

Regulation of steroid synthesis and apoptosis by insulin-like growth factor I and insulin-like growth factor binding protein 3 in human corpus luteum during the midluteal phase

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The aim of the present study was to investigate the action of insulin-like growth factor I (IGF-I) and insulin-like growth factor-binding protein 3 (IGFBP-3) on steroidogenesis and apoptosis in human corpus luteum during the midluteal phase. Slices from corpora lutea were incubated for 4 h with IGF-I or IGFBP-3. Progesterone, oestradiol, androstenedione and testosterone concentrations were determined by radioimmunoassay; caspase 3 expression was assessed by immunohistochemistry; *bcl-2*, *bax* and *P_{450arom}* expression were assessed by RT-PCR; and apoptosis was detected by *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling. The results showed that addition of IGF-I stimulated progesterone

production (150%, $P < 0.01$), oestradiol production (65%, $P < 0.05$) and *bcl-2* gene expression (approximately 200%, $P < 0.05$), but decreased apoptosis ($P < 0.05$). In contrast, IGFBP-3 reduced steroid production and increased *bax* gene expression and the percentage of apoptotic cells ($P < 0.05$). Neither IGF-I nor IGFBP-3 had an effect on *P_{450arom}* expression or on the concentrations of its substrates. However, maximum expression of caspase 3 was detected in corpus luteum during the midluteal phase. In conclusion, these results indicate that IGF-I and IGFBP-3 act as regulatory peptides of the function of the human corpus luteum during the midluteal phase. This action may be direct or mediated by steroid production or by *bcl-2*–*bax* expression.

Introduction

The human corpus luteum is an endocrine gland of transient feature in a non-fertile cycle, which has recognizable stages of growth, maximum development and involution. The cell composition of the corpus luteum (Lei *et al.*, 1991; Bukovsky *et al.*, 1995; Carrasco *et al.*, 1996) and its secreted compounds contribute to the rapid development, maturity and regression of the corpus luteum.

Several molecules act as intraovarian regulators of the lifespan of the human corpus luteum, including cytokines, growth factors, reactive oxygen species (Vega *et al.*, 1995; Johnson *et al.*, 1996, 1999; Brannstrom *et al.*, 1999), peptide hormones, steroids (Vega *et al.*, 1994a) and members of the Bcl-2 family (Rodger *et al.*, 1995, 1998; Sugino *et al.*, 2000). Maximum steroidogenic activity in the corpus luteum occurs during the midluteal phase, as evidenced by progesterone and oestradiol production, and, thereafter, a gradual decline in steroid synthesis occurs, representing the involution period of the corpus luteum (Vega *et al.*, 1994b).

Insulin-like growth factor I (IGF-I), IGF-II, IGF receptor type 1 (RIGF-1) and IGF-binding proteins 1–6 (IGFBP-1–6)

are present in the human corpus luteum throughout the luteal phase (Iñiguez *et al.*, 2001). However, IGFBP-3 is the most abundant circulating binding protein for IGFs (Drop *et al.*, 1992). IGF-I regulates human luteal steroidogenesis in a cell culture system by an increase in progesterone (Devoto *et al.*, 1995) and oestradiol production (Johnson *et al.*, 1996). Furthermore, IGF-I has an important anti-apoptotic effect in various tissues (Morales *et al.*, 2000; Remacle *et al.*, 2000) and regulates the relative concentrations of some members of the Bcl-2 family (Dews *et al.*, 2000).

Several authors have suggested that luteal regression in many species, including humans (Shikone *et al.*, 1996; Vega *et al.*, 2000), occurs by programmed cell death or apoptosis. Addition or withdrawal of steroid hormones from target tissues may induce apoptosis (Rotello *et al.*, 1992; Tenniswood *et al.*, 1992). In addition, oestradiol exerts an anti-apoptotic effect in several tissues and cell lines (Gompel *et al.*, 2000; Razandi *et al.*, 2000), although it is not known whether oestradiol exhibits an anti-apoptotic action in the human corpus luteum. However, the action of progesterone on luteal cell viability is controversial. In fact, some studies have shown that progesterone is able to decrease Bcl-2 concentrations, exerting a pro-apoptotic action (Gompel *et al.*, 2000). In contrast, Matsuo *et al.* (1997) showed an anti-apoptotic action of progesterone in human uterine leiomyoma.

