Regulation and role of the insulin-like growth factor I system in rat luteal cells

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The relationship between insulin-like growth factor I (IGF-I), a hormone which has potent metabolic effects and stimulates protein synthesis, and prolactin and oestradiol was examined to investigate a possible mechanism for the luteal cell hypertrophy that is responsible for the increase in size of the corpus luteum. A luteal cell line (GG-CL) derived from large luteal cells of the pregnant rat corpus luteum was used. IGF-I, IGF-I receptor and oestrogen receptor β mRNA contents were determined by semiquantitative RT-PCR. The results revealed that prolactin upregulates the expression of IGF-I mRNA in luteal cells, but not that of its receptor. IGF-I had no effect on the expression of its receptor but caused a dose-related increase in the expression of oestrogen receptor β . Furthermore, whereas IGF-I upregulated oestrogen receptor β expression, oestradiol downregulated expression of mRNA for both IGF-I and its receptor. This effect of oestradiol is not mediated through progesterone which is stimulated by oestradiol in the corpus luteum. The developmental studies indicate that mRNA for IGF-I and its receptor are not expressed in tandem throughout pregnancy. Whereas the receptor mRNA is expressed at higher concentrations in early pregnancy, that of its ligand is highly expressed close to parturition. Collectively, the results indicate that prolactin stimulates luteal IGF-I production, which in turn acts on the luteal cell to stimulate expression of oestrogen receptor β . Luteal cells with increased oestrogen receptor β can respond fully to oestradiol, leading to cell hypertrophy.

Introduction

Many tropic hormones act on steroidogenic glands to increase not only steroidogenesis but also the size of their target tissues (Gibori, 1993; Juengel *et al.*, 1995, 1997). In rats, the corpus luteum more than doubles in size with the advance of pregnancy, mainly due to hypertrophy of the large luteal cells (McLean *et al.*, 1992). It is well established that prolactin and luteal oestradiol act in concert to cause hypertrophy of the rat corpus luteum (Alloiteau, 1959). However, the mechanism by which these tropic hormones affect corpus luteum hypertrophy is still unclear. The observation that only the large luteal cell responds to insulin-like growth factor I (IGF-I) stimulation and contains the bulk of the receptors for IGF-I (Parmer *et al.*, 1991) led to the proposal that this growth hormone may mediate, at least in part, prolactin and oestradiol action. IGF-I has potent metabolic effects and stimulates protein synthesis. Expression of IGF-I in the rat liver is stimulated by prolactin (Murphy *et al.*, 1988) and the expression of its receptor is upregulated by oestradiol in a variety of tissues (Murphy

and Ghahary, 1990; Adesanya *et al.*, 1996; Clarke *et al.*, 1997). These observations led to the hypothesis that prolactin stimulates IGF-I expression, whereas oestradiol upregulates expression of the IGF-I receptor. However, administration of oestradiol *in vivo* caused a marked decrease in IGF-I receptor expression in the corpus luteum of pregnant rats (Parmer *et al.*, 1991). This oestradiol-induced downregulation may be due to progesterone, since oestradiol stimulates progesterone production by the rat corpus luteum and progesterone is known to inhibit expression of the IGF-I receptor and its ligand in several tissues (Papa *et al.*, 1991; Strowitzki *et al.*, 1996; Manikkam and Rajamahendran, 1997). Although oestradiol does not upregulate IGF-I receptor expression, it acts synergistically with IGF-I to stimulate progesterone production by the large luteal cell (Parmer *et al.*, 1991). The fact that oestradiol stimulation depends upon the presence of IGF-I indicates that this growth factor may sensitize the luteal cell to oestradiol action by upregulating oestradiol receptor expression. Therefore, the aims of this investigation in luteal cells were to examine (i) whether oestradiol inhibition of IGF-I and IGF-I receptor expression is due to progesterone, (ii) whether prolactin stimulates IGF-I mRNA expression, and (iii) whether IGF-I, in turn, upregulates expression of the oestradiol receptor.

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Materials and Methods

Materials

Progesterone, oestradiol, D-glucose and recombinant human IGF-I were purchased from Sigma Chemical Co. (St Louis, MO). RPMI-1640 medium, antibiotic antimycotic solution, non-essential amino acids and sodium pyruvate were from Mediatech (Washington, DC). Fetal bovine serum (FBS) was from HyClone (Logan, UT). [α - 32 P]deoxycytidine triphosphate (dCTP) was from Amersham (Arlington Heights, IL). Culture flasks (25 or 75 cm²) were from Becton Dickinson Co. (Franklin Lakes, NJ). *Taq* DNA polymerase was from Perkin-Elmer Co. (Foster City, CA). Lipofectin and G418 sulfate (Geneticin) were from Life Technologies Inc. (Grand Island, NY).

