha}$ on PRL-R expression in vitro

To determine whether $PGF_{2\alpha}$ decreases PRL-R expression acting on luteal cells directly, we used LGC, which were treated with different doses of $PGF_{2\alpha}$. As shown in Fig. 4A, treatment with a low dose of $PGF_{2\alpha}$ caused maximal inhibition of PRL-R expression, yet this dose had no effect on 20αHSD expression. Higher concentrations of $PGF_{2\alpha}$ in the culture medium induced 20αHSD expression. Furthermore, pretreatment of LGC with PRL did not affect $PGF_{2\alpha}$ stimulation of 20αHSD (data not shown), suggesting that $PGF_{2\alpha}$ stimulation of 20αHSD is dissociated from its effect on PRL-R. To examine this possibility, we transfected LGC with an expression vector for a constitutively active PRL-R ($PRL\text{-}R_{CA}$). This receptor has been previously shown to activate constitutively PRL signaling and to regulate the transcription of PRL-regulated genes (9). Cells were then treated with either vehicle or $PGF_{2\alpha}$. $PRL\text{-}R_{CA}$ expression prevented neither the $PGF_{2\alpha}$-mediated inhibition of PRL-R expression nor the stimulation of 20αHSD (Fig. 4B). Nevertheless, $PRL\text{-}R_{CA}$ alone led to an increase in PRL-R expression, in accord with our previous finding demonstrating an up-regulation of PRL-R by PRL (5). Primers used to determine PRL-R do not amplify the mRNA transcribed from the $PRL\text{-}R_{CA}$ (10).

Discussion

In rodents, the CL of pregnancy is highly dependent on the action of PRL and PRL-like hormones to hypertrophy and produce progesterone needed for the maintenance of gestation. Accordingly, PRL-R increases during the differentiation from granulosa cell into luteal cell (11) and remains elevated until before parturition. Early binding studies have shown a sharp drop in PRL-R between d 21 and 22 of pregnancy (11). Telleria *et al.* (6) demonstrated that this loss in PRL binding is most likely due to a decrease in receptor protein subsequent to a sharp fall in mRNA for both forms of the PRL-R. This renders the CL unresponsive to PRL and PRL-like hormones of placental origin. A similar phenomenon also occurs in mice CL, in which receptor expression drops on d 19 and 20, 2 d before parturition (this investigation). However, the factor(s) that causes the drop in luteal PRL-R expression was unknown. Our present findings, obtained with $PGF_{2\alpha}$-R knockout mice as well as pregnant rats and luteal cell culture, indicate clearly that $PGF_{2\alpha}$ inhibits rapidly the expression of both forms of the PRL-R and that the physiological drop in PRL-R expression seen at the end of pregnancy is due to $PGF_{2\alpha}$.