Members of the Bcl-2 family may be of importance in

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controlling the rate of apoptosis (Bissonette *et al.*, 1992; Yin *et al.*, 1994); for example, Bcl-2 is known to protect cells from cell death. The activity of Bcl-2 is determined by its interaction with Bax, a pro-apoptotic protein related to the Bcl-2 family; thus, the relative ratio of Bcl-2 to Bax expression, rather than the absolute concentrations, is the critical determinant for a cell to enter apoptosis.

Immunohistochemical (Rodger *et al.*, 1995; Sugino *et al.*, 2000) and biochemical studies (Vega *et al.*, 2000) have shown that Bcl-2 and Bax are present in human corpus luteum. Rodger *et al.* (1995) reported that the expression of the product of the proto-oncogene *bcl-2* remains unchanged throughout the luteal phase, but Sugino *et al.* (2000a) reported that *bcl-2* mRNA concentrations were highest in the corpus luteum during the midluteal phase, and that *bax* mRNA concentrations were highest in the late human corpus luteum. Members of the Bcl-2 family, via dimeric interactions with other molecules that regulate cell death (Monney *et al.*, 1996; Zou *et al.*, 1997; Hu *et al.*, 1998), are important for regulating activation of members of the caspase family (Alnemri *et al.*, 1996), which serve as regulators and effectors of apoptosis (Thornberry, 1997; Thornberry *et al.*, 1997). Many members of the caspase family are expressed in the ovary (Maravei *et al.*, 1997), including in the human corpus luteum, where caspase-3 has been reported to be abundantly expressed, and thus it has been speculated that this protease is important for luteal regression (Krajewska *et al.*, 1997). Nevertheless, little is known regarding the function of caspases in the human corpus luteum.

The mechanisms of regulation of the function of the human corpus luteum during the midluteal phase were investigated by studying the effect *in vitro* of IGF-I and IGFBP-3 on progesterone, oestradiol, androstenedione and testosterone production, and on the transcription of Bcl-2 family genes related to apoptosis, such as *bcl-2* and *bax*, in human corpora lutea obtained during the midluteal phase.

Materials and Methods

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO); culture media were obtained from Gibco BRL Life Technologies (Grand Island, NY). Human recombinant IGF-I was obtained from NHPP, NIDDK and A. F. Parlow (National Hormone and Peptide Program, Torrance, CA), and IGFBP-3 was obtained from Research Diagnostic Inc. (Flanders, NJ). Progesterone, oestradiol, androstenedione and testosterone concentrations were measured by radioimmunoassay from Diagnostic System Laboratories (Webster, TX) and the concentration of LH was measured by radioimmunoassay from Diagnostic Products Corporation (Los Angeles, CA). Primers, DNase I (Amp Grade), TRIzol, SuperScript RT II, and Taq DNA Polymerase were purchased from Gibco BRL Life Technology (Bethesda, MD). Apoptosis was detected by the fluorescein kit (Promega, Madison, WI). Monoclonal anti-active caspase 3 (mAb C92-265) was obtained from PharMingen (San Diego,

CA), and the second antibody and reagents for the detection system were obtained from Dako Corp. (Carpinteria, CA).

Subjects

Corpora lutea were obtained from seven women aged 35.6 ± 1.34 years (range 31–40 years) with regular menstrual cycles, with a high parity, undergoing mini-laparotomy for tubal sterilization at the San Borja Arriarán Clinical Hospital, University of Chile (National Health Service, Santiago, Chile). None of the patients had experienced infertility or endometriosis, or had received any hormone therapy during the previous 3 months. The patients gave their written informed consent to participate in this study, which was approved by the Hospital Ethics Committee. The date of the cycle of each woman was confirmed by several criteria, including biopsies of the corpora luteal and endometrium (Noyes *et al.*, 1950; Corner, 1956), ultrasonography, occurrence of the preovulatory LH peak and progesterone serum concentrations. The corpora lutea classified as midluteal were obtained 5–9 days after ovulation ($n = 7$); serum progesterone concentrations (mean \pm SEM) in the midluteal phase were 30.5 ± 6.9 nmol l⁻¹. The preovulatory LH peak serum concentration fluctuated between 21.9 iu l⁻¹ and 56.6 iu l⁻¹ (first International Reference Preparation number 68/40).

