Involvement of nuclear factor kappa B in the regulation of rat luteal function: potential roles as survival factor and inhibitor of 20 α -hydroxysteroid dehydrogenase

C M Telleria¹, A A Goyeneche¹, C O Stocco and G Gibori

Department of Physiology and Biophysics, University of Illinois, Chicago, Illinois 60612, USA

¹Division of Basic Biomedical Sciences, University of South Dakota School of Medicine, Vermillion, South Dakota 57069, USA

(Requests for offprints should be addressed to G Gibori; Email: ggibori@uic.edu)

Abstract

Nuclear factor kappa B (NF κ B) is an important intracellular conveyor of extracellular signals and modulates a number of gene responses. Due to the potential significance of NF κ B in regulating ovarian gene expression, we examined in the rat: (i) whether NF κ B is activated and developmentally regulated in the corpus luteum (CL) throughout pregnancy; (ii) the proteins forming the NF κ B complex in luteal cells; and (iii) the role of this transcription factor in luteal function. Western analysis and immunohistochemistry revealed that p65 and p50 were highly expressed throughout pregnancy and were located in both the nucleus and cytoplasm of luteal cells. In addition, because NF κ B is maintained in the cytoplasm bound to I κ B, whose phosphorylation allows NF κ B translocation to the nucleus, we studied the developmental expression of phosphorylated and nonphosphorylated forms of I κ B α . Western analysis revealed that I κ B α was present and phosphorylated throughout pregnancy in the CL whereas by protein/DNA array and electromobility shift assays we found that luteal nuclear extracts bind to an NF κ B consensus sequence, and that the binding activity decreased along pregnancy. The specific binding was supershifted only by an anti-p65 antibody and not by antibodies against p50, p52, cRel, or RelB. Using day 4 postpartum ovaries, we found higher NF κ B binding activity in the newly formed CL than in old CL of pregnancy. Furthermore, NF κ B DNA binding activity was enhanced by prolactin in luteinized granulosa cells. In our first functional study, blockade of NF κ B/p65 binding to DNA with the sesquiterpene lactone helenalin in luteinized granulosa cells correlated with induction of cell death in a dose-dependent manner. In a second functional study, overexpression of NF κ B/p65 in luteal cells resulted in inhibition of 20 α -hydroxysteroid dehydrogenase (20 α HSD) promoter activity as well as endogenous 20 α HSD mRNA expression. In summary, we have shown that: (i) NF κ B is expressed within the CL, primary luteinized granulosa cells, and a rat luteal cell line; (ii) NF κ B activation within the CL is developmentally regulated in pregnancy, depends on the age of the gland, and can be upregulated by prolactin; (iii) inhibition of NF κ B/p65 binding to an NF κ B DNA consensus sequence correlates with induction of cell death in ovarian luteinized granulosa cells; and (iv) overexpression of NF κ B in luteal cells inhibits 20 α HSD gene expression. The results further support a role for NF κ B as a survival factor in the CL.

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Introduction

Nuclear factor kappa B (NF κ B) proteins are an important class of transcriptional regulators that affect expression of genes involved in the immune and inflammatory responses, cell growth and

differentiation, and apoptosis (Blackwell & Christman 1997, Delfino & Walker 1999, Mercurio & Manning 2000). The NF κ B family is composed of five mammalian homologous, cRel, RelA (p65), RelB, p50/p105 (NF κ B1), and p52/p100 (NF κ B2). NF κ B proteins form an array of homo- and

hetero-dimers (Mercurio & Manning 2000). The classic NFκB transcriptional activator is a heterodimer composed of p65 and p50. In most cells, NFκB exists as an inactive form found predominantly in the cytoplasm bound to the inhibitory proteins IκBs (Siebenlist *et al.* 1994, Baldwin 1996). The main role of IκB is to maintain cytoplasmic localization of NFκB dimers and to inhibit NFκB binding to DNA both by masking the DNA-binding regions of the NFκB dimers and by promoting nuclear export of NFκB. Nuclear translocation of transcriptionally active NFκB dimers is induced by stimulation of cells with a large array of NFκB inducers, damaging agents, and oxidative stress (Siebenlist *et al.* 1994). These stimuli initiate the activation of signal transduction pathways that culminate in the phosphorylation of IκB at amino-terminal serine residues by the IκB kinase complex. Phosphorylation of IκB targets it for ubiquitination and subsequent degradation by the 26S proteasome. Degradation of IκB allows import and accumulation of NFκB binding within the nucleus, and activation of target gene expression (Delfino & Walker 1999).

