GnRH agonist treatment decreases progesterone synthesis, luteal peripheral benzodiazepine receptor mRNA, ligand binding and steroidogenic acute regulatory protein expression during pregnancy

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ABSTRACT

We have demonstrated that continuous administration of a gonadotropin-releasing hormone agonist (GnRH-Ag) suppresses luteal steroidogenesis in the pregnant rat. We further demonstrated that the peripheral-type benzodiazepine receptor (PBR) and the steroidogenic acute regulatory protein (StAR) play key roles in cholesterol transport leading to steroidogenesis. The purpose of this study was to understand the cellular and molecular mechanisms involved in the suppression of luteal steroidogenesis leading to a fall in serum progesterone levels in GnRH-Ag-treated rats during early pregnancy. Pregnant rats were treated individually starting on day 8 of pregnancy with 5 µg/day GnRH-Ag using an osmotic minipump. Sham-operated control rats received no treatment. At 0, 4, 8 and 24 h after initiation of the treatment, rats were killed and corpora lutea (CL) were removed for PBR mRNA, protein and radioligand binding analyses, immunoblot 1-D gel analysis of

StAR, P450 scc and 3β-hydroxysteroid dehydrogenase as well as 2-D gel analysis of StAR. The treatment decreased the luteal PBR mRNA expression at all time periods starting at 4 h compared with that in corresponding sham controls. GnRH-Ag also reduced, in the CL, the PBR protein/ligand binding, the StAR protein and P450 scc protein and its activity as early as 8 h after the treatment and they remained low compared with those in corresponding sham controls. The data from 2-D gel studies suggest that the majority of the decrease in StAR protein appears to be in the phosphorylated forms of StAR. Thus, we have demonstrated, for the first time, the presence of PBR and StAR in the pregnant rat CL and that the coordinated suppression of these proteins involved in the mitochondrial cholesterol transport along with P450 scc by GnRH-Ag leads to reduced ovarian steroidogenesis.

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INTRODUCTION

We have previously demonstrated that continuous administration of a gonadotropin-releasing hormone agonist (GnRH-Ag) in a pregnant rat suppresses serum levels of pregnenolone and progesterone; however, the treatment had no effect on the content of free cholesterol in the corpus luteum (CL)

(Sridaran *et al.* 1995). These data suggest that the mechanism(s) underlying cholesterol transport into the mitochondria may be impaired.

During the last decade two proteins have been shown to be involved in the regulation of the movement of cholesterol across the mitochondrial membranes, the rate-determining step in steroidogenesis, the peripheral-type benzodiazepine

receptor (PBR) (Papadopoulos *et al.* 1997a) and the steroidogenic acute regulatory protein (StAR) (Stocco & Clark 1997).

PBR is an 18 kDa protein that is present in all steroidogenic tissues (Papadopoulos *et al.* 1997a) including the rat ovarian granulosa cells (Fares *et al.* 1988, Amsterdam & Suh 1991), where it is primarily located on the outer mitochondrial membrane (Anholt *et al.* 1986). Detailed studies demonstrated that PBR ligand-induced stimulation of pregnenolone synthesis was due to PBR-mediated translocation of cholesterol from the outer to the inner mitochondrial membrane (Krueger & Papadopoulos 1990, Papadopoulos *et al.* 1990). Moreover, PBR was shown to participate in the hormone-induced cholesterol transport in Leydig and adrenocortical cells (Papadopoulos *et al.* 1991). Furthermore, inhibition of PBR expression both *in vivo* (Amri *et al.* 1996) and *in vitro* (Papadopoulos *et al.* 1997b) resulted in decreased steroid production.

Recently, Stocco & Clark (1997) suggested that StAR, a family of mitochondrial proteins, is present exclusively in steroidogenic tissues and is indispensable in regulating cholesterol transfer to the inner mitochondrial membrane. It has also been shown that StAR protein is present in pseudopregnant rabbit CL (Townson *et al.* 1996) and pseudopregnant rat ovaries (Ronen-Fuhrmann *et al.* 1998). The presence of StAR mRNA has been demonstrated in mouse (Clark *et al.* 1995), human (Sugawara *et al.* 1995) and pseudopregnant rat (Sandhoff & McLean 1996, Ronen-Fuhrmann *et al.* 1998) ovaries and in ovine CL (Juengel *et al.* 1995). Further, it has been reported that StAR protein can be found localized to the mitochondrial inner membrane as well as to sites of contact between the outer and inner membranes (Cherradi *et al.* 1997). In the rabbit CL, the gene for StAR is regulated by the steroid hormone, estrogen (Townson *et al.* 1996). Stocco & Clark (1997) suggested that tropic hormone-stimulated phosphorylation of StAR is required for cholesterol transport to the inner mitochondrial membrane. More recently, the data presented by Arakane *et al.* (1997) suggested that this post- or co-translational event accounts, in part, for the immediate effects of cAMP on steroid production.