Tissue preparation

After removal from the ovary, the corpora lutea were collected in sterile PBS and transported immediately to the laboratory at room temperature. One piece of each corpus luteum was immediately placed in 4% (w/v) formalin and PBS for histological evaluation (Corner, 1956). The rest of the tissue was cut into slices (15–60 mg wet mass). Two or three slices from each corpus luteum (explants) for each experimental condition (basal, IGF-I or IGFBP-3) were incubated separately.

Incubation procedures

Tissue slices were incubated as reported by Vega *et al.* (2000). In brief, slices of corpus luteum from the midluteal phase were incubated with Hank's media, supplemented with 0.1 mg glutamine ml⁻¹, 1 mg BSA ml⁻¹, 26 mmol NaHCO₃ l⁻¹, 25 mmol Hepes l⁻¹, antibiotics (60 iu penicillin ml⁻¹ and 5 mg streptomycin ml⁻¹) (pH 7.4), to give a final volume of 0.5 ml. Basal and treated conditions (3.2–6.4 nmol IGF-I l⁻¹ and 6.9–13.8 nmol IGFBP-3 l⁻¹) were carried out in duplicate. Incubations were terminated at 4 h and the media were stored at –20°C until assay for progesterone, oestradiol, androstenedione and testosterone by specific radioimmunoassay. After incubation, one tissue slice was stored at –70°C for expression studies of mRNA for *bcl-2*, *bax*, *P_{450arom}* and β -actin, and another slice was fixed and embedded in paraffin wax for immunohistochemical identification of caspase 3 and for *in situ* detection of cell death.

Radioimmunoassay

Progesterone and oestradiol concentrations were determined as described by Iñiguez *et al.* (2001) and the intra-assay and interassay coefficients of variation (CV) determined in the culture medium were, respectively, for progesterone: 3.8 and 5.5%; and for oestradiol: 4.1 and 6.7%. Androstenedione and testosterone were determined using commercial kits (DSL) and intra-assay and interassay CVs determined in culture medium were, respectively, for androstenedione: 3.2 and 6.1%; and for testosterone: 6.2 and 8.1%.

Immunohistochemical study

The immunohistochemical study for caspase 3 was performed in duplicate on human corpora lutea from the midluteal phase. Tissue sections (5–6 μm) were deparaffinized in xylene, hydrated in 100, 95, 85, 70 and 50% ethanol. Endogenous peroxidase activity was inhibited using 3% (w/v) hydrogen peroxide for 15 min, and then sections were blocked with PBS–2% (w/v) BSA for 60 min. The primary antibody for caspase 3 (applied at 1:100 dilution) is a purified anti-active caspase 3 monoclonal antibody that recognizes the active form of caspase 3 in human and mouse cells (Thornberry and Lazebnik, 1998). After incubation for 1 h at 37°C, the second antibody, a biotinylated anti-mouse-anti-rabbit-anti-goat mixture immunoglobulin, was applied for 30 min. The reaction was evaluated by streptavidin–biotin peroxidase conjugate using 3,3'-diaminobenzidine (DAB) as the chromogen, and cell nuclei were stained with haematoxylin. PBS–1% (w/v) BSA was used instead of the primary antibody for the negative control, and no specific immunoreactivity was detected in these sections. The slides were analysed in a Nikon optical microscope (Nikon Inc., Melville, NY).

RNA preparation, cDNA synthesis and RT–PCR assessment

Total RNA was extracted from frozen corpora lutea from the midluteal phase, which were incubated in the absence (basal condition) or in the presence (treated conditions) of IGF-I or IGFBP-3. The extraction procedure of mRNA and the method of cDNA synthesis were those described by Johnson *et al.* (1997). A PCR fragment of 383 bp was generated with primers based on sequences 1894–1914 upstream (5'-AGG ATT GTG GCC TTC TTT GAG-3') and 2276–2256 downstream (5'-CCT GCA GCT TTG TTT CAT GGT-3') of a human follicular lymphoma *bcl-2* cDNA (Tsujimoto and Croce, 1986). Another PCR fragment of 334 bp was generated with primers based on sequences 39–56 upstream (5'-CAC CAG CTC TGA GCA GAT-3') and 373–356 downstream (5'-CAG CCT TGA GCA CCA GTT-3') of a human *εbax* cDNA (NIDig 4558699). Amplification was performed by adding 2 μl cDNA to a total volume of 25 μl PCR buffer containing 3 mmol MgCl_2 l^{-1} , 0.625 U Taq DNA polymerase, and 0.25 μmol l^{-1} of each primer. *bcl-2*