Whereas NFκB has been shown to be involved in the transcriptional regulation of genes within cells of the immune system (Blackwell & Christman 1997), less information is available on the expression, activation status, and physiological roles of this transcription factor in reproductive tissues. One of the primary reports in this matter demonstrated a stage-specific nuclear expression of NFκB in the mammalian testis (Delfino & Walker 1998). These authors showed nuclear expression of NFκB in Sertoli and germ cells in a stage-specific manner, suggesting a role for this transcription factor in regulating spermatogenesis. In mouse mammary gland it was shown that NFκB activity was developmentally regulated, increasing during pregnancy and declining with the onset of lactation (Geymayer & Doppler 2000). In the human endometrium, a role for NFκB has been attributed during menstrual induction (Brenner *et al.* 2002). In rat ovarian granulosa cells NFκB was activated by follicle-stimulating hormone (Wang *et al.* 2002) and prevented apoptosis induced by tumor necrosis factor-α (TNF-α) (Xiao *et al.* 2001). The NFκB-mediated antiapoptotic effect in granulosa cells appears to involve the stimulation of at least two antiapoptotic proteins: X-linked inhibitor of apoptosis (XIap) (Xiao *et al.* 2001), a direct inhibitor of

executioner caspase-3 and caspase-7 (Tamm *et al.* 2003), and Flice-like inhibitor protein (FLIP) (Xiao *et al.* 2002), an inhibitor of death receptor-mediated activation of caspase-8 (Krueger *et al.* 2001). In human ovarian epithelial cancer cells, a role for NFκB has been proposed as mediator of resistance to apoptosis induced by TNF-α (Xiao *et al.* 2003). In the corpus luteum, there is little information regarding the expression and function of NFκB. A moderate expression of NFκB in the human corpus luteum, which remained relatively constant throughout the luteal phase, was recently reported (Vaskivuo *et al.* 2002). It is not yet known, however, whether NFκB is activated during luteal development, what are the members of the NFκB family of proteins forming the NFκB complex in luteal cells, and what role this transcription factor may have in regulating luteal function.

In the present studies we have examined the expression and activation status of NFκB in the rat corpus luteum during development along pregnancy, and defined the luteal NFκB subunits having DNA binding capacity. We have also determined that NFκB activation is regulated by the main tropic hormone in the rat corpus luteum, i.e. prolactin (PRL) (Risk & Gibori 2001), and have shown that NFκB may be involved in the regulation of luteal apoptosis and in the expression of the 20α-hydroxysteroid dehydrogenase (20αHSD) gene, which encodes for a key enzyme that metabolizes progesterone during luteal regression (Wiest *et al.* 1968, Albarracin *et al.* 1994).

Materials and methods

Chemicals

DMEM/F12 (1:1), RPMI 1640, nonessential amino acids, sodium pyruvate, trypsin-EDTA, antibiotics and antimycotics were purchased from Mediatech (Herndon, VA, USA). Fetal bovine serum (FBS) was from Hyclone Laboratories Inc. (Logan, UT, USA). Ovine PRL (oPRL) (PRL-18, 30 IU/mg) was from NIDDK (NIH, Bethesda, MD, USA). Helenalin was from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). The oligonucleotides used in the RT-PCR and electromobility shift assays (EMSAs) were obtained from Invitrogen Life Technologies Inc. (Frederik, MD, USA). Aprotinin and protease inhibitor cocktail tablets were from Roche Diagnostics

GmbH (Mannheim, Germany). [γ - 32 P]ATP and [α - 32 P]dCTP were purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA). PMSG, human chorionic gonadotropin (hCG), 0.45% D-glucose, EGTA, Hepes, sucrose, dithiothreitol, leupeptin, pepstatin-A, phenylmethylsulfonyl fluoride, and all other reagent grade chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA). Nuclear extracts derived from treated phorbol 12-myristate 13-acetate (TPA) Jurkat cells were purchased from Geneka Biotechnology Inc. (Montreal, Quebec, Canada).

Animals and tissue preparation

Pregnant (day 1 = sperm-positive), adult males, and immature female (day 25 of age) Sprague–Dawley rats were obtained from Sasco Animal Labs (Madison, WI, USA). The animals were housed at 22–24 °C on a 14 h light:10 h darkness cycle and allowed free access to standard rat chow and water. All experiments were conducted in accordance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Illinois Animal Care and Use Committee. For the developmental studies, rats were killed at various stages of pregnancy from day 7 to day 21 (the day before parturition), and on day 4 after parturition. Corpora lutea were dissected from the ovaries under a stereoscopic microscope. On day 4 postpartum the isolated corpora lutea were classified as old (i.e. corpora lutea of the previous pregnancy) or new (i.e. newly formed corpora lutea after postpartum ovulation) as previously reported (Goyeneche *et al.* 2003a). Spleen, prostate and epididymis were obtained from three different male rats. All tissues were frozen in liquid nitrogen and stored at –80 °C until processed for protein isolation. For the immunohistochemical studies whole ovaries from pregnant (day 15) animals were obtained and fixed for 1 h at room temperature in a solution of 10% (v/v) phosphate-buffered neutral formalin, dehydrated in ethanol series, cleared in xylene, and embedded in paraffin. To determine the effect of lipopolysaccharide (LPS) *in vivo* on NF κ B activation animals were injected i.p. with either 5 mg LPS (*E. coli* 055:B5; Sigma) or vehicle on day 9 of pregnancy, and were killed 1 h later. Corpora lutea were isolated, frozen in liquid nitrogen, and stored at –80 °C until protein isolation.