The presence of PBR or StAR in the CL or ovary of the pregnant rat has not yet been established. In this study, we present evidence that PBR and StAR proteins are present in the CL of the rat during early pregnancy and that inhibition of the expression of these two proteins involved in cholesterol transport, by the *in vivo* administration of GnRH-Ag, may lead to suppressed luteal steroidogenesis. In addition, these data reveal a coordinated

regulation of the expression of key elements of the steroidogenic machinery in the CL of pregnancy. (This work was presented at the 29th Annual Meeting of the Society for the Study of Reproduction in London, Ontario, Canada and at the 30th Annual Meeting of the Society for the Study of Reproduction in Portland, OR, USA.)

MATERIALS AND METHODS

Materials

GnRH-Ag ([pyro]-Glu-His-Trp-Ser-Tyr-D-Trp-NMeLeu-Arg-Pro-ethylamide- luteinizing hormone-releasing hormone; Wyeth-40972) was a gift from Wyeth-Ayerst Laboratories (Philadelphia, PA, USA). Osmotic minipumps (Model 1003D) were obtained from Alza Corp. (Palo Alto, CA, USA). The RIA kit for progesterone was obtained from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA). [N-methyl-³H]PK 11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide; specific activity 83·50 Ci/mmol) and [α -³²P] dCTP (3000 Ci/mmol) were obtained from Du Pont-New England Research Products (Boston, MA, USA). PK 11195 was obtained from Research Biochemicals, Inc. (Natick, MA, USA). 22R-hydroxycholesterol was obtained from Sigma (St Louis, MO, USA). Protein markers that were used for the estimation of molecular sizes of the proteins (18 kDa for PBR, 30 kDa for StAR, 42 kDa for 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and 49 kDa for P450 scc) were acquired from Bio-Rad (Hercules, CA, USA). The primary antibodies for 3 β -HSD and P450 scc were gifts from Drs V Luu-The (CHUL Research Center, Quebec City, Quebec, Canada) and M J Soares (University of Kansas, Kansas City, KS, USA) respectively. The primary antibodies used for PBR (Amri *et al.* 1996) and StAR (Clark *et al.* 1994) detection were obtained as previously described. All other chemicals were of analytical quality and were obtained from various commercial sources.

Animals

Timed-pregnant Holtzman Sprague-Dawley rats were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN, USA). They were housed at the animal facilities of Morehouse School of Medicine in a temperature- (23–25 °C) and light- (14 h light: 10 h darkness) controlled room. Purina rodent chow and tap water were freely accessible. The day of insemination, identified by a sperm plug, was designated as day 1 of pregnancy. All procedures

involving animals were approved by the Institutional Animal Care and Use Committee at the Morehouse School of Medicine and in accordance with the principles and procedures of the US National Institutes of Health (NIH) guide for the care and use of laboratory animals.

Experiment

GnRH-Ag (5 µg/day) was administered continuously using osmotic minipumps starting on the morning of day 8 of pregnancy and sham-operated control rats received no treatment (Sridaran 1987, Sridaran *et al.* 1998). Briefly, each rat was implanted s.c. in the dorsal surface of the neck with an osmotic minipump under metofane anesthesia. These pumps were incubated in saline overnight prior to implantation. Rats were killed at 0, 4, 8 and 24 h after the commencement of treatment. Prior to killing, under metofane anesthesia, blood from the jugular vein was obtained for the measurement of progesterone. At autopsy, ovaries were removed; CL from the ovaries of each animal were separated and snap-frozen in liquid nitrogen and stored at -70°C for PBR mRNA analysis and radioligand binding assays. Since the data obtained from untreated rats (time 0) were not different from those from control rats, to conserve the number of animals used in this study, following the NIH guidelines, rats were killed in subsequent experiments only at 4, 8 and 24 h for the immunoblot analysis of StAR, P450 scc and 3β -HSD.

