Effect of nitric oxide on the expression of insulin-like growth factors and insulin-like growth factor binding proteins throughout the lifespan of the human corpus luteum

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The presence of insulin-like growth factors (IGF), IGF binding proteins (IGFBP) and IGF receptor type 1 (IGF-IR) in the human corpus luteum was investigated by examining the expression and production of related proteins throughout the lifespan of the corpus luteum and the action of nitric oxide upon their production. The expression of proteins in corpora lutea from the early, mid- and late luteal phases was assessed by immunohistochemistry, evaluated by a semi-quantitative analysis and the functional study was performed in corpus luteum explants incubated with nitric oxide donors. IGF-I and -II and IGFBP-1 and -3 were measured in the culture media by specific immunoassays. The results showed that IGF-I and -II, IGFBP-1 to -6 and IGF-IR were detected in the human corpus luteum throughout the luteal phase. Moreover, the expression and production of IGF-I and IGFBP-1 increased progressively from corpora lutea from the early to late

luteal phases ($P < 0.05$), whereas the expression and production of IGFBP-2, -4 and -5 were significantly higher in corpora lutea from the mid-luteal phase ($P < 0.05$). No differences were observed in the expression of IGF-II, IGFBP-3 and -6 and IGF-IR throughout the lifespan of the corpus luteum. However, functional studies showed that nitric oxide donors elicited a stimulatory action on production of IGF-I in corpora lutea from the early luteal phase (80%) and on production of IGFBP-1 in corpora lutea from the late luteal phase (50%) ($P < 0.05$), whereas production of IGF-II and IGFBP-3 was not affected by nitric oxide. In conclusion, the components of the IGF-IGFBP system are expressed in the human corpus luteum throughout its lifespan. Nitric oxide regulates IGF-I and IGFBP-1 production, indicating that the growth factors may serve, at least in part, as mediators of the action of nitric oxide in the human corpus luteum.

Introduction

The corpus luteum is a transitory gland formed by distinct types of cell, including steroidogenic cells, endothelial cells, fibroblasts and leucocytes, which interact via their secreted products. Some of these products act as intra-ovarian regulators of the menstrual cycle (Remohi *et al.*, 1996; Castro *et al.*, 1998), for example the insulin-like growth factors, IGF-I and -II (Remohi *et al.*, 1996). The biological actions of IGF-I and -II, which include proliferation and cellular differentiation, are regulated by six binding proteins named IGFBP-1 to -6 (Feld and Hirschberg *et al.*, 1996).

The transcripts of IGF-I and its receptor (IGF-IR) have been detected in the ovary of several species, including rats (Parmer *et al.*, 1991), cows (Einspanier *et al.*, 1990; Sauerwein *et al.*, 1992), pigs (Perks *et al.*, 1995) and women

(Johnson *et al.*, 1996). Functional studies have shown that IGF-I stimulates progesterone production by the rat corpus luteum (Parmer *et al.*, 1991), which increases when the cells are stimulated simultaneously with IGF-I and hCG (Talavera and Menon, 1991). Similar results have been reported in human luteal cells, in which IGF-I enhances both basal and hCG-stimulated progesterone production (Devoto *et al.*, 1995). In addition, IGF-I induces an increase in oestradiol secretion by human luteinized granulosa cells and by luteal cells in culture (Erickson *et al.*, 1989; Johnson *et al.*, 1996). These results indicate that IGF-I has a role in the regulation of the steroidogenic function of the human corpus luteum. Furthermore, IGF-I has an important anti-apoptotic effect in various tissues (Janiga *et al.*, 2000; Morales *et al.*, 2000; Remacle *et al.*, 2000) and regulates the relative concentrations of several members of the Bcl-2 family (Dews *et al.*, 2000).

The production of IGFBP-2 to -5 has been detected in the corpora lutea of pigs (Gadsby *et al.*, 1996) and cows (Kirby *et al.*, 1996), and in the normal ovaries of women, where IGFBP-1 to -5 have been detected in dominant follicles (El-Roeiy *et al.*, 1994). As yet, few studies have

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been performed regarding IGFBPs in the human corpus luteum. However, the production and secretion of IGFBP-1 in human luteinized granulosa cells has been reported (Angervo *et al.*, 1991; Holst *et al.*, 1997). In addition, IGFBP-3 mRNA is expressed in the endothelial cells of the human corpus luteum (Fraser *et al.*, 2000).