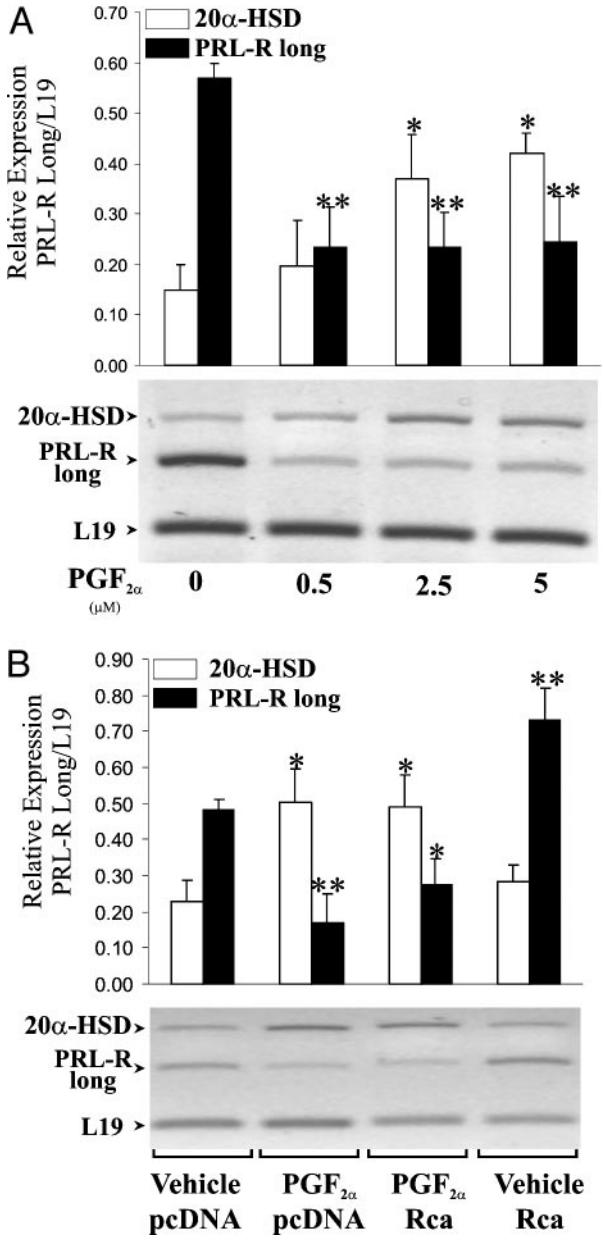

FIG. 4. Effect of $PGF_{2\alpha}$ on the expression of PRL-R in LGC. A, Cells were treated with different doses of $PGF_{2\alpha}$ for 12 h. B, Cells transfected with an empty plasmid or a constitutively active PRL-R (Rca) were treated with $PGF_{2\alpha}$ or vehicle for 12 h. In both experiments, gene expression was determined as described in *Materials and Methods*. Normalized mRNA levels are graphically represented in the *top panel*. *Bars* represent means ± SEM, n = 3. *, $P < 0.05$; and **, $P < 0.01$ *vs.* control (0 μM or vehicle).

This decrease in PRL-R expression may also contribute to the decline in progesterone secretion seen at this time. The fall in progesterone before parturition depends on the expression of the enzyme 20αHSD (4). We have shown that $PGF_{2\alpha}$-induced 20αHSD expression is at the transcriptional level and is mediated by a rapid stimulation of the transcription factor Nur77. *nur77* is an early gene that is rapidly induced; however, its expression is also shut down very quickly. We have observed that, despite the fact that Nur77 expression falls to undetectable levels after 6 h of $PGF_{2\alpha}$

administration, 20αHSD expression keeps increasing with time (4). These results and our finding that down-regulation of PRL-R expression precedes the stimulation of 20αHSD induced by $PGF_{2\alpha}$ suggested to us that $PGF_{2\alpha}$ may stimulate 20αHSD by both inducing Nur77 expression and by preventing PRL signaling in the luteal cells. We thought that Nur77 may be necessary to stimulate rapidly the expression of 20αHSD but that, once Nur77 expression falls, the decrease in PRL signaling induced by $PGF_{2\alpha}$ may ensure high levels of 20αHSD because PRL is a potent inhibitor of 20αHSD (3). However, our finding that overexpression of PRL-R_{CA} does not prevent the induction of 20αHSD nor the inhibition of PRL-R expression caused by $PGF_{2\alpha}$ suggests rather that the effect of $PGF_{2\alpha}$ on 20αHSD expression is independent of PRL signaling in luteal cell. In support of this hypothesis is our previous finding that $PGF_{2\alpha}$ activates 20αHSD promoter activity in the absence of PRL action (4).

The possibility that $PGF_{2\alpha}$ may prevent the inhibitory effect of PRL on 20αHSD by affecting the intracellular signaling of PRL has been proposed (12). It has been demonstrated that an analog of $PGF_{2\alpha}$, cloprostenol, increases the expression of the suppressor of cytokine signaling 3, which in turn prevents the activation of signal transducers and activators of transcription 5 (Stat5) transcription factor by PRL. However, it is not yet clear whether Stat5 mediates the inhibitory effect of PRL on 20αHSD expression.

Although the molecular mechanism by which $PGF_{2\alpha}$ stimulates 20αHSD has been investigated in detail (13), that of $PGF_{2\alpha}$ inhibition of PRL-R expression is not known. $PGF_{2\alpha}$ was shown to induce the expression of *nur77* through a Ca^{2+}-calmodulin-dependent mechanism. The Ca^{2+}-calmodulin complex formed upon $PGF_{2\alpha}$ treatment causes activation of ERK1/2, which phosphorylates the transcription factor JunD constitutively bound to its cognate binding site in the *nur77* gene, increasing its transactivational activity. The Nur77 generated then acts to activate the 20αHSD gene expression. Whether this pathway is involved in $PGF_{2\alpha}$-mediated inhibition of PRL-R appears unlikely because both the time course and responsiveness to $PGF_{2\alpha}$ differs. Because the expression PRL-R depends on active Stat5 (14), it is possible that the induction of suppressor of cytokine signaling 3 by $PGF_{2\alpha}$ described by Curlewis *et al.* (12) causes the decrease in PRL-R expression observed in the present study.

In conclusion, the results of this investigation have revealed that $PGF_{2\alpha}$ acts on luteal cells to inhibit the expression of the PRL -R and is responsible for the physiological decline in both forms of this receptor that occurs at the end of pregnancy in the CL. The results further suggest that $PGF_{2\alpha}$-mediated stimulation of 20αHSD is independent of $PGF_{2\alpha}$-induced inhibition of PRL signaling in luteal cels.

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