and *bax* were confirmed to correspond to human genes by direct sequencing using an ABI PRISM 310 analysis system (Perkin Elmer). The amplified fragments for *P_{450arom}* and reaction conditions were as described by Vega *et al.* (2000). A fragment of 260 and 661 bp of β -actin were amplified under the same conditions as described above to act as an internal control. Both *bcl-2* and *bax* cDNA amplifications were repeated for 30 cycles and β -actin and *P_{450arom}* cDNA amplifications for 25 cycles, as described by Vega *et al.* (2000). All gene amplifications were within the linear range. cDNA synthesis and PCR variability were checked using control RNA with the respective control primers, as indicated by the manufacturer (Gibco BRL). The concentrations of *bcl-2*, *bax*, *P_{450arom}* and β -actin obtained for RT–PCR (semi-quantitative assay) were evaluated by computerized analysis of absorbance of the gel using Kodak 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY).

In situ detection of apoptosis by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) method

Nuclear DNA fragmentation was performed as described by Vega *et al.* (2000). In brief, sections of corpora lutea from the midluteal phase, 5–6 μm in thickness, were mounted on silane-coated slides, deparaffinized with xylene and rehydrated, and TdT-mediated dUTP nick-end labelling (TUNEL) was performed using the apoptosis detection fluorescein kit, according to the specifications of the manufacturer. Slides were analysed by fluorescence microscopy with a wide band excitation barrier filter suitable for analysing both green (fluorescein-labelled fragmented DNA) and red (propidium iodide counterstain identifying cell nuclei) fluorescence. The different optical fields (magnification \times 400) were selected randomly, and at least 2000 cells were counted for each sample. Positive control tissues were rat breast carcinoma and corpora lutea samples pretreated with DNase (5 μg ml^{-1} , type I). Negative controls (sections treated without the TdT enzyme) were included in all assays to estimate non-specific binding and auto fluorescence. Necrosis was not observed as assessed by morphological criteria.

Statistical analysis

The data are presented as mean \pm SEM and the results were analysed using the non-parametric test of ANOVA and the Newman–Keuls multiple comparison test. Differences were considered significant at $P < 0.05$.

Results

Effect of IGF-I or IGFBP-3 on progesterone production in human corpus luteum from the midluteal phase

Explants of human corpus luteum from the midluteal phase were cultured for 4 h with or without different

Table 1. Effect of insulin-like growth factor I (IGF-I) or IGF-binding protein 3 (IGFBP-3) on progesterone and oestradiol concentrations in culture media of explants of human corpora lutea during the midluteal phase

Addition (nmol l ⁻¹)	Progesterone	Oestradiol
Basal	14.1 ± 2.1 (15)	267.8 ± 24.6 (15)
IGF-I (3.2)	23.9 ± 2.9 (12) ^a	297.7 ± 65.3 (13)
IGF-I (6.4)	35.06 ± 3.19 (14) ^{ab}	441.0 ± 60.0 (14) ^c
IGFBP-3 (6.9)	5.4 ± 0.5 (4) ^a	250.9 ± 40.9 (5)
IGFBP-3 (13.8)	6.5 ± 0.5 (8) ^a	156.1 ± 21.9 (8) ^a

Values are expressed as nmol per g of corpus luteum ± SEM.

Value in parentheses is the number of explants studied in five basal corpora lutea, five corpora lutea treated with IGF-I and in four corpora lutea treated with IGFBP-3.