Nitric oxide (NO) is produced in most tissues and may regulate different physiological functions, some of which are associated with reproductive processes (Norman and Cameron, 1996). Endothelial and inducible NO synthases are expressed in the human corpus luteum throughout its lifespan (Vega *et al.*, 1998). As has been described in other tissues (Dimmeler and Zeiher, 1997), NO has an anti-steroidogenic action (Johnson *et al.*, 1999) and induces apoptosis in the human corpus luteum (Vega *et al.*, 2000). The aims of the present study were to investigate the presence and production of IGFs and IGFBPs in the human corpus luteum throughout the luteal phase and to determine whether NO induces a modification in the concentration of these proteins in luteal tissue explants.

Materials and Methods

Materials

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO) and culture media were obtained from GibcoBRL, Life Technologies (Grand Island, NY). Human recombinant IGF-I and IGF-II were obtained from Bachem (Torrance, CA). Polyclonal anti-IGF-I (NIH-AFP 4892898) was obtained from NHPP, NIDDK and Dr Parlow (National Hormone and Peptide Program, Torrance, CA). Polyclonal anti-IGF-II, anti-IGFBP-1, anti-IGFBP-2 and anti-IGFBP-3 were obtained from GroPep (North Adelaide, SA), monoclonal anti-IGFBP-4, anti-IGFBP-5 and anti-IGFBP-6 were obtained from Austral Biologicals (San Ramon, CA), and monoclonal anti-IGF-IR (Ab-1) was obtained from Oncogene Research Products (Cambridge, MA). IGF-I and IGF-II were determined as described by García *et al.* (1996). In brief, recombinant IGFs were iodinated using the Chloramine T method (vanObberghen-Schilling and Pouyssegur, 1983). Radiolabelled peptides were separated from the free iodine by gel filtration on Sephadex G-50 columns (1 cm × 50 cm). The specific activity was approximately 300 mCi mg⁻¹ for IGF-I and 190 mCi mg⁻¹ for IGF-II, and the corresponding standard curves were established using the same recombinant peptides. The concentrations of IGFBP-1 and IGFBP-3 were measured by immunoradiometric assay, and progesterone and oestradiol were measured by radioimmunoassay from Diagnostic System Laboratories (Webster, TX).

Subjects

Corpora lutea were obtained from 12 women aged 33–45 with normal menstrual histories who were undergoing minilaparotomy for tubal sterilization at the San Borja-Arriarán Clinical Hospital, University of Chile,

National Health Service (Santiago, Chile). The study was approved by the Institutional Ethics Committee and an informed written consent was obtained from all patients before surgery. The day of the menstrual cycle of each woman on which the tissue had been obtained was confirmed by ultrasonographic and histological analysis of endometria, according to the criteria of Noyes *et al.* (1950). After removal of the corpus luteum, the tissue was placed in a sterile PBS and transported immediately to the laboratory at room temperature. The tissues were then cut into slices, the masses of which ranged from 15 to 60 mg (wet mass). Two or three slices (explants) from each corpus luteum were incubated separately for each experimental condition (basal or L-arginine treatment). One piece of each corpus luteum was placed in 4% (v/v) formalin–PBS (0.1 mol l⁻¹, pH 7.2–7.4) for histological evaluation and immunohistochemistry, and the corpora lutea were classified as from the early (days 1–4 after ovulation), mid- (days 5–9 after ovulation) and late (days 10–14 after ovulation) luteal phases.