Progesterone RIA

Serum progesterone levels were measured in one assay. The coefficient of intra-assay variation was 4.7%.

Radioligand binding assays

CL were homogenized in 20 mM Tris-HCl (pH 7.4) containing 0.32 M sucrose and 0.1 mM polymethylsulfonyl fluoride (PMSF), centrifuged at 15 000 g for 20 min and the pellet resuspended in PBS. Aliquots of 100 µg protein were used for [^3H]PK 11195 binding studies performed at 4°C , in a final incubation volume of 0.3 ml, using the radioligand in the concentration range 0.019–20 nM and 200-fold excess of unlabeled ligand, as previously described (Krueger & Papadopoulos 1990, Papadopoulos *et al.* 1990, 1991). After 120 min incubation, assays were stopped by filtration through FP-100 Whatman GF/B fired filters (Brandel, Gaithersburg, MD, USA) and washed with ice-cold PBS. Radioactivity trapped on the

filters was determined by liquid scintillation counting at 50% efficiency. The dissociation constant (K_d) and the number of binding sites (B_{max}) were determined by Scatchard plot analysis of the data using the LIGAND program (Munson & Rodbard 1980) (Kell, v. 4.0, Biosoft, Inc., Ferguson, MO, USA).

RNA (Northern) blot analysis

Total RNA from the luteal tissue was isolated by the acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987) using the RNASAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA). RNA electrophoresis, transfer, probe labeling and membrane hybridization were performed as previously described by us (Amri *et al.* 1996). Briefly, RNA was size-fractionated by electrophoresis and transferred to derivatized nylon membranes (Nytran Plus, Schleicher and Schuell, Keene, NH, USA). The blots were then hybridized against a [^{32}P]cDNA probe for PBR labeled by the random priming technique. The 623 bp probe for PBR mRNA used was prepared as we previously described (Garnier *et al.* 1994). Screen-enhanced autoradiography was performed by exposing Kodak X-OMAT AR films to the blots at -80°C for 48 h. Image and membrane analysis of the ethidium bromide staining of 28S rRNA and the bands on the X-ray film was performed using Sigmagel software (Jandel Scientific, San Rafael, CA, USA).

Cytochrome P450 scc activity

The P450 scc activity was examined using the hydrosoluble derivative of cholesterol, 22R-hydroxycholesterol, which has direct access to the active site of the enzyme. Thus, it bypasses the transport mechanisms, allowing direct access to the P450 scc enzyme. Mitochondria were isolated either from sham control rats or from animals treated with GnRH-Ag for 8 h and were then frozen. Upon thawing, mitochondria were resuspended at a concentration of 1.0 mg/ml in 250 mM sucrose, 20 mM KCl, 15 mM triethanolamine hydrochloride (pH 7.0), 10 mM K_3PO_4 and 5 mM MgCl_2 containing 5 µM trilostane, an inhibitor of pregnenolone metabolism (Papadopoulos *et al.* 1990). 22R-hydroxycholesterol was then added at a concentration of 10 µM. The mixture was pre-incubated for 5 min at 37°C , and the reaction was initiated by addition of 10 mM isocitrate and 0.5 mM NADP. The incubation was continued for 20 min at 37°C . The reaction was stopped with ethanol and steroids were extracted with four

volumes of diethylether and the organic phase evaporated to dryness. Pregnenolone was measured by a specific RIA as previously described (Papadopoulos *et al.* 1990).

Subcellular fractionation of the CL

CL were homogenized in 1 ml homogenizing buffer (50 mM Tris-HCl pH 7.4 containing 25 mM sucrose, 2 mM EDTA, 1 mM PMSF and 1 mM dithiothreitol) using a glass homogenizer. The homogenates were fractionated as described previously (Fleischer & Kervina 1974). Briefly, the homogenates were placed on a 1.9 M sucrose cushion and centrifuged at 7000 *g* for 45 min. The supernatant and cushion interface were transferred and centrifuged at 25 000 *g* for 10 min to isolate the mitochondrial pellet. This pellet was resuspended in 1 ml homogenizing buffer.

SDS-PAGE

Mitochondrial fractions (40 µg for PBR, 20 µg for StAR and 15 µg for P450 scc) and unfractionated homogenates (40 µg for 3β-HSD) were resolved by discontinuous, 1-D electrophoresis as described by Laemmli (1970). Fractionated proteins were electrophoretically transferred to Polyscreen polyvinylidene difluoride membranes (Dupont, Boston, MA, USA).