Animals

Pregnant Sprague–Dawley rats (day 1 = sperm positive) purchased from Sasco Animal Labs (Madison, WI) were housed at 24°C with a 14 h light: 10 h dark photoperiod (lights on 05:00 h–19:00 h) and allowed free access to Purina rat chow and water. The rat care and handling conformed to the NIH guidelines for animal research. The experimental protocol was approved by the Institutional Animal Care and Use Committee.

For the developmental studies, rats were obtained at various stages of pregnancy from days 4 to 22, the day of parturition and the day after parturition. Corpora lutea were dissected from the ovaries under a stereoscopic microscope. All tissues were frozen in liquid nitrogen and stored at –80°C until RNA isolation.

Cell culture

The luteal cell line termed GG-CL, which was recently generated in this laboratory (Sugino *et al.*, 1998), was used in this study. Originally, large luteal cells from corpora lutea of rats on day 14 of pregnancy were purified to homogeneity by flow cytometry as reported by Nelson *et al.* (1992). Cells were infected with a temperature sensitive simian virus 40 (SV-40 *tsA209*) according to Chou (1985). Transformed cells were maintained at the permissive temperature (33°C) until colonies were identified. Several colonies of the transformed cells were isolated and passaged. One clone, designated GG-CL cells, was used in this study. GG-CL cells were cultured in 25 or 75 cm² flasks with the incubation medium (RPMI-1640 medium containing 2 × antibiotic antimycotic solution, 1 × non-essential amino acids, 1 × sodium pyruvate, 0.5% (w/v) D-glucose and 10% FBS) at permissive (33°C) and non-permissive (39°C) temperatures under an atmosphere of 5% CO₂:95% air. The FBS used in this study was heat-inactivated. GG-CL cells show the normal morphological differentiated phenotype similar to that of primary luteal cells at 39°C and express many genes that are characteristic of primary cells, although they do not produce progesterone (Sugino *et al.*, 1998).

Transfection of GG-CL cells with the prolactin receptor

GG-CL cells transfected stably with the long form of the prolactin receptor were used for the experiments with prolactin. Transfection was performed according to the procedure of Felgner *et al.* (1987) with a slight modification as described by Sugino *et al.* (1998). Briefly, GG-CL cells were plated in six-well Falcon dishes and grown at 33°C to 33% confluence in RPMI-1640 medium containing 5% FBS. Cells were washed with serum-free medium with no antibiotics and were transfected using Lipofectin according to the manufacturer's protocol. The cells were transfected with 10 µg of the expression vector pMT2poly containing the long form of the prolactin receptor cDNA and with pSV2neo vector. After transfection, the medium was replaced with growth medium containing 5% FBS and antibiotics and incubated for 48 h. After 48 h, the medium was again replaced with fresh growth medium and treated with 100 µg G418 sulfate ml⁻¹. Addition of G418 sulfate was continued once every 2 days until G418 sulfate resistant colonies were identified. These colonies were picked and cultured in the growth medium containing 5% FBS until the cells were confluent. For identification of successful stable transfection with prolactin receptor, cells were grown and passaged several times. Prolactin receptor mRNA expression in these cells was determined by RT-PCR using prolactin receptor specific primers as reported by Sugino *et al.* (1998).

Treatment of GG-CL cells

GG-CL cells were cultured at 33°C until 50% confluent and then at 39°C for 2 days to examine the effects of IGF-I on oestrogen receptor β mRNA expression and the effects of oestradiol or progesterone on IGF-I and IGF-I receptor mRNA expression. Cells were then treated with either IGF-I, oestradiol or progesterone. To examine the effect of prolactin in GG-CL cells GG-CL cells transfected with prolactin receptor were cultured at 33°C until 50% confluent and then at 39°C for 2 days. Cells were then treated with ovine prolactin (NIDDK oPRL-20, 31 iu mg⁻¹). Cells were cultured in the presence of 1% FBS during the treatment at 39°C as serum is necessary for survival at 39°C. Changes in morphology were not observed after hormonal treatments in GG-CL cells and in cells transfected with prolactin receptor. After culture, the cells were washed with PBS several times and stored at –80°C for RNA isolation.