Incubation procedures

For experiments designed to test the influence of NO on production of oestradiol, progesterone, IGF-I and -II, and IGFBP-1 and -3, tissue explants were incubated as described by Vega *et al.* (2000). In brief, corpus luteum slices were placed into four-well plates with Hank's (L-Arginine-free) medium, supplemented with 0.1 mg glutamine ml⁻¹, 1 mg BSA ml⁻¹, 26 mmol NaHCO₃ l⁻¹, 25 mmol Hepes l⁻¹ and 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 were carried out in duplicates or triplicates. NO synthase substrate, 1.0 mmol L-Arginine l⁻¹ was then added to some plates. Incubations were terminated at 4 h and the media were stored at –70°C until assayed for the different compounds. The sensitivity of the immunoassays was: 0.018 pmol oestradiol ml⁻¹; 0.318 pmol progesterone ml⁻¹; 0.129 pmol IGF-I ml⁻¹; 1.298 pmol IGF-II ml⁻¹; 0.0036 pmol IGFBP-1 ml⁻¹ and 0.017 pmol IGFBP-3 ml⁻¹. The intra- and interassay coefficients of variation were, respectively: oestradiol: 4.1 and 6.7%; progesterone: 3.8 and 5.5%; IGF-I: 8.6 and 10.2%; IGF-II: 8.2 and 11.8%; IGFBP-1: 3.5 and 4.2%; and IGFBP-3: 1.1 and 1.8%.

Immunohistochemical detection of IGFs, IGFBPs and IGF-IR

The expression of IGF-I and -II, IGFBP-1 to -6 and IGF-IR was examined in histological paraffin wax sections (4–6 µm) of human corpora lutea of different ages. The antibodies and dilutions used are shown (Table 1). The second antibody was a biotinylated anti-mouse–anti-rabbit–anti-goat mixture immunoglobulin. The reaction was evaluated by streptavidin–biotin peroxidase conjugate using diaminobenzidine as the chromogen and cell nuclei were stained with haematoxylin. The slides were analysed in a Nikon optical microscope (Nikon Inc., Melville, NY).

Table 1. Antibodies and dilutions used in the immunohistological study

	Antibody	Dilution
IGF-I	Polyclonal	1:2000
IGF-II	Polyclonal	1:250
IGFBP-1	Polyclonal	10 µg ml ⁻¹
IGFBP-2	Polyclonal	1:250
IGFBP-3	Polyclonal	10 µg ml ⁻¹
IGFBP-4	Monoclonal	1:150
IGFBP-5	Monoclonal	1:150
IGFBP-6	Monoclonal	1:150
IGF-IR	Monoclonal	1:300

IGF: insulin-like growth factor; IGFBP: insulin-like growth factor binding protein.

The immunochemical evaluation for each protein was performed by a semi-quantitative analysis described by Lessey *et al.* (1992) named HScore (HS), which corresponds to: $[P](i + 1) / 100$, where $[P]$ is the percentage of positively stained cells and i is the intensity of the staining on a scale of 1–3 (1: low intensity; 2: mid-intensity; and 3: high intensity). Each protein was evaluated by three independent observers and the positive staining was assessed in at least 500 cells.

Statistical analysis

Data are expressed as means $\pm$ SEM. For comparison between basal concentrations and immunohistochemical analysis, an unpaired Student's *t* test was used. A paired Student's *t* test was used to compare basal and stimulated conditions for production of IGFs and IGFBPs. $P < 0.05$ was considered statistically significant.

Results

Detection of IGFs, IGFBPs and IGF-IR

The presence of IGF-I and -II was detected in human corpora lutea throughout the luteal phase (Fig. 1a,b). The number of positively stained cells and the intensity of the immunostaining (HS) for IGF-I increased progressively in luteal tissue throughout the luteal phase (Table 2). In corpora lutea from the early luteal phase, nearly 80% of the parenchyma cells showed positive staining for IGF-I, whereas in corpora lutea from the mid- and late luteal phases, 91 and 93%, respectively, of the cells were positively stained. The HS for IGF-I in corpora lutea from the late luteal phase was significantly different from that for corpora lutea from the early luteal phase ($P < 0.05$) (Table 2). Almost 95% of the luteal cells were positively immunostained for IGF-II, regardless of the stage of the luteal phase studied.