Immunoblot analysis

The transferred blots were washed with 5% non-fat dried milk for 1 h. After washing in 1% PBS-Tris, the membrane was incubated with a 1:2000 dilution of rabbit antiserum raised against purified human placental 3β-HSD (Luu-The *et al.* 1989) for 1 h. The blots were washed and incubated with a 1:16 000 dilution of anti-rabbit IgG-peroxidase conjugate. Blots were then incubated with chemiluminescent reagent for 30 s. Membranes were then wrapped in saran wrap and exposed to reflection autoradiography film for 30 s using an intensifying screen. Film was processed using a Kodak M35A X-Omat processor. The intensity of the signal emitted recognizing the 3β-HSD protein was quantitatively analyzed for integrated intensity using the Bio Image Visage 2000 computer-assisted image analyzer (Bio Image Products, Ann Arbor, MI, USA). The same procedure was followed for P450 scc, PBR and StAR using different primary and secondary antibodies. The primary antibody for P450 scc was used at a dilution of 1:2000 and the secondary antibody used was the same as described for 3β-HSD (Roby *et al.* 1991). The primary

FIGURE 1. Serum progesterone levels (means $\pm$ S.E.M.) at points timed after initiation of the treatment. *n*=at least 5/group. **P*<0.0001 compared with corresponding sham controls.

antibody for PBR was used at 1:1000 and the secondary antibody at 1:6000 (Amri *et al.* 1996). Immunodetection of StAR was made following incubation with rabbit sera containing the specific antipeptide antibodies at a dilution of 1:2000 followed by the secondary antibody, donkey anti-rabbit IgG conjugated with horseradish peroxidase (Clark *et al.* 1994). Densitometric analyses of the immunoblot spots were quantitated using the Bio Image analyzer.

Two-dimensional gel electrophoresis

The procedure used here is described in detail previously (Clark *et al.* 1994).

Protein measurement

Microgram amounts of protein were quantitated by the dye-binding assay of Bradford (1976) with either BSA or γ-globulin as standard.

Statistical analyses

The data were analyzed by one-way ANOVA followed by Scheffe's test when differences were significant. A *P* value less than 0.05 was considered statistically significant. All results, with the exception of representative autoradiograms, are presented as means $\pm$ S.E.M. of combined data from the replicate experiments. In these cases, the sample size was between three and six rats per time point. All the Western and Northern blot studies were conducted first in samples from one set of experiments and these data were confirmed by repeating these studies once more in an additional

set of experiments. Only one set of data is presented in the Results section.

RESULTS

Effect of GnRH-Ag treatment on serum progesterone levels

GnRH-Ag treatment suppressed serum progesterone levels as early as 8 h following initiation of the treatment; further reduction in serum progesterone levels occurred at 24 h (Fig. 1). These data are in agreement with our earlier data in a similar model (Sridaran 1987, Sridaran *et al.* 1995, 1998). It should be noted that prolonged administration of GnRH-Ag (48 and 72 h) resulted in further decrease in serum progesterone levels (Sridaran 1987).

Effect of GnRH-Ag treatment on the luteal PBR mRNA expression

Northern blot analysis followed by autoradiography (Fig. 2; bottom) revealed that GnRH-Ag treatment suppressed the luteal mRNA expression of PBR at all time periods compared with that in corresponding sham controls. The loading of the samples was assessed by ethidium bromide staining of the 28 and 18S rRNA. Following densitometric/image analysis of the RNA data we concluded that the relative expression of PBR mRNA/28S rRNA (Fig. 2; top) was suppressed by the GnRH-Ag treatment at all time periods compared with that in corresponding sham controls. The inhibition of PBR mRNA expression by the GnRH-Ag appears to be time dependent with the maximum effect seen at 24 h after initiation of treatment.