^a*P* < 0.01 versus corresponding basal values; ^b*P* < 0.001 versus IGF-I (3.2 nmol l⁻¹); ^c*P* < 0.05 versus corresponding basal condition.

concentrations of IGF-I or IGFBP-3 to determine whether IGF-I or IGFBP-3 had an effect on luteal progesterone production. In the presence of IGF-I, progesterone production by explants of human corpus luteum from the midluteal phase increased by 69.5 ± 0.9% with 3.2 nmol IGF-I l⁻¹ (*P* < 0.01) and by 148.6 ± 0.16% with 6.4 nmol IGF-I l⁻¹ (*P* < 0.01) compared with basal values (Table 1). Progesterone production in the presence of 6.4 nmol IGF-I l⁻¹ was significantly higher than it was with 3.2 nmol IGF-I l⁻¹ (*P* < 0.01), indicating a dose-dependent stimulatory action of IGF-I on progesterone production by luteal explants. In contrast, IGFBP-3 elicited a decrease in progesterone production of 61.7 ± 0.2% with 6.9 nmol IGFBP-3 l⁻¹ and of 53.9 ± 0.2% with 13.8 nmol IGFBP-3 l⁻¹ (Table 1, *P* < 0.01).

Effect of IGF-I or IGFBP-3 on oestradiol production in human corpus luteum from the midluteal phase

Explants of human corpus luteum from the midluteal phase were cultured for 4 h with or without different concentrations of IGF-I or IGFBP-3 to determine whether IGF-I or IGFBP-3 had an effect on luteal oestradiol production. An increase of 64.7 ± 16.35% in oestradiol concentration was observed with 6.4 nmol IGF-I l⁻¹ compared with basal values (*P* < 0.05), whereas no stimulatory effect was observed with a lower dose of IGF-I (Table 1). However, oestradiol concentration decreased 41.7 ± 6% (*P* < 0.01) in the presence of 13.8 nmol IGFBP-3 l⁻¹, and no effect was observed with 6.9 nmol IGFBP-3 l⁻¹ (Table 1).

Effect of IGF-I or IGFBP-3 on androstenedione and testosterone production and on P_{450arom} expression in human corpus luteum from the midluteal phase

Explants from human corpora lutea from the midluteal phase were cultured for 4 h with or without IGF-I (6.4 nmol l⁻¹) or IGFBP-3 (13.8 nmol l⁻¹) to determine whether these substances had an effect on luteal androstenedione and testosterone production or on P_{450arom} gene expression. No

significant effects of IGF-I or IGFBP-3 were observed on androstenedione (basal: 0.84 ± 0.3 nmol per g of corpus luteum) or testosterone production (basal: 2.8 ± 0.9 nmol per g of corpus luteum). Similarly, expression of normalized P_{450arom} mRNA was not changed significantly in the presence of IGF-I (1.29 ± 0.09 arbitrary units) or IGFBP-3 (1.5 ± 0.13 arbitrary units) compared with basal values (1.29 ± 0.26 arbitrary units).

Expression of caspase 3 in human corpus luteum from the midluteal phase

The presence of caspase 3 was detected in human corpus luteum from the midluteal phase (Fig. 1a). Positive cells for active caspase 3, indicated by abundant punctuate cytoplasmic staining, were observed within the parenchyma of all corpora lutea examined. In addition, some luteal cells of the stroma were weakly stained and endothelial cells displayed negative staining. Sections for negative control were free from staining (Fig. 1b).

Effect of IGF-I and IGFBP-3 on bcl-2 and bax gene expression in human corpus luteum from the midluteal phase

Explants from human corpora lutea from the midluteal phase were cultured for 4 h with or without IGF-I (6.4 nmol l⁻¹) or IGFBP-3 (13.8 nmol l⁻¹) to determine whether these substances had an effect on expression of *bcl-2* and *bax* mRNA. The expression of *bcl-2* mRNA was increased by IGF-I by approximately 200% (*P* < 0.05), whereas in the presence of IGFBP-3, *bcl-2* mRNA expression was similar to the basal condition (Fig. 2a). Expression of *bax* was not modified by the action of IGF-I, but IGFBP-3 induced a significant increase in *bax* mRNA expression during culture (*P* < 0.05) (Fig. 2b). Accordingly, the ratio of *bcl-2* to *bax* mRNA expression increased significantly by 150% in the presence of IGF-I (*P* < 0.05), whereas a 50% decrease was observed in the presence of IGFBP-3 (Fig. 2c).