The positive staining for IGFBP-1 to -6 is shown (Fig. 1c–h). The immunohistochemical evaluation for IGFBP-1 showed that 70% of luteal cells were positively stained (Fig. 1c), in addition to some endothelial cells, although smooth muscle cells were negative for IGFBP-1. The

percentage of positively stained cells increased in corpora lutea from the mid- and late luteal phases, but this increase was not significant, whereas the HS value increased gradually in corpora lutea from the early to the mid- and late luteal phases (HS early versus corpora lutea from the late luteal phase: $P < 0.05$; Table 2). Ninety per cent of cells were positively stained for IGFBP-2 during the three stages of the luteal phase, although the HS value for IGFBP-2 increased significantly in corpora lutea from the early to the mid-luteal phases ($P < 0.05$; Table 2). In contrast, the HS value for IGFBP-3 and the positive immunostaining (almost 100%) did not show modifications throughout the luteal phase. Although a similar percentage of cells (95%) stained positively for IGFBP-4 and IGFBP-5 throughout the luteal phase, the HS value for both binding proteins was significantly higher in corpora lutea from the mid- than from the early luteal phase ($P < 0.05$; Table 2). The immuno-histochemical study for IGFBP-6 showed a non-significant increase in positively stained cells in corpora lutea from the early to the mid- and late luteal phases (from 77 to 95%) and a similar HS value for this binding protein was observed throughout the luteal phase.

No changes were observed in the number of positively stained cells or in the HS value for IGF-IR throughout the luteal phase (Fig. 1i, Table 2). However, the distribution of staining differed with age of corpora lutea. In corpora lutea from the early and late luteal phases, the positive staining for the receptor was predominantly near the cell membrane, whereas in corpora lutea from the mid-luteal phase, staining was distributed homogeneously in the cytoplasm and plasma membrane.

Basal production of oestradiol, progesterone, IGF-I, IGF-II, IGFBP-1 and IGFBP-3

Slices of human corpora lutea of different ages were incubated for 4 h to assess the production of oestradiol and progesterone. Oestradiol production increased significantly in corpora lutea from the mid- and late luteal phases (2.6- and 1.9-fold, respectively, $P < 0.05$) compared with basal production by corpora lutea from the early luteal phase (Table 3). Similarly, progesterone production increased in corpora lutea from mid- (2.9-fold) and late (2.0-fold) phases ($P < 0.05$) compared with basal production by corpora lutea from the early luteal phase.

Production of IGF-I increased significantly ($P < 0.05$) with age of corpus luteum by 2.5-fold and 5.9-fold in corpora lutea from the mid- and late luteal phases, respectively, compared with basal production by corpora lutea from the early luteal phase (Table 3). No significant change in IGF-II production was observed in corpora lutea of different ages, although IGF-II production was significantly lower in corpora lutea from the mid- and late luteal phases than IGF-I basal production ($P < 0.05$).

Similar to that of IGF-I, the production of IGFBP-1 increased progressively throughout the luteal phase (Table 3). Basal production of IGFBP-1 was enhanced significantly

Fig. 1. Immunohistochemical detection of insulin-like growth factors (IGFs), IGF binding proteins (IGFBPs) and IGF-I receptor (IGF-IR) in paraffin wax sections of human corpus luteum from the mid-luteal phase. Intense positive staining of the cytoplasm was observed for (a) IGF-I, (b) IGF-II, (c) IGFBP-1, (d) IGFBP-2 and (e) IGFBP-3 when polyclonal antibodies against the respective proteins were used, and for (f) IGFBP-4, (g) IGFBP-5 and (i) IGF-IR when monoclonal antibodies were used. Weaker immunostaining for (h) IGFBP-6 was observed in luteal parenchyma. (j,k) Negative controls (non-immune rabbit serum and non-immune mouse serum, respectively). Scale bars represent 30 μ m.

($P < 0.05$) in corpora lutea from the mid- (1.5-fold) and late (5.5-fold) luteal phases compared with basal production by corpora lutea from the early luteal phase. Basal production of IGFBP-3 was unchanged throughout the luteal phase, although its production was significantly higher than that of IGFBP-1 at all stages of the luteal phase ($P < 0.05$) (Table 3).