Effect of GnRH-Ag treatment on luteal PBR protein/ligand binding sites

The expression of a bioactive PBR protein in these luteal tissue samples was examined by radioligand binding assays using the radiolabeled isoquinolone ligand PK 11195, which binds specifically to the 18 kDa PBR protein (Krueger & Papadopoulos 1990). The results obtained were analyzed using Scatchard plot analysis. GnRH-Ag treatment reduced the number of PBR binding sites ($B_{\max}$) in the CL as early as 8 h after initiation of the treatment and they remained low thereafter compared with those in corresponding sham controls (Fig. 3). No significant effect on the PBR receptor affinity (K_d) could be seen among the groups (data not shown). The densitometric analysis of the immunoblot for PBR (Fig. 4) demonstrates further

FIGURE 2. Bottom: autoradiogram of the PBR mRNA blot. RNA was purified from luteal tissue, isolated from animals treated with or without (sham) GnRH-Ag for the indicated time periods, and analyzed as described in Materials and Methods. Ethidium bromide staining indicating equal loading of the samples. Similar results were obtained in two independent experiments. U, untreated; S, sham; G, GnRH-Ag. Top: relative intensity of the 18 kDa PBR mRNA/28S rRNA estimated by densitometric/image analysis of the spots.

that GnRH-Ag suppressed the luteal PBR protein content at 8 h after treatment compared with that of sham controls. Thus the effect of GnRH-Ag treatment on the number of PBR binding sites is indeed due to decreased protein expression and is very similar to its effect on serum progesterone levels, in that both were suppressed starting 8 h after initiation of the treatment. Interestingly, although at 4 h the GnRH-Ag began suppressing PBR mRNA expression (Fig. 2), this effect was translated into an effect at the protein level at 8 h as determined by immunoblot (Fig. 4) and ligand binding assays (Fig. 3).

Effect of GnRH-Ag treatment on the luteal cytochrome P450 scc activity

Administration of 22R-hydroxycholesterol to a mitochondrial preparation *in vitro* stimulated

FIGURE 3. Luteal PBR PK 11195 ligand binding sites (B_{max}) quantitated by Scatchard plot analysis of the saturation isotherms using the LIGAND program. Ligand binding studies were performed on luteal tissue membrane preparations, from animals treated with or without (sham) GnRH-Ag for the indicated time periods, using radiolabeled PK 11195 as described in Materials and Methods. Values given are means $\pm$ s.e.m. ($n=3$). * $P<0.0001$ by ANOVA compared with corresponding sham controls.

FIGURE 4. Densitometric (top) and immunoblot (bottom) analyses of the luteal PBR protein content at 8 h after commencement of treatment. GnRH-Ag (G) suppressed the PBR protein levels compared with those in corresponding sham (S) controls.

a 2.8-fold increase in pregnenolone production in sham control rats compared with no change in GnRH-Ag-treated rats (Table 1). Interestingly, there is no difference between the control values from sham control and GnRH-Ag-treated samples. Thus, it appears that GnRH-Ag treatment impaired the P450 scc activity. Thus, we conclude that the

TABLE 1. Effect of GnRH-Ag treatment on luteal cytochrome P450scc activity (production of pregnenolone (ng/mg protein per 20 min, $\pm$ s.e.m. $n=4$))

Treatment of animals	Treatment of mitochondria	
	Control	22R-hydroxycholesterol (10 μ M)
Sham	722 $\pm$ 155	2016 $\pm$ 315*
GnRH-Ag	846 $\pm$ 137	848 $\pm$ 124

* $P<0.0001$ compared with corresponding control.

FIGURE 5. Densitometric (top) and immunoblot (bottom) analyses of the luteal StAR protein content at points timed after commencement of treatment. GnRH-Ag (G) suppressed StAR protein levels at 8 and 24 h after the treatment compared with those in corresponding sham (S) controls. Maximum suppression occurred at 8 h.

treatment with GnRH-Ag affects not only the cholesterol transport but also the cytochrome P450 scc activity.

Effect of GnRH-Ag treatment on the luteal content of StAR protein

The densitometric analysis of the 1-D immunoblot for StAR (Fig. 5) demonstrated that GnRH-Ag suppressed the luteal StAR protein content at 8 and 24 h after treatment while it increased the luteal StAR protein content at 4 h compared with that in corresponding sham controls. The maximum suppressive effect of GnRH-Ag occurred at 8 h. Two-dimensional gel electrophoresis was performed to determine if the phosphorylated forms of StAR are suppressed preferentially at the time (8 h) that the maximum suppressive effect of GnRH-Ag

FIGURE 6. Immunoblot (left) and densitometric (right) analyses of the luteal StAR protein content 8 h after commencement of treatment. GnRH-Ag suppressed the phosphorylated more than the non-phosphorylated forms of StAR protein compared with those in corresponding sham controls.

was noticed. The densitometric analysis of the 2-D immunoblot for StAR (Fig. 6) suggested that GnRH-Ag suppressed the phosphorylated more than the non-phosphorylated forms of the StAR protein compared with those in corresponding sham controls.