Primary luteinized granulosa cells

Twenty-five-day-old immature rats were treated with 15 IU PMSG i.p. followed 2 days later by 15 IU hCG i.p. Eight hours after hCG injection, follicles were incubated sequentially in DMEM/F12 (1:1) containing respectively 6 mM EGTA and 0.5 M sucrose, and luteinized granulosa cells were harvested by needle pricking the follicles. The cells were cultured for 72 h at 37 °C in an atmosphere consisting of 5% CO₂/95% air in DMEM/F12 (1:1) with 15 mM Hepes, 1% FBS, and 100 IU/ml penicillin G, 100 µg/ml streptomycin, and 0.25 mg/ml amphotericin B. Medium was then replaced and cells were treated as depicted in figure legends. To evaluate the morphology of the luteinized granulosa cells *in situ*, cells were fixed in 4% paraformaldehyde and stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Roche). Alteration of the chromatin was observed using a Leica DM/IRB microscope (Leica Microsystems, Bannockburn, IL, USA).

Luteal cell line

The simian virus 40-transformed temperature-sensitive rat luteal cell line GG-CL was used (Sugino *et al.* 1998a). Cells were cultured under 5% CO₂/95% air in RPMI 1640 medium supplemented with 1 × glutamine, 2 × antibiotic-antimycotic solution, 1 × nonessential amino acids, 1 × sodium pyruvate, 0.45% D-glucose, and 10% FBS. Cells were cultured at 33 °C to 60% confluence and differentiated at 39 °C over 48 h before harvesting.

Western blot analysis

Isolated corpora lutea, prostate, epididymis, and spleen were homogenized in 2 ml ice-cold homogenization buffer containing 25 mM Tris–HCl (pH 7.4), 2 mM MgCl₂, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 µM leupeptin, 1 µM pepstatin-A, and 1 µg/ml aprotinin. Homogenates were assayed for protein content by the Lowry method, appropriately diluted in 6 × concentrated electrophoresis sample buffer, boiled for 10 min, and stored at –80 °C until electrophoresed. Equivalent amounts of protein (50 µg) were separated through 8% SDS-PAGE gels and electrotransferred to nitrocellulose membranes (Schleicher & Schuell Inc.,

Keene, NH, USA). Immunoblotting was performed by blocking nonspecific binding with 5% nonfat milk in Tris-buffered saline (TBS) (0.15 M NaCl, 0.05 M Tris, pH 7.6) containing 0.1% Tween 20. Blots were then incubated overnight with 0.5–1 µg/ml of the following antibodies (all obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA): NFκB p65 rabbit polyclonal (A), NFκB p50 (H-119) rabbit polyclonal, IκBα rabbit polyclonal (C-21) and p-IκBα mouse monoclonal (B-9). The membranes were washed and incubated with a secondary antibody (goat anti-rabbit or goat anti-mouse; 1:1000 dilution) linked to horseradish peroxidase (HRP) (Dako Corp., Carpinteria, CA, USA) for 90 min. After extensive washing, blots were developed by chemiluminescence and exposed for 15–120 s to X-ray films. The molecular size of immunoreactive bands was estimated by the co-migration of a prestained SDS-PAGE molecular mass standard (Benchmark; Gibco BRL, Gaithersburg, MD, USA).

Immunohistochemistry

Serial paraffin sections 5 µm thick were mounted on 3-aminopropyltriethoxysilane-coated slides. The immunohistochemical procedure was performed using a highly sensitive strept ABCComplex/HRP detection system (Dako). The sections were deparaffinized with xylene and rehydrated in alcohol solutions of increasing water content, then washed in TBS. Endogenous peroxidases were blocked in 3% H₂O₂ for 20 min at room temperature. To reduce nonspecific background staining, the sections were blocked with 3% normal goat serum for 20 min. The sections were then incubated with the anti-NFκB p65 antibody (Santa Cruz Biotechnology; 1 µg IgG/ml) overnight at 4 °C in a moist incubation chamber. The sections were washed in TBS and incubated successively with biotinylated goat anti-rabbit IgG (2.5 mg/ml; Dako) for 30 min at room temperature, and strept ABCComplex/HRP (1:50 dilution) for 30 min at room temperature. Each incubation was followed by two 10 min washes in TBS. After the final wash, sections were incubated in the dark for 10 min with diaminobenzidine (DAB) solution (0.05 M Tris buffer, pH 7.6, containing 0.6 mg/ml DAB and 0.03% H₂O₂) for developing peroxidase activity. After washing with tap water the sections were counterstained with hematoxylin, dehydrated in

serial alcohols to xylene, and mounted. In the negative control slides the primary antibody was replaced with TBS. The tissue samples were observed and photographed with a Zeiss IM 35 microscope (Carl Zeiss, Oberkochen, Germany).

Protein/DNA array

Activation of several transcription factors in the corpus luteum was screened using the TranSignal protein/DNA array procedure according to the manufacturer's instructions (Panomics, Inc., Redwood City, CA, USA). Activation of transcription factors other than NFκB was assessed for other studies. For the present study we focused on the binding capacity to an NFκB DNA consensus sequence of nuclear extracts obtained from corpora lutea of rats on day 5 of pregnancy. In brief, the nuclear extracts were incubated with a set of biotin-labeled DNA binding oligonucleotides, one of them containing the NFκB consensus sequence, to allow the formation of protein/DNA complexes. Then the protein/DNA complexes were separated from the free probes, and the probes in the complexes were isolated from the proteins and hybridized to the TranSignal Array (Panomics). Detection of the signals was obtained by chemiluminescence.