Effect of nitric oxide on production of oestradiol, progesterone, IGF-I, IGF-II, IGFBP-1 and IGFBP-3

Explants of human corpus luteum of different ages were incubated in the presence or absence of NO synthase

substrate, L-Arginine. The addition of L-Arginine had an inhibitory effect on oestradiol production in corpora lutea from the mid- (70%; $P < 0.05$) and late luteal phases (60%; $P < 0.05$) compared with the corresponding basal values (Fig. 2a). The same effect was observed for progesterone production, for which there was a 60 and a 62% decrease in corpora lutea from the mid- and late luteal phase, respectively ($P < 0.05$) (Fig. 2b). No effect of the presence of L-Arginine was detected on the production of oestradiol or progesterone in corpora lutea from the early luteal phase. Similar results were obtained in corpus luteum explants incubated with sodium nitroprusside, an NO-generating

Table 2. Immunohistochemical detection of insulin-like growth factors (IGF), IGF binding proteins (IGFBP) and IGF-I receptors (IGF-IR) in human corpora lutea from the early, mid- and late luteal phases

	HScore		
	Early corpus luteum	Mid-corpus luteum	Late corpus luteum
IGF-I	1.8 ± 0.07	2.4 ± 0.25	3.0 ± 0.29*
IGF-II	2.5 ± 0.20	3.0 ± 0.20	3.1 ± 0.26
IGFBP-1	1.7 ± 0.27	2.6 ± 0.28	2.9 ± 0.15*
IGFBP-2	1.8 ± 0.20	2.7 ± 0.12 [†]	2.7 ± 0.15*
IGFBP-3	3.1 ± 0.03	3.1 ± 0.03	3.1 ± 0.06
IGFBP-4	2.8 ± 0.03	3.1 ± 0.06 [†]	2.8 ± 0.17
IGFBP-5	2.5 ± 0.09	3.2 ± 0.17 [†]	2.9 ± 0.29
IGFBP-6	2.7 ± 0.03	2.6 ± 0.09	2.6 ± 0.06
IGF-IR	2.7 ± 0.24	2.4 ± 0.05	2.8 ± 0.37

Tissue slices were obtained from human corpora lutea at different stages of the luteal phase and immunostained with different antibodies for the proteins studied.

HScore corresponds to the percentage of positively stained cells $[P] (i + 1) / 100$, where i is the intensity of the staining on a scale of 1–3 (1: low intensity; 2: mid-intensity; and 3: high intensity).

Values are expressed as HScore (mean ± SEM); * $P < 0.05$, early corpus luteum versus late corpus luteum; [†] $P < 0.05$, early corpus luteum versus mid-corpus luteum.

Table 3. Concentrations of oestradiol, progesterone, insulin-like growth factor I (IGF-I) and IGF-II, IGF binding proteins 1 (IGFBP-1) and IGFBP-3 in the incubation media of human corpora lutea of different ages

	Early corpus luteum	Mid-corpus luteum	Late corpus luteum
Oestradiol (pmol g ⁻¹ corpus luteum)	413.9 ± 38.8	1.083 ± 61.9*	770.9 ± 72.5*
Progesterone (nmol g ⁻¹ corpus luteum)	13.4 ± 2.6	38.8 ± 2.9*	27.0 ± 1.6*
IGF-I (pmol g ⁻¹ corpus luteum)	31.2 ± 15.4	108.6 ± 11.8*	215.4 ± 37.0*
IGF-II (pmol g ⁻¹ corpus luteum)	50.9 ± 4.2	54.9 ± 10.1 [†]	59.6 ± 9.6 [†]
IGFBP-1 (pmol g ⁻¹ corpus luteum)	0.19 ± 0.1	0.48 ± 0.1*	1.24 ± 0.4*
IGFBP-3 (pmol g ⁻¹ corpus luteum)	29.7 ± 6.6 [‡]	37.8 ± 5.2 [‡]	37.9 ± 5.9 [‡]

Explants from human corpora lutea of different ages (three early corpora lutea, $n = 8$ slices; five mid-corpora lutea, $n = 12$ slices; and four late corpora lutea, $n = 10$ slices) were incubated in supplemented Hank's (L-Arg free) medium for 4 h and the concentration of proteins in the incubation media was determined by specific immunoassays.

Data are presented as means ± SEM.

* $P < 0.05$ compared with early corpus luteum; [†] $P < 0.05$ compared with IGF-II production at the same luteal stage;

[‡] $P < 0.05$ compared with IGFBP-1 production.

drug (data not shown), in agreement with the results of previous studies (Johnson *et al.*, 1999; Vega *et al.*, 2000).