Isolation of total RNA and RT-PCR

Total RNA was isolated from corpora lutea by homogenization in guanidinium thiocyanate and centrifugation through a caesium chloride cushion (Chirgwin *et al.*, 1979), whereas total RNA from the cultured cells was isolated by the guanidinium isothiocyanate–phenol–chloroform extraction procedure (Chomczynski and Sacchi, 1987). For mRNA analysis by RT-PCR, oligonucleotide primers for IGF-I (5'-CACATCTCTTCTACCTGGCA-3' and 5'-TGAGTCTTGGGCATGTCAGT-3'), IGF-I receptor

(5'-TCCACCATAGACTGGTCTCT-3' and 5'-ACGAAGCC-ATCTGAGTCACT-3') and oestrogen receptor β (5'-GCCAATCATGTGCACCAGTTCCTT-3' and 5'-AAAGCCAAGAG-AAACGGTGGGCAT-3') were designed on the basis of each cDNA sequence (Roberts *et al.*, 1987; Werner *et al.*, 1989; Kuiper *et al.*, 1996). Each reaction also included primers (5'-CTGAAGGTCAAAGGGAATGTG-3' and 5'-GGACAGAG-TCTTGATGATCTC-3') to amplify ribosomal protein L19 or primers (5'-CGTTCACCTTGATGAGCCCAT-3' and 5'-TCCAAGGGTCCGCTGCAGTC-3') to amplify ribosomal protein S16. Both L19 and S16 were used as internal controls (Chan *et al.*, 1987; Batra *et al.*, 1991). The predicted sizes of the PCR-amplified products were 319 bp for IGF-I, 434 bp for IGF-I receptor, 203 bp for oestrogen receptor β , 194 bp for L19, and 100 bp for S16. Total RNA (3 μ g) was reverse transcribed at 42°C in a 20 μ l reaction mixture (1 $\times$ PCR buffer, 2.5 mmol deoxynucleoside triphosphates l^{-1} , 5 μ mol random hexamer primers l^{-1} , 1.5 mmol $MgCl_2$ l^{-1} , and 200 U Moloney murine leukaemia virus reverse transcriptase (Life Technologies Inc., Grand Island, NY)). For PCR amplification, a mixture containing oligonucleotide primers (50 pmol), [α - ^{32}P]dCTP (2 μ Ci at 3000 Ci $mmol^{-1}$), and *Taq* DNA polymerase (2.5 U) was added to each reaction. The total volume was increased to 90 μ l with 1 $\times$ PCR buffer, and the samples were overlaid with mineral oil. Amplifications were carried out for 20 cycles with 62°C annealing temperature for IGF-I, for 20 cycles with 63°C annealing temperature for IGF-I receptor, and for 30 cycles with 65°C annealing temperature for oestrogen receptor β in a Perkin-Elmer Cetus thermal cycler. The conditions were such that amplification of the product was in

the exponential phase, and the assay was linear with respect to the amount of input RNA. Reaction products were separated by electrophoresis on an 8% (w/v) polyacrylamide non-denaturing gel. Each RT-PCR reaction included L19 or S16 ribosomal protein mRNA primers to normalize the data. After autoradiography, data were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

Data were examined by ANOVA and Duncan's new multiple range test. Differences were considered to be significant if $P < 0.05$.

Results

Effects of oestradiol and progesterone on IGF-I and IGF-I receptor mRNA expression

GG-CL cells were cultured in the presence of either oestradiol or progesterone to examine whether the oestradiol inhibition of IGF-I and IGF-I receptor observed *in vivo* (Parmer *et al.*, 1991) also occurs in cell culture and whether the oestradiol effect is mediated by progesterone. Oestradiol treatment caused a marked inhibition in both IGF-I and IGF-I receptor mRNA expression despite the fact that these cells do not produce progesterone (Fig. 1). In addition, progesterone treatment of GG-CL cells had no inhibitory effect on the expression of IGF-I and IGF-I receptor mRNA (Fig. 2).

Fig. 1. Effect of oestradiol on (a) insulin-like growth factor I (IGF-I) and (b) IGF-I receptor mRNA expression in a luteal cell line (GG-CL) derived from the large luteal cells of the pregnant rat corpus luteum. GG-CL cells were incubated with oestradiol (10^{-8} , 10^{-7} and 10^{-6} mol l^{-1}) for 8 h at 39°C. Total RNA was isolated and subjected to RT-PCR. The quantification data are mean $\pm$ SEM of three different experiments. C, control. a, $P < 0.01$ versus control.