Nuclear extract preparation and EMSAs

To prepare nuclear cell extracts from either GG-CL cells or primary luteinized granulosa cells, 100 mm plates at 70–80% of confluence were used. Cells were washed in cold PBS and quickly frozen by placing the plate on dry ice. Then, plates with cells were kept at –80 °C until nuclear extract preparation. Cells were harvested at 4 °C by scraping in 300 µl solution A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 µg/ml aprotinin, 1 × protein inhibitor cocktail), transferred to 1.5 ml Eppendorf tubes, and placed in an orbital rocker for 15 min at 4 °C. After rocking, 25 µl Nonidet P-40 were added and the tubes were vigorously vortexed for 30 s. The nuclear pellet was obtained by centrifugation at 14 000 *g* at 4 °C for 1 min and resuspended in solution B, which was similar to solution A except that it contained 420 mM NaCl and 25% (v/v) glycerol, and lacked KCl. The solution was

vigorously vortexed for 30 min at 4 °C and then centrifuged at 14 000 *g* at 4 °C for 5 min. The supernatant containing the nuclear extract was divided into aliquots, snap frozen in liquid nitrogen, and stored at –80 °C. To prepare nuclear extracts from corpora lutea, 30 mg tissue were homogenized in 200 μ l solution A using a Dounce homogenizer (B type pestle). The homogenate was transferred to 1.5 ml Eppendorf tubes, placed on ice for 15 min, and centrifuged at 400 *g* for 10 min at 4 °C. The pellet was resuspended in 80 μ l solution B, once again homogenized, rocked vigorously for 20 min at 4 °C, vortexed for 1 min at 4 °C, and then centrifuged at 14 000 *g* for 20 min at 4 °C. The supernatant constituted the luteal nuclear extract. Protein concentration was determined by the bicinchoninic acid method (Pierce Biotechnology Inc., Rockford, IL, USA). The following oligonucleotides and their complements were used as probes and competitors: 5'-AGCTTGGGGTATTTCCAGCCG-3' (Rel probe) and 5'-AGATTGGCATAGGTCCAGCCG-3' (mutant probe). The underlined portions represent the consensus sequences. Double-strand DNA oligonucleotides were ³²P labeled with [γ -³²P]ATP and T4 polynucleotide kinase (Invitrogen Life Technologies, Carlsbad, CA, USA). Nuclear extracts (3–8 μ g) were incubated in reaction buffer (10 mM Tris–HCl, pH 7.5, 1.2 mM MgCl₂, 0.6 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 0.25 μ g/ μ l poly(dI–dC), and 4% glycerol) for 20 min at 20–22 °C in the presence or absence of excess unlabeled oligonucleotide or 1–3 μ l of the following specific antibodies: p65 (A), p50 (H-119), p52 (K-27), cRel (N) or Rel B (C-19) (all from Santa Cruz Biotechnology). The labeled probe was added and the incubation was continued for 20 min at 20–22 °C. The DNA/protein complexes were separated from the unbound DNA probe by nondenaturing PAGE (4% gel) at 4 °C, in Tris–borate EDTA buffer (0.5 $\times$; pH 8) and detected by autoradiography.

RNA isolation and RT-PCR analysis

Total RNA from GG-CL cells was purified using TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using the Advantage RT for PCR kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). The PCR reaction to

amplify the 20 α HSD mRNA was performed as described previously (Stocco *et al.* 2000).

Transient transfection, plasmids and reporter assays

GG-CL were trypsinized and suspended in OPTI-MEM (Life Technologies Inc.) at 1×10^6 cells/0.8 ml and transfected by electroporation with 1 μ g 20 α HSD-Luc construct and 3 μ g of a control β -galactosidase expression vector and a different amount of RelA (p65) expression plasmid using the gene pulser (Bio-Rad, Hercules, CA, USA) at a capacitance setting of 950 μ F and 280 V. The total amount of DNA per well was kept constant by the addition of an empty vector when necessary. After each electroporation, cells were pooled and resuspended in RPMI 1640 medium containing 10% FBS and plated into six-well plates at a density of 0.8×10^6 cells/well. Cells were cultured overnight and then the medium was changed to phenol red-free RPMI 1640 with 1% FBS. Cells were maintained for a maximum of 48 h after electroporation. To harvest cells, each well was washed twice with ice-cold PBS. Reporter lysis buffer (100 μ l) (Promega, Madison, WI, USA) was added into each well; 20 μ l cell lysate were used to measure firefly luciferase activity driven by the 20 α HSD promoter using a luciferase reporter assay system (Promega), and 20 μ l cell lysate were used to measure β -galactosidase activity using a luminescence β -galactosidase detection kit (Clontech). Measurements were conducted in a Lumat LB 9507 Lumimeter (EG & G Berthold, Oak Ridge, TN, USA). Relative light units were obtained by dividing the luciferase activity by the β -galactosidase activity. The 20 α HSD-Luc construct used contains a 2.5 kb 5' flanking region of the rat ovarian 20 α HSD gene fused with a luciferase reporter plasmid (pGL3-Basic; Promega) (Zhong *et al.* 1998). The pSV- β -galactosidase control vector was commercially obtained (Promega), whereas the RelA (p65) expression vector was kindly provided by Dr J Anrather (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA).