Effect of IGF-I and IGFBP-3 on apoptosis in human corpus luteum from the midluteal phase

The TUNEL method was used to detect the percentage of cells from human corpora lutea from the midluteal phase with apoptotic positive signals. This percentage decreased 43 ± 8.3% in the presence of 6.4 nmol IGF-I l⁻¹ and increased 140 ± 14.5% in the presence of 13.8 nmol IGFBP-3 l⁻¹, compared with basal values (*P* < 0.05) (Fig. 3).

Discussion

Maximum steroidogenic activity in the corpus luteum occurs during the midluteal phase and a gradual decline in steroid synthesis represents the involution period of the tissue. Luteal regression is necessary for the cyclicity of the reproductive process, and occurs by the synchronous loss of cellular function and subsequent cell death by apoptosis.

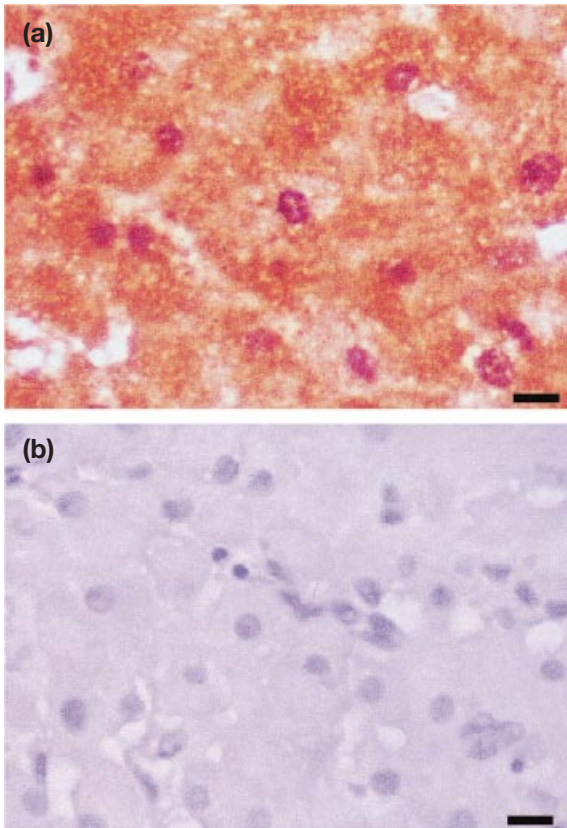


Fig. 1. Immunohistochemical staining for caspase 3 in explants of human corpus luteum from the midluteal phase. (a) Representative corpus luteum explants with positive immunostaining for caspase 3 protein in paraffin wax sections of three corpora lutea from the midluteal stage. (b) Negative control. Scale bars represent 30 μm .

This process is triggered by the activation of a proteolytic cascade of cysteine aspartate-specific proteases, namely caspases, and caspase 3 is the main downstream effector of cell death (Boone and Tsang, 1998). The results of the present study showed a high expression of active caspase 3 in corpus luteum from the midluteal phase, indicating that the active enzyme is important for luteal regression and that programmed cell death may be triggered during the midluteal phase, as proposed by Vega *et al.* (1994b).

The components of the IGF-IGFBP system are expressed in the human corpus luteum and play a role in the regulation of its lifespan (Iñiguez *et al.*, 2001). The present results indicate that IGF-I, a multifunctional monomeric peptide, regulates the steroidogenic function in human corpus luteum during the midluteal phase, increasing progesterone and oestradiol production. Párrizas and Loroith (1997) suggested that IGF-I acts as a cell survival factor and inhibits apoptosis in a number of types of cell through modulation of the activity of Bcl-2 family members, with the concomitant regulation of the apoptotic mechanism in most cells (O'Connor *et al.*, 2000). The protective action of Bcl-2 in cells is determined by its interaction with Bax, a pro-apoptotic gene; the ratio of bcl-2 to bax expression, rather than