Fig. 2. Effect of progesterone on (a) insulin-like growth factor I (IGF-I) and (b) IGF-I receptor mRNA expression in a luteal cell line (GG-CL) derived from the large luteal cells of the pregnant rat corpus luteum. GG-CL cells were incubated with progesterone (10^{-7} , 10^{-6} and 10^{-5} mol l⁻¹) for 24 h at 39°C. Total RNA was isolated and subjected to RT-PCR. The quantification data are mean ± SEM of three different experiments.

Effects of prolactin on IGF-I and IGF-I receptor mRNA expression

The effect of prolactin on IGF-I and IGF-I receptor mRNA expression was examined by incubating GG-CL cells expressing the prolactin receptor with different concentrations of prolactin. Prolactin stimulated the expression of IGF-I mRNA significantly, but not that of IGF-I receptor (Fig. 3).

Effects of IGF-I on oestrogen receptor β and IGF-I receptor mRNA expression

GG-CL cells were incubated with IGF-I at different concentrations to examine the effects of IGF-I on oestrogen receptor β mRNA. The result indicates that IGF-I elicits a dose-dependent increase in oestrogen receptor β expression (Fig. 4). Urban *et al.* (1994) reported that IGF-I downregulates IGF-I receptor mRNA expression in pig granulosa cells. Thus, the effect of IGF-I on IGF-I receptor mRNA expression was also examined. IGF-I had no effect on IGF-I receptor mRNA expression in GG-CL cells (data not shown).

Developmental changes in IGF-I and IGF-I receptor mRNA concentrations in the corpus luteum throughout pregnancy and after parturition

Corpora lutea were isolated on different days of pregnancy, the day of parturition, or the day after parturition

to investigate the developmental changes in IGF-I and IGF-I receptor mRNA. IGF-I mRNA is constitutively expressed in the corpus luteum throughout pregnancy, but increases significantly from day 20 (Fig. 5a). In contrast, the concentration of IGF-I receptor mRNA reaches a peak at day 9, and gradually decreases thereafter (Fig. 5b).

Discussion

In pregnant rats, two luteal cell populations, small and large luteal cells, form the corpus luteum (Gibori, 1993). Although the origin of the two luteal cell populations is uncertain, it is hypothesized that luteal cell size does not depend on follicular origin, but rather on the responsiveness of individual luteal cells to growth signals (Gibori, 1993). It has been reported that large luteal cells respond to IGF-I stimulation and contain the bulk of the IGF-I receptors (Parmer *et al.*, 1991), and that it is this population that undergoes hypertrophy during the development of the corpus luteum (McLean *et al.*, 1992). The results of the present study indicate a possible mechanism for luteal cell hypertrophy. Prolactin stimulates luteal production of IGF-I, which acts on luteal cells expressing the IGF-I receptor, such as the large luteal cells, to stimulate oestradiol receptor expression. Greater numbers of oestrogen receptors may allow the cells to respond fully to oestradiol stimulation, leading to cell hypertrophy and an increase in the size of the corpus luteum.

Oestradiol upregulates mRNA expression for both IGF-I and IGF-I receptor in non-steroidogenic tissues (Murphy and

Fig. 3. Effect of prolactin on (a) insulin-like growth factor I (IGF-I) and (b) IGF-I receptor mRNA expression in a luteal cell line (GG-CL) derived from the large luteal cells of the pregnant rat corpus luteum. GG-CL cells stably transfected with the long form of the prolactin receptor were incubated with prolactin (0.01, 0.1 and 1.0 µg ml⁻¹) for 8 h at 39°C. Total RNA was isolated and subjected to RT-PCR. The quantification data are mean ± SEM of three different experiments. a, $P < 0.01$ versus control.

Fig. 4. Effect of insulin-like growth factor I (IGF-I) on oestrogen receptor β mRNA expression in a luteal cell line (GG-CL) derived from the large luteal cells of the pregnant rat corpus luteum. GG-CL cells were incubated with recombinant human IGF-I (5, 50 and 100 ng ml⁻¹) for 8 h at 39°C. Total RNA was isolated and subjected to RT-PCR. The quantification data are mean ± SEM of three different experiments. b, $P < 0.05$ versus control.