Results

NF κ B/p50 and p65 proteins are expressed in the corpus luteum throughout pregnancy

To explore the potential role of NF κ B in the function of the corpus luteum, the expression of the

best-characterized NFκB components essentially present in all cells, the p65 and p50 proteins (Siebenlist *et al.* 1994, Delfino & Walker 1999), was studied by Western analysis in total protein extracts obtained from corpora lutea of rats on different days of pregnancy. In the corpus luteum, the antibodies recognized proteins of the same sizes as those found in spleen, which was used as positive control tissue (Zhou *et al.* 2003). Other tissues included in the study, such as prostate, expressed only p50, whereas epididymis displayed a higher expression of p65 than p50 (Fig. 1A). In addition, both p65 and p50 proteins were highly expressed in the corpus luteum throughout pregnancy (Fig. 1B).

NFκB proteins have cytoplasmic and nuclear localization within the luteal cells

In order to define the state of activation of NFκB, the localization of the proteins within the luteal cells was established. Both p65 and p50 were mainly located within the cytoplasm of the luteal cells and showed some nuclear localization as well, with no staining whatsoever in the membrane fraction (Fig. 2A). These results were confirmed by immunohistochemistry using an antibody capable of recognizing the p65 protein. The results presented in Fig. 2B showed nuclear as well as cytoplasmic localization for p65 in luteal cells, whereas the endothelial cells surrounding the capillaries did not stain for the protein. The pattern of intracellular localization, however, varied among luteal cells. Some luteal cells showed more intense nuclear staining compared with its cytoplasmic counterpart, other cells showed only cytoplasmic staining, whereas some cells showed comparable nuclear and cytoplasmic immunostaining.

Nuclear extracts obtained from luteal cells are capable of binding an NFκB consensus sequence

Once the intracellular localization of NFκB was demonstrated, we examined the expression of phosphorylated and unphosphorylated IκB. IκB maintains the transcription factor NFκB within the cytoplasm, but once phosphorylated allows its translocation to the nucleus (Siebenlist *et al.* 1994, Delfino & Walker 1999, Mercurio & Manning 2000). Constitutive luteal expression of both unphosphorylated and phosphorylated IκBα was

Figure 1 Tissue specificity and changes in NFκB/p65 and p50 immunoreactive proteins in the rat corpus luteum during pregnancy. (A) Corpora lutea (CL) were obtained from rats on day 15 of pregnancy, and prostate (P), epididymis (E), and spleen (S) were isolated from adult males. (B) Corpora lutea were obtained from rats killed on days 7, 12, 15, 20, 21, and 22 of pregnancy. Total protein extract was obtained from the different tissues and equal amounts of proteins (50 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blot as described in Materials and methods. Depicted are autoradiographs from a representative experiment.

found by Western blot throughout pregnancy (Fig. 3A). Phosphorylation of IκBα indicates that most probably translocation of NFκB to the nucleus is continuously taking place in the corpus luteum during pregnancy, opening the possibility for a physiological role of the transcription factor in the regulation of luteal function. To demonstrate this possibility further, we studied the capacity of nuclear luteal extracts to bind an NFκB DNA consensus sequence. Nuclear extracts obtained from corpora lutea of day 5 pregnant rats were

Figure 2 Cellular localization of NF κ B/p65 and p50 proteins in the rat corpus luteum. (A) Corpora lutea were isolated from rats on day 15 of pregnancy: T, total extract; N, nuclear extract; C, cytosolic extract; M, membrane extract. Total extracts from spleen (S) and prostate (P), were used as positive and negative control respectively for NF κ B/p65 according to the results obtained and shown in Fig. 1. Western blot was conducted as detailed in Fig. 1 and in Materials and methods. (B) Ovaries were obtained from rats on day 15 of pregnancy and analyzed by immunohistochemistry for the presence of NF κ B/p65 as detailed in Materials and methods. NF κ B/p65 depicts nuclear (short arrows) as well as cytoplasmic (arrowheads) localization in luteal cells, whereas no staining is observed in the endothelial cells surrounding the capillaries (long arrows). The inset depicts absence of immunostaining in luteal cells when the ovarian section was incubated with TBS instead of NF κ B/p65 antibody. Magnification $\times 400$.

capable of binding an NF κ B DNA consensus sequence, as demonstrated by either protein/DNA array (Fig. 3B) or EMSA (Fig. 3C). To explore the specificity of the binding of nuclear luteal proteins to the NF κ B probe, we performed gel shift analysis with specific antibodies for the different subunits capable of forming the NF κ B complex (Siebenlist *et al.* 1994, Mercurio & Manning 2000). As shown

in Fig. 4, we found a strong shift of the NF κ B probe induced by the nuclear extract. This binding was specific as demonstrated by their competition with excess cold probe, and by the fact that the binding was not competed by a mutant probe. The nature of the proteins bound to the NF κ B probe was revealed by using specific antibodies for the known components of the NF κ B family of proteins.

Figure 3 Expression of $\text{I}\kappa\text{B}\alpha$ in the rat corpus luteum during pregnancy and binding of luteal nuclear extracts to an $\text{NF}\kappa\text{B}$ DNA consensus sequence. (A) Corpora lutea were obtained from rats killed on days 7, 12, 15, 20, 21, and 22 of pregnancy. Cytosolic extracts were obtained from the corpora lutea and equal amounts of proteins (50 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blot as described in Materials and methods. Depicted are autoradiographs from representative experiments. (B) Binding of nuclear extracts obtained from corpora lutea of three rats on day 5 of pregnancy and subjected to protein/DNA array as described in Materials and methods. (C) Binding to an $\text{NF}\kappa\text{B}$ DNA consensus sequence of the same nuclear extracts analyzed in (B) and evaluated by EMSA.