Addition of L-Arginine had a stimulatory effect on IGF-I production in corpora lutea from the early luteal phase (80%; $P < 0.05$) (Fig. 3a) compared with corresponding basal values, whereas no significant modification was observed in corpora lutea from the mid- or late luteal phases. In contrast, NO did not change the production of IGF-II *in vitro* by the human corpus luteum (Fig. 3b). However, L-Arginine induced a 50% increase in the concentration of IGFBP-1 in the incubation media of corpora lutea from the late luteal phase explants ($P < 0.05$) (Fig. 4a). In contrast, IGFBP-3 production by human corpora

lutea of different ages was not modified by L-Arginine (Fig. 4b).

Discussion

Several molecules may regulate the morphological and functional changes in the corpus luteum throughout its lifespan acting via endocrine, paracrine or autocrine mechanisms. Among these molecules, NO has been suggested as a regulator of human corpus luteum function (Vega *et al.*, 1998, 2000; Johnson *et al.*, 1999). The NO–NO synthase system is expressed throughout the lifespan of the human corpus luteum eliciting an anti-steroidogenic effect

Fig. 2. Effect of nitric oxide on (a) oestradiol and (b) progesterone production by human corpus luteum during the luteal phase. Tissue explants (three corpora lutea from the early luteal phase, $n = 8$ slices; five corpora lutea from the mid-luteal phase, $n = 12$ slices; and four corpora lutea from the late luteal phase, $n = 10$ slices) were treated with L-Arginine (1 mmol l^{-1}) for 4 h. Results are expressed as percentages $\pm$ SEM of basal values (Table 3). * $P < 0.05$ versus corresponding basal value.

in vitro, mainly upon oestradiol production. When NO synthase activity was inhibited by N^G-monomethyl-L-arginine L-NMMA (a competitive inhibitor of NO synthase), steroid production was enhanced, indicating an inhibitory effect of endogenous NO on luteal steroidogenesis. Furthermore, when dispersed luteal cells were co-incubated with L-Arginine and L-NMMA, no differences in steroid production were observed (Vega *et al.*, 1998, 2000; Johnson *et al.*, 1999). However, IGFs and IGFBPs have been considered to elicit a regulatory role in the function of the corpus luteum (Parmer *et al.*, 1991; Davis *et al.*, 1996; Niswender *et al.*, 2000). In this regard, the present

Fig. 3. Effect of nitric oxide on (a) insulin-like growth factor I (IGF-I) and (b) IGF-II production by human corpus luteum throughout the luteal phase. Tissue explants (three corpora lutea from the early luteal phase, $n = 8$ slices; five corpora lutea from the mid-luteal phase, $n = 12$ slices; and four corpora lutea from the late luteal phase, $n = 10$ slices) were treated with L-Arginine (1 mmol l^{-1}) for 4 h. Results are expressed as percentages $\pm$ SEM of basal values (Table 3). * $P < 0.05$ versus corresponding basal value.

investigation showed that the expression of several proteins of the IGF-IGFBP system changes during the lifespan of the human corpus luteum. Immunohistochemistry showed that maximum expression of IGFBP-2, -4 and -5, and the detection of IGF-IR in the cell cytoplasm, occurred in mid-stage corpora lutea. The biological relevance of the increase in these binding proteins and the immunolocalization of the

Fig. 4. Effect of nitric oxide on (a) insulin-like growth factor binding protein 1 (IGFBP-1) and (b) IGFBP-3 production by human corpus luteum of different ages. Tissue explants (three corpora lutea from the early luteal phase, $n = 8$ slices; five corpora lutea from the mid-luteal phase, $n = 12$ slices; and four corpora lutea from the late luteal phase, $n = 10$ slices) were treated with L-Arginine (1 mmol l^{-1}) for 4 h. Results are expressed as percentages $\pm$ SEM of basal values (Table 3). * $P < 0.05$ versus corresponding basal value.

IGF receptor was not addressed directly in the present study, although the data indicate that the binding proteins and the receptor regulate the metabolic actions of IGFs in corpora lutea from the mid-luteal phase. IGF-I exerts a luteotrophic action by stimulating progesterone and oestradiol synthesis in cultures of luteal cells from the mid-luteal phase (Devoto *et al.*, 1995; Niswender *et al.*, 2000).