Effect of GnRH-Ag treatment on the luteal content of the steroidogenic enzymes

Densitometric analysis of the immunoblot for P450 scc (Fig. 7) demonstrates that GnRH-Ag suppressed P450 scc at 8 and 24 h after treatment at the same time points as the treatment suppressed the StAR protein (Fig. 5), which is in agreement with the suppressed P450 scc activity at 8 h after the treatment (Fig. 4). However, densitometric analysis of the immunoblot for β -HSD (Fig. 8) suggested that GnRH-Ag had no significant effect on β -HSD at any time period.

Of the three proteins (PBR, StAR, P450 scc) measured in the CL that are involved in the steroidogenic pathway, GnRH-Ag treatment seems to affect PBR most profoundly, while its effect on P450 scc is the least.

DISCUSSION

The results of the present investigation demonstrate that PBR and StAR are present in the CL during early pregnancy and that *in vivo* administration of a GnRH-Ag suppresses luteal steroidogenesis during early pregnancy by decreasing the PBR mRNA, PBR protein, the number of PBR binding sites, StAR protein and P450 scc enzyme in the CL. The

FIGURE 7. Densitometric (top) and immunoblot (bottom) analyses of the luteal P450 scc enzyme content. GnRH-Ag (G) suppressed P450 scc enzyme levels at 8 and 24 h after treatment compared with those in corresponding sham (S) controls.

FIGURE 8. Densitometric (top) and immunoblot (bottom) analyses of the luteal β -HSD enzyme content. GnRH-Ag (G) had no significant effect on β -HSD enzyme at any time period. S, sham controls.

number of PBR binding sites in the CL was quantitated using the radiolabeled isoquinolone PK 11195, which binds specifically to the 18 kDa PBR protein. Therefore, these data further suggest that the PBR binding sites present in the CL of the pregnant rat are functional; the expression of these PBR sites is suppressed as early as 8 h after initiation of GnRH-Ag treatment. The data also suggest that the majority of the decrease in StAR protein appears to be in the phosphorylated forms

of StAR. This is a significant observation in light of recent studies which have shown that the phosphorylated forms of the StAR protein are required for maximal steroid production when expressed in COS-cells (Arakane *et al.* 1997).

The results obtained further suggest that the inhibitory effect of GnRH-Ag on PBR expression is rapid, because 4 h after the beginning of GnRH-Ag treatment PBR mRNA expression was inhibited. This effect was translated at the PBR protein level 4 h later (at 8 h). We have previously demonstrated, in a similar model, that both serum progesterone and pregnenolone levels fall 8 h after the commencement of the treatment (Sridaran 1987, Sridaran *et al.* 1995, 1998). These data were confirmed in the present study. In addition, we have shown that GnRH-Ag treatment does not affect the luteal content of free cholesterol (Sridaran *et al.* 1995). These observations indicate that the total amount of the cholesterol substrate available for steroid synthesis is the same in sham control and GnRH-Ag-treated rats. It was earlier demonstrated that PBR ligand-induced stimulation of pregnenolone was due to PBR-mediated translocation of cholesterol from the outer to the inner mitochondrial membrane (Krueger & Papadopoulos 1990) and that reduction of PBR expression in Leydig cells *in vitro* (Papadopoulos *et al.* 1997b) or in adrenal cells *in vivo* (Amri *et al.* 1996) results in reduced steroid synthesis and decreased circulating steroid levels.