Figure 4 Identification of NFκB complexes in the corpus luteum. EMSA was performed using a probe containing the sequence of a classical NFκB binding site (Rel) and equal amounts of nuclear extracts in the presence and absence of antibodies (Ab), a 100-fold excess of unlabeled NFκB probe (UP) or 100-fold excess of unlabeled NFκB mutant probe as indicated at the upper left margin of the figure. Arrows in the left margin mark the positions of the NFκB-specific complexes. A supershifted complex is also indicated. DNA/protein complexes were resolved by nondenaturing PAGE and detected by autoradiography. The unbound probe is shown at the bottom of the gel (free probe). The experiment depicted was performed in triplicate with consistent results.

The specific binding was supershifted by only the antibody directed against the NFκB/p65 protein. No supershift whatsoever was seen when antibodies directed against p50, cRel, p52, or RelB were used.

This result indicates that p65 is the major NFκB component within the corpus luteum. Analogous information was obtained when nuclear extracts were isolated from the luteal cell line GG-CL

Figure 5 Identification of NFκB complexes in the GG-CL luteal cell line. EMSA was performed using a probe containing the sequence of a classical NFκB binding site (Rel) and equal amounts of nuclear extracts in the presence and absence of antibodies (Ab), a 100-fold excess of unlabeled NFκB probe (UP) or 100-fold excess of unlabeled NFκB mutant probe as indicated at the upper left margin of the figure. Arrows in the left margin mark the positions of the NFκB-specific complexes. A supershifted complex is also indicated. DNA/protein complexes were resolved by nondenaturing PAGE and detected by autoradiography. The unbound probe is shown at the bottom of the gel (free probe). The experiment depicted was performed in triplicate with consistent results.

(Fig. 5). Similarly to what was observed in the corpus luteum, in GG-CL cells p65 was the only component of the NFκB family of proteins capable of binding the NFκB consensus sequence.

This was demonstrated by the supershifted band obtained when the protein extract and the probe were incubated in the presence of the anti-p65 antibody.

Figure 6 Developmental regulation of NF κ B binding to an NF κ B consensus sequence in the corpus luteum of pregnancy. EMSA was performed using a probe containing the sequence of a Rel binding site and equal amounts of nuclear extracts. (A) Nuclear extracts were obtained from corpora lutea of rats killed on days 5, 8, 15, and 21 of pregnancy; (B) nuclear extracts were from old corpora lutea of pregnancy (Old) or newly formed corpora lutea after postpartum ovulation (New), which were isolated from rats killed on day 4 postpartum; (C) nuclear extracts were isolated from rats killed on day 15 of pregnancy that were treated with either saline vehicle (Veh) or LPS (5 mg i.p.) 2 h before being killed. The arrow in the right margin marks the position of the NF κ B-specific complexes. The unbound probe is shown at the bottom of the gel (free probe). Experiments shown were performed in triplicate with consistent results.

Binding of NF κ B to an NF κ B consensus sequence is developmentally regulated in pregnancy, varies with the age of the corpus luteum, and can be further activated by LPS and PRL

Once we had established that p65 is the major member of the NF κ B family of proteins binding DNA in the corpus luteum, we decided to determine whether such binding is developmentally regulated in pregnancy, and whether it is affected by the ubiquitous NF κ B stimulator LPS (Manna & Aggarwal 1999), and the luteal regulatory hormone PRL (Risk & Gibori 2001). The binding of nuclear proteins obtained from corpora lutea of different days of pregnancy decreased while gestation advanced, demonstrating that the activation of this transcription factor is developmentally regulated in pregnancy (Fig. 6A). The binding appeared to be age-dependent if comparing the weak binding

elicited by nuclear proteins obtained from the old corpus luteum of pregnancy harvested on day 4 postpartum, with the stronger binding elicited by nuclear extracts obtained from newly formed corpora lutea after postpartum ovulation and harvested on day 4 postpartum as well (Fig. 6B). Nuclear extracts obtained from corpora lutea of day 15 of pregnancy, which showed lower binding to DNA as compared with that of days 5 and 8 of pregnancy (Fig. 6A), had a remarkably increased binding to the NF κ B consensus sequence after *in vivo* challenge with LPS (Fig. 6C).