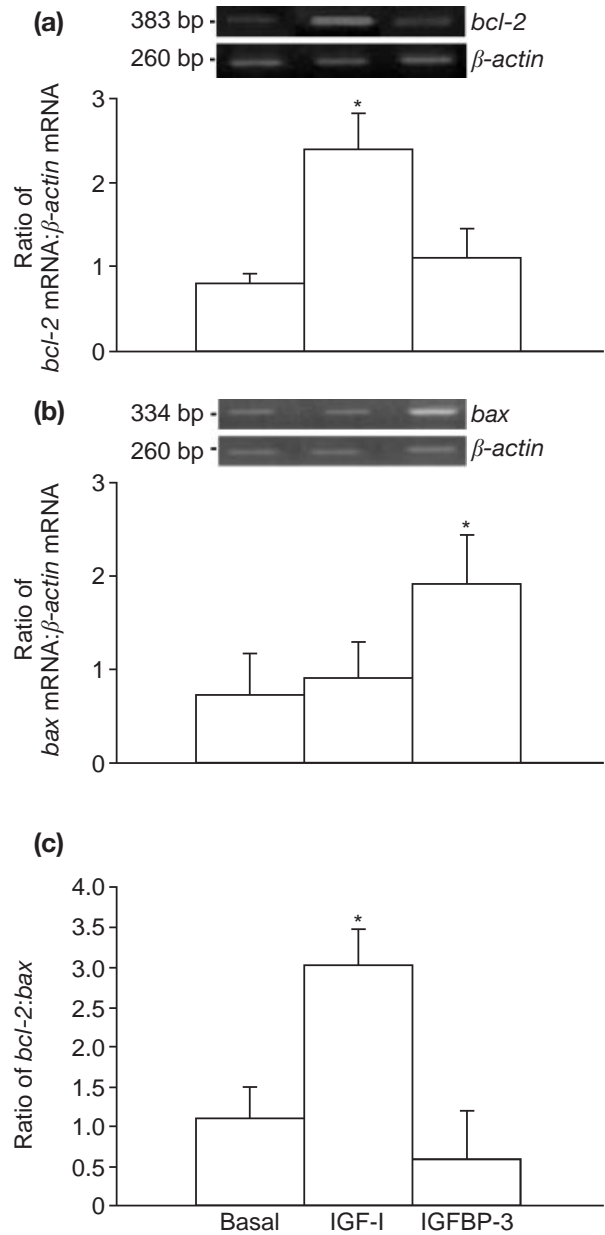


Fig. 2. Effect of insulin-like growth factor I (IGF-I) or IGF-binding protein 3 (IGFBP-3) on *bcl-2* and *bax* mRNA expression in human corpus luteum from the midluteal phase. Normalized yield of (a) *bcl-2* and (b) *bax* PCR fragments relative to β -actin PCR products. (c) Relative ratio of *bcl-2* to *bax* mRNA expression in human corpus luteum from the midluteal phase. Tissue explants were incubated for 4 h without (basal: *bcl-2*, three corpora lutea, $n = 4$ slices; *bax*, four corpora lutea, $n = 7$ slices) or with IGF-I (*bcl-2*, three corpora lutea, $n = 5$ slices; *bax*, two corpora lutea, $n = 3$ slices) or IGFBP-3 (*bcl-2*, two corpora lutea, $n = 4$ slices; *bax*, two corpora lutea, $n = 2$ slices). The ratio of *bcl-2* to *bax* was calculated from each sample in (a) and (b). * $P < 0.05$ compared with basal values.

the absolute concentration, is the critical determinant of cell fate. These reports are in agreement with the results obtained in the present study indicating that IGF-I facilitates cell survival in corpus luteum during the midluteal phase,

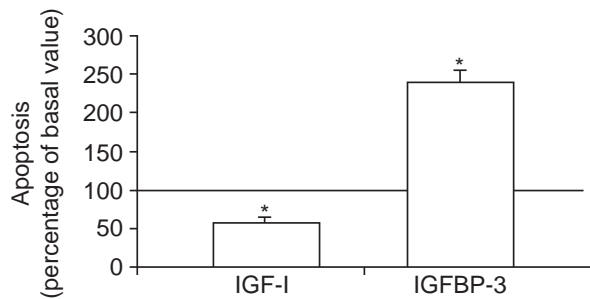


Fig. 3. Apoptotic cell death *in situ* was assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) in human corpus luteum from the midluteal phase in the presence of insulin-like growth factor I (IGF-I) or IGF-binding protein 3 (IGFBP-3). Tissue explants obtained from human corpora lutea from the midluteal phase ($n = 5$) were incubated for 4 h in the presence of 6.4 nmol IGF-I l^{-1} or 13.8 nmol IGFBP-3 l^{-1} . Values are expressed as percentage \pm SEM of positive green fluorescence luteal cells (a mean of 2000 cells counted for each corpus luteum). * $P < 0.05$ compared with basal values.