The present results further suggest that the inhibitory effect of GnRH-Ag on StAR is rapid, since at 8 h after the commencement of treatment GnRH-Ag suppressed both the phosphorylated and non-phosphorylated forms. It is important to note that in the present studies it is the 30 kDa form of the StAR protein which is detected by Western analysis. This form represents the mature protein which is processed from a 37 kDa precursor protein and is found inside mitochondria. While it is believed that once inside mitochondria the 30 kDa form is no longer active in supporting cholesterol transfer for steroidogenesis, the amount of 30 kDa protein present is, nevertheless, a good indication of the amount of cholesterol transfer and steroidogenesis which has occurred during the time period measured. In addition, the amount of 37 kDa precursor present in the cell at any time is very low, probably as a result of its very short half-life (Epstein & Orme-Johnson 1991), making its measurement virtually impossible. It should be noted that while the luteal P450 scc enzyme protein, as well as its activity, is suppressed by GnRH-Ag treatment the luteal 3 β -HSD levels were not affected by the treatment. Therefore, we interpret

the present data to mean that the GnRH-Ag-induced rapid suppression of the mitochondrial membrane PBR mRNA, PBR protein and/or StAR protein results in the reduction of the amount of the substrate, cholesterol. Thus, less cholesterol will become available to the inner mitochondrial membrane cytochrome P450 scc leading to decreased pregnenolone formation and subsequent progesterone production by the CL. The apparent partial recovery in luteal StAR levels, 24 h after initiation of the treatment with GnRH-Ag, requires further investigation.

The suppressive effect of GnRH-Ag *in vivo* on the luteal P450 scc is similar to the suppressive effect of GnRH-Ag *in vitro* on this enzyme in the CL as has been demonstrated by us previously (Srivastava *et al.* 1994). Therefore, we propose the hypothesis that the suppressive effect of GnRH-Ag on the CL of the pregnant rat is due to the direct effect of the agonist. In support of this hypothesis, the CL of the rat has been shown to have high-affinity, low-capacity binding sites for GnRH (Clayton *et al.* 1979), and more recently the human granulosa-lutein cells and the ovary have been described as expressing mRNA for GnRH receptor (Minaretzis *et al.* 1995). Furthermore, the demonstration of GnRH mRNA expression in granulosa cells by RT-PCR (Oikawa *et al.* 1990) and the expression of the GnRH gene (Goubau *et al.* 1992) in different components of the ovary by *in situ* hybridization histochemistry (Clayton *et al.* 1992) strongly implies a paracrine or autocrine function for GnRH, which may be actively participating under normal physiological conditions in modulating ovarian steroidogenesis. These observations support an emerging concept that an intrinsic GnRH system, comprising the ligand, receptor and biological response, exists in the ovary. Currently, these aspects are under investigation in our laboratory. A Ca²⁺-dependent, protein kinase C-mediated pathway has been demonstrated in rat granulosa and luteal cells and thus implicated in the mechanism of GnRH action on ovarian steroidogenesis (Leung & Wang 1989). Further studies are in progress to understand the mechanism of action of GnRH including its direct effect on PBR, StAR and P450 scc proteins that are involved in the regulation of steroid biosynthesis in the CL.

The suppressive effect of GnRH-Ag *in vivo* on luteal steroidogenesis could also be mediated by a direct action on the pituitary gonadotropin secretion. However, serum luteinizing hormone (LH) levels remain almost 300% higher even after 72 h upon the administration of GnRH-Ag compared with those in sham-treated rats during early

pregnancy (Sridaran 1987). Nonetheless, GnRH-Ag treatment does not alter cAMP levels in the CL (R Sridaran and CJ Smith, unpublished observations). In addition, we have demonstrated that luteal production of progesterone during early pregnancy is LH dependent, mediated via testosterone production in the CL (Sridaran *et al.* 1981). However, the luteal synthesis and release of testosterone is not impaired by the GnRH-Ag treatment (Sridaran *et al.* 1988). In conclusion, these observations taken together suggest that the suppression of luteal steroidogenesis by GnRH-Ag is most likely due to its direct effect on the CL and that it is a post-cAMP-mediated mechanism.

Collectively, the data presented in this study provide evidence that PBR and StAR proteins are present in the rat CL during early pregnancy and that inhibition of these proteins, involved in cholesterol transport, by *in vivo* administration of GnRH-Ag, leads to suppressed luteal steroidogenesis. Based on the results of the present study, we propose a model for cholesterol transport wherein the GnRH-Ag suppresses the expression of the PBR protein on the outer mitochondrial membrane and also suppresses the StAR protein. This would lead to reduced cholesterol availability to P450 scc which is present in the inner mitochondrial membrane resulting in reduced pregnenolone and consequently reduced progesterone production by the CL. Additional reduction of P450 scc activity by the GnRH-Ag treatment will further contribute to the reduced amount of steroid formed. In this model, an interaction of StAR with PBR may be responsible for the supply of cholesterol to the P450 scc.

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