In order to study the regulation of NF κ B activation by PRL in ovarian cells, we took advantage of an *in vitro* approach. We used primary luteinized granulosa cells in culture which respond to PRL with increased production of progesterone (Jones *et al.* 1983, Risk & Gibori 2001). As shown in Fig. 7, cellular extracts from these cells bind

Figure 7 Identification of NFκB complexes in primary luteinized granulosa cells. (A) EMSA was performed using a probe containing the sequence of a classical NFκB binding site (Rel) and equal amounts of nuclear extracts in the presence and absence of antibodies (Ab), a 100-fold excess of unlabeled NFκB probe (UP) or 100-fold excess of unlabeled NFκB mutant probe as indicated at the upper left margin of the figure. Arrows in the right margin mark the positions of the NFκB-specific complexes. A supershifted complex is also indicated. DNA/protein complexes were resolved by nondenaturing PAGE and detected by autoradiography. The unbound probe is shown at the bottom of the gel (free probe). The experiment shown was performed in triplicate with consistent results. (B) Binding of nuclear extract from TPA-treated Jurkat T-cells and the Rel consensus DNA sequence. Note that the supershifted band by the anti-p65 antibody observed in Jurkat T-cells migrates similarly to that in luteinized granulosa cells.

specifically to the NFκB binding site since this binding was competed by a cold but not by a mutated NFκB probe. Similarly to what we observed in the corpus luteum, p65 was the only component of the NFκB family of proteins recognized in the protein/DNA complex in

luteinized granulosa cells (Fig. 7A). Nuclear extracts obtained from Jurkat T-cells treated with TPA and calcium ionophore were run in parallel and showed similar bands to those obtained in ovarian cells. The band supershifted in the presence of the anti-p65 antibody was similar in both cell types

Figure 8 Nuclear extracts from luteinized granulosa cells treated with PRL have a time-dependent increase in binding to a Rel consensus sequence. Primary luteinized granulosa cells were treated with 0.5 μ g/ml oPRL for the times depicted. DNA/protein complexes were resolved by nondenaturing PAGE and detected by autoradiography. The unbound probe is shown at the bottom of the gel (free probe). Experiment shown was performed in triplicate with consistent results.

(Fig. 7A and B). When luteinized granulosa cells were treated with PRL for various times (Fig. 8), an increase in NF κ B binding activity was observed within 15 min and remained elevated for 6 h. A decrease in binding activity was observed after 24 h.

Blocking NF κ B/p65 binding to an NF κ B response element correlates with cell death in luteinized granulosa cells

In order to determine a role for NF κ B in luteal function, we took advantage of the previous observation that p65 is the main component of NF κ B able to bind DNA in luteal as well as luteinized granulosa cells. We cultured primary luteinized granulosa cells in the presence of helenalin, a sesquiterpene lactone that inhibits NF κ B by specific and irreversible alkylation of the p65 subunit, thereby blocking DNA binding (Lyss *et al.* 1998). To confirm that helenalin blocked

binding of NF κ B to DNA we performed a time-course experiment in luteinized granulosa cells in the presence of different doses of the compound. As expected, helenalin was capable of blocking binding of nuclear extracts to the NF κ B probe within 1 h of treatment, whereas the inhibition persisted for at least 6 h (Fig. 9). After confirming that helenalin blocked binding of NF κ B/p65 to DNA, we examined whether inhibition of NF κ B activity causes ovarian cell death as previously reported for other cell types (Yamamoto & Gaynor 2001, Orlowski & Baldwin 2002). The results shown in Fig. 10 indicate that helenalin was capable of inducing ovarian cell death in a dose-dependent manner. Observations on the morphology of the cells in a bright field indicate that the ovarian cells in culture most probably undergo cell death in the presence of helenalin, and the condensation of the chromatin after DAPI staining clearly confirms such a previous presumption.

Figure 9 Time-course inhibition of binding of nuclear extracts of luteinized granulosa cells by treatment with helenalin. Nuclear extracts from luteinized granulosa cells treated with either helenalin or vehicle were analyzed by EMSA using a probe containing the Rel response element. Experiments were repeated three times, and results from one representative experiment are shown.

Overexpression of NFκB/p65 in GG-CL luteal cells inhibits the expression of the 20αHSD gene

Previous data from our laboratory indicated that the potent NFκB activator LPS decreased the expression of the progesterone-catabolizing enzyme 20αHSD in rat luteinized granulosa cells (Telleria *et al.* 2000). In addition, our laboratory has previously cloned the cDNA encoding 20αHSD (Mao *et al.* 1994). This gene is highly induced before parturition when luteal regression is taking place, and its induction is crucial in order for parturition to occur (Wiest *et al.* 1968). We have also cloned the 5' flanking region of the 20αHSD gene (Zhong *et al.* 1998), which revealed the presence of three putative NFκB response elements at positions -2393, -1484, and -222 from the transcription starting site. The sequence of the NFκB response element located between pos-

itions -222 and -213 contains a perfect Rel sequence known to bind NFκB/p65 (GGGGTAT TCC). In order to determine the possible role of NFκB as regulator of 20αHSD gene expression, we transfected GG-CL luteal cells with the full-length 2.5 kb 20αHSD promoter linked to a luciferase reporter gene, and incubated the cells in the presence of increasing concentrations of an NFκB/p65 expression plasmid. We found that transfection of the NFκB/p65 expression plasmid induced a decrease in the activity of the 20αHSD promoter in a DNA-dependent fashion (Fig. 11A) together with a decrease in the endogenous levels of mRNA encoding 20αHSD (Fig. 11B and C).