In this context, in the human corpus luteum, a potential direct effect of IGFBPs may operate independently from the effect of IGFs described in other systems (Ferry *et al.*, 1999). However, a gradual increase in the number of positively stained cells and the intensity of immunostaining for IGF-I and IGFBP-1 was detected with increasing age of corpus luteum, in agreement with the amounts of IGF-I and IGFBP-1 found in culture media. Collectively, these data indicate that IGF-I and IGFBP-1 play a role in the regulation of the lifespan of the human corpus luteum.

Maximum steroidogenic activity in the corpus luteum occurs during the mid-luteal phase, as evidenced by oestradiol and progesterone production and, thereafter, a gradual decline in steroid synthesis, representing the involution period or late luteal phase of the corpus luteum. The involution may be due, in part, to a progressive decrease in the mRNA that encodes steroidogenic enzymes, to a decrease in the activities of these enzymes, or to a decrease in the number of cells composing the corpus luteum. Previous studies indicate that programmed cell death mechanisms may be involved in corpus luteum involution (Vega *et al.*, 2000). Apoptosis occurs in cells of different organisms in physiological and pathological conditions, and allows the maintenance of homeostasis of the relevant organ (Schwartzman and Cidlowsky, 1993). In the human corpus luteum, apoptosis increases with age and is enhanced by NO, which exhibits pro-apoptotic activity (Vega *et al.*, 2000).

IGF-I exerts anti-apoptotic action in several tissues (Janiga *et al.*, 2000; Morales *et al.*, 2000; Remacle *et al.*, 2000). One of the mechanisms by which IGF-I promotes cell survival in rat fibroblasts is via downregulation of the pro-apoptotic protein Bad by activation of phosphatidylinositol 3-kinase and Akt, leading to the sequestration of phospho-Bad in the cytosol (Kulik and Weber, 1998). In PC12 cells, IGF-I mediates the expression of the anti-apoptotic protein Bcl- x_L , which plays a role in cell survival (Párrizas and LeRoith, 1997), and IGF-I also inhibits the downregulation of Bcl-2 protein induced by hypoxia in cortical neurone cultures (Pugazhenthil *et al.*, 1999a). The precise mechanism by which IGF-I sustains the expression of *bcl-2* has not been elucidated. IGF-I apparently increases *bcl-2* expression at transcription, because *bcl-2* promoter is positively regulated by the nuclear transcription factor CREB, which can be activated by IGF-I (Pugazhenthil *et al.*, 1999b). NO causes a decrease in Bcl-2 concentrations in corpora lutea from the mid- and late luteal phases (Vega *et al.*, 2000) but does not induce changes in corpora lutea from the early luteal phase. In the present study, NO increased IGF-I concentration in early stage corpora lutea. It is not known whether these two issues are linked in the regulation of the lifespan of the human corpus luteum, although it is possible that, in the corpus luteum in the early luteal phase, the presence of IGF-I represents a protective mechanism that maintains Bcl-2 expression in response to the action of pro-apoptotic agents such as NO.

The reduction in steroid production observed during

luteal regression was coincident with the increase in IGFBP-1 concentration in both basal and NO-treated corpora lutea. Moreover, the gradual increase in IGFBP-1 concentration in human corpora lutea during the luteal phase is in agreement with studies in bovine corpora lutea, in which IGFBP-1 expression increased during luteolysis (Sayre *et al.*, 2000). Furthermore, hypoxia in HepG2 cells stimulated the expression of IGFBP-1 (Tazuke *et al.*, 1998). NO increases IGFBP-1 concentration, particularly in corpora lutea from the late luteal phase, indicating that in the stress condition associated with corpora lutea from the late luteal phase (Vega *et al.*, 1994), the concentration of IGFBP-1 increases as a regulatory mechanism to restrict IGF-I bioavailability.

In summary, the present investigation revealed that the expression of growth factors and their binding proteins undergoes modification throughout the luteal phase and that NO regulates IGF and IGFBP protein concentrations. Collectively, these data indicate that growth factors may mediate NO action in the human corpus luteum and, therefore, the lifespan of the corpus luteum. Moreover, the increased expression of IGF-I and IGFBP-1 in the human corpus luteum from the late luteal phase, in addition to the effect of NO on IGFBP-1 concentration, indicate that these proteins are involved in human luteal regression, and are likely to be associated with a mechanism of programmed cell death.

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