Ghahary, 1990; Adesanya *et al.*, 1996; Clarke *et al.*, 1997), but downregulates expression of both mRNAs in the rat corpus luteum (Parmer *et al.*, 1991). A luteal cell line (GG-CL) derived from the large luteal cells of the pregnant rat corpus luteum (Sugino *et al.*, 1998) was used to examine whether oestradiol-induced downregulation is mediated by progesterone. GG-CL cells express IGF-I, IGF-I receptor, oestradiol receptor β and the glucocorticoid receptor that serves as a receptor for progesterone in the rat corpus luteum (Zilberstein *et al.*, 1991; Sugino *et al.*, 1997, 1998; Telleria *et al.*, 1998). The advantage of GG-CL cells is that they do not

secrete progesterone (Sugino *et al.*, 1997, 1998) and thus the possibility that oestradiol action is mediated by progesterone is eliminated. Another advantage of these cells is that they are responsive to progesterone (Sugino *et al.*, 1997). The results of the present study indicate that oestradiol downregulation of IGF-I and IGF-I receptor mRNA expression in luteal cells is not mediated by progesterone.

Parmer *et al.* (1991) reported a synergistic effect of IGF-I and oestradiol on progesterone production by large luteal cells. The present study indicates that this synergistic effect involves, at least in part, IGF-I-stimulated oestrogen receptor

Fig. 5. Developmental expression of (a) insulin-like growth factor I (IGF-I) and (b) IGF-I receptor mRNA in the rat corpus luteum throughout pregnancy and after parturition. Total RNA isolated on each day was subjected to RT-PCR. RNA samples were collected from more than three animals on each day and in each experiment. P0 and P1 are day 0 and day 1 post partum, respectively. The quantification data are mean $\pm$ SEM of three different experiments. a, $P < 0.01$ versus day 4–18; b, $P < 0.01$ versus day 4, 6 and 12; c, $P < 0.01$ versus day 15–P1.

mRNA expression. Therefore, IGF-I may sensitize luteal cells to oestradiol action by upregulating oestradiol receptor expression. This is supported by the finding that ligands for IGF-I receptor modulate the regulation of oestrogen receptor expression in a breast cancer cell line (Clayton *et al.*, 1997).

Prolactin is known to enhance overall protein synthesis at the translational level (Albarracin *et al.*, 1994) and to upregulate the expression of the oestradiol receptor (Telleria *et al.*, 1998). The results of the present study show that prolactin upregulates IGF-I mRNA expression and that IGF-I stimulates oestradiol receptor expression in luteal cells. This indicates that prolactin stimulation of both protein synthesis and oestradiol receptor expression is mediated, at least in part, by IGF-I.

The present study also examined the developmental changes in IGF-I and IGF-I receptor mRNA in the corpus luteum throughout pregnancy. The results reveal that mRNA for IGF-I and its receptor are not expressed in tandem. Whereas concentrations of IGF-I mRNA increase substantially at the end of pregnancy, beginning from day 20, concentrations of IGF-I receptor mRNA are higher in early pregnancy and decrease from day 12. Tamada *et al.* (1995) reported that expression of IGF-I mRNA is high at the end of pregnancy. In rats, prolactin and placental lactogens (rPL-I and -II) secreted by the placenta may be responsible for maintaining the concentration of IGF-I mRNA until day 20. However, these hormones are not responsible for the increase in IGF-I mRNA expression after day 20 since, at this stage, a decrease in prolactin receptor expression occurs in the corpus luteum, which becomes non-responsive to prolactin and placental

lactogen stimulation (Telleria *et al.*, 1997). The increase in IGF-I expression at the end of pregnancy and after parturition, at a time when the corpus luteum is undergoing both functional and structural luteolysis, is intriguing. However, the increase in IGF-I binding protein 3 in corpora lutea undergoing luteolysis (Erickson *et al.*, 1993) probably neutralizes the action of IGF-I. The expression of IGF-I receptor mRNA in the corpus luteum appears to be the opposite to that of IGF-I. However, the decline in the number of receptors does not appear to be induced by IGF-I, since IGF-I had no effect on IGF-I receptor expression, and it may be due to oestradiol produced locally by the corpus luteum. Oestradiol concentrations in the corpus luteum increase in the second half of pregnancy (Taya and Greenwald, 1981) and oestradiol inhibits IGF-I receptor expression both *in vivo* (Parmer *et al.*, 1991) and *in vitro*, as observed in the present study.

In summary, the results of the present study indicate that prolactin stimulates the expression of IGF-I mRNA in luteal cells, which in turn upregulates the expression of oestradiol receptor β . The results also indicate that there is a feedback mechanism between IGF-I and oestradiol action. Whereas IGF-I stimulates oestradiol receptor expression, oestradiol downregulates the concentration of the IGF-I receptor and its ligand.

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