mainly by a positive action on *bcl-2* gene expression. Even though the expression studies were performed using RT-PCR, a semi-quantitative technique, this finding is consistent with data showing that, in the presence of 6.4 nmol IGF-I l^{-1} , the number of luteal cells undergoing apoptosis decreases in corpus luteum from the midluteal phase. These results indicate that there is a relationship between *bcl-2* expression and apoptosis in corpus luteum during the midluteal phase. It will be important to determine the mechanism by which IGF-I sustains the expression of *bcl-2* in the human corpus luteum during the midluteal phase.

IGFBP-3 is the principal carrier of IGF-I in the circulation and the primary regulator of the amount of free IGF-I available to interact with the IGF-I receptor. IGFBP-3 is produced in large amounts by the human corpus luteum during its lifespan (Iñiguez *et al.*, 2001). However, there is accumulating evidence that IGFBP-3 can also cause apoptosis in a manner that is independent of IGF (Grimberg, 1999). The results obtained in the present study showed that addition of 6.4 nmol IGFBP-3 l^{-1} reduced progesterone and oestradiol production and increased the percentage of cells with apoptotic positive signals, coincident with the increase in *bax* gene expression, which is elicited by IGFBP-3. These results indicate that IGFBP-3 inhibits the protective action of endogenous IGF-I, although a potential IGF-I-independent action of IGFBP-3 on luteal cell viability cannot be ruled out.

One of the mechanisms involved in the enhancement or diminution of oestradiol production in human corpus luteum by IGF-I and IGFBP-3 may be attributable to an effect on the transcription of the cytochrome $P_{450arom}$. $P_{450arom}$ mRNA is expressed in human corpora lutea of different ages and exhibits a decreased profile throughout the luteal phase (Vega *et al.*, 2000). Nevertheless, it is possible that aromatase activity was affected in the experimental

system used in the present study. Addition of 6.4 nmol IGF-I l^{-1} or 13.8 nmol IGFBP-3 l^{-1} did not affect $P_{450arom}$ gene expression in corpus luteum from the midluteal phase; however, IGF-I increased, and IGFBP-3 decreased, oestradiol production. In this regard, it is probable that the incubation period used in the experimental model of the present investigation did not allow visualization of an effect of IGF-I and IGFBP-3 on aromatase RNA expression. However, in human corpus luteum from the midluteal phase, other stimuli have been shown to regulate the expression of $P_{450arom}$ mRNA in the same experimental system (Vega *et al.*, 2000). These findings raised the question of whether locally produced IGFBPs can affect cell response to IGF-I.

Furthermore, production of androstenedione and testosterone, which are $P_{450arom}$ substrates, was not changed by the action of IGF-I and IGFBP-3. Therefore, in the experimental model used in the present study, the increase in oestradiol production probably reflects the stimulatory action of IGF-I upstream of the steroidogenic pathway, in the conversion of cholesterol to pregnenolone and the concomitant increase in progesterone synthesis. Steroidogenic acute regulatory (StAR) protein stimulates the translocation of cholesterol from the outer to the inner mitochondrial membrane, and IGF-I is known to stimulate StAR mRNA and protein expression in human granulosa-lutein cells (Devoto *et al.*, 1999).

In conclusion, the results of the present study indicate that IGF-I and IGFBP-3 act as regulatory peptides of human corpus luteum function during the midluteal phase. This action may be direct or mediated by the regulation of progesterone and oestradiol synthesis or by the expression of genes related to cell survival, such as *bcl-2* and *bax*. Collectively, this information provides knowledge about the various regulatory mechanisms of human corpus luteum from the midluteal phase. A better understanding of the molecules that regulate corpus luteum function at the time of implantation may contribute to future studies of recurrent pregnancy loss of unknown aetiology.

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