Discussion

The corpus luteum is a transient endocrine gland whose life span is limited to accomplish a specific reproductive function. While functioning, the cells composing the corpus luteum appear to be terminally differentiated and committed to the production of progesterone. When progesterone is no longer needed the corpus luteum regresses through a process that involves loss of progesterone-producing capacity and cell death. To achieve its mission luteal cells are subjected to a fine-tuned regulation by hormones. In the pregnant rat, the corpus luteum is maintained in function by PRL, placental lactogens, luteinizing hormone, progesterone, androgens and estrogens (Risk & Gibori 2001). In the present study, we introduced the transcription factor NFκB as a putative mediator of hormonally controlled mechanisms involved in luteal cell function and survival. The fact that NFκB activation in the corpus luteum was developmentally regulated in pregnancy suggests that such activation is under hormonal control. This reasoning is supported by the results showing that the basal activation status of NFκB in cultured luteinized cells was increased by the presence of PRL. These results also suggest that PRL may control the expression of certain luteal genes by regulating NFκB activation. In this regard, we have demonstrated that NFκB is capable of silencing the expression of the 20αHSD gene, which has been shown to be inhibited by PRL in the corpus luteum (Albarracin *et al.* 1994, Zhong *et al.* 1997); however, whether PRL inhibition of 20αHSD in luteal cells involves NFκB activation requires further

Figure 10 Culture of primary luteinized granulosa cells in the presence of different concentrations of the p65 inhibitor helenalin. The cells were treated with vehicle or 1, 5, or 10 μ M helenalin for 6 h. At the end of the incubation, the cells were fixed and stained with DAPI. Images were taken in parallel in bright fields (top panels) and fluorescence fields (DAPI; bottom panels). Magnification $\times 200$.

investigation. In addition, we have shown that by inactivating NF κ B, cell death can be induced in luteinized cells, opening the possibility for a role of NF κ B in controlling the expression of genes involved in cell survival and apoptosis.

The p50 and p65 proteins are essentially present in all cells (Siebenlist *et al.* 1994) and this was also true for luteal cells. In the corpus luteum we showed that both p50 and p65 proteins are expressed in the cytoplasm and nucleus of the luteal cells. Even though the p50/p65 dimer is the most frequent NF κ B complex in different cell types, including reproductive organs such as testis (Delfino & Walker 1998) and mammary gland (Geymayer & Doppler 2000), our results indicate that p65 is the major member of the NF κ B family of proteins capable of binding DNA in the corpus luteum, most probably as a p65/p65 homodimer. Even if p50 does not bind DNA associated with p65, it may have a role in luteal function by forming complexes with other members of the NF κ B family of proteins, and regulating gene expression by binding other response elements distinct from the classical NF κ B DNA element (Rel) tested in our study. Therefore the function of p50 in luteal cells needs further investigation. It is also interesting to note from our immunohistochemistry studies that the presence of p65 within

luteal cells varied from cell to cell. Whereas p65 in some cells showed strong nuclear localization, it was less abundant in other surrounding cells. Additionally, the abundance of p65 in the cytoplasm also varied between cells. This may indicate that the activation status of NF κ B in luteal cells is not synchronized along the entire gland and that in each particular luteal cell the transcription factor might be regulating physiological processes in need of being controlled on a cell-to-cell basis. The absence of p52, cRel and RelB within the protein/DNA complexes in the corpus luteum was not surprising since the expression of these NF κ B subunits is mainly restricted to hematopoietic organs (Beauparlant & Hiscott 1996).

Despite p65 appearing to be constitutively expressed in the corpus luteum throughout pregnancy, its activation status (i.e. its capacity to bind an NF κ B DNA consensus sequence) varied along pregnancy. It is clear that the binding capacity of p65 to DNA decreased while pregnancy advanced, suggesting that NF κ B activation depends on the age of the corpus luteum. This was confirmed by the abundant binding to the DNA probe of nuclear extracts obtained from young corpora lutea as compared with nuclear extracts obtained from old corpora lutea of pregnancy, both coexisting in the same ovaries of rats 4 days after

parturition. As we demonstrated that p65 binding to DNA is important for cell survival and for inhibition of 20αHSD, it is tempting to speculate that early on in pregnancy young corpora lutea having NFκB highly activated are better protected against insults that threaten normal luteal function; one such insult could be the activation of 20αHSD gene expression, which leads to increased progesterone catabolism, luteal regression, and termination of pregnancy (Wiest *et al.* 1968).

The decline in NFκB DNA binding at the end of pregnancy in the rat corpus luteum does not appear to be due to a decline in p65 protein expression and/or to IκB phosphorylation. Whether it involves protein–protein interaction between NFκB and Stat5b or NFκB and the glucocorticoid receptor, as previously shown for other cell types (May & Ghosh 1998, Luo & Yu-Lee 2000), remains to be investigated. The loss of sensitivity of the corpus luteum to PRL at the time of luteal regression may contribute to the low activation status of NFκB found at the end of pregnancy in our studies. At the end of pregnancy in the rat, PRL does no longer target the corpus luteum. This is first due to the lack of sensitivity caused by the rapid expression of inhibitor of cytokine signaling molecules that interfere with PRL signaling (Curlew *et al.* 2002), and second, because of the disappearance of PRL receptors (Telleria *et al.* 1997), most probably due to luteolytic signals triggered by prostaglandin F2α (Stocco *et al.* 2003). The fact that PRL was capable of stimulating NFκB in luteinized cells is consistent with the tropic action of PRL through most of pregnancy, which may well be stimulating NFκB. The decrease in NFκB binding activity at the end of pregnancy (day 21) coincides with the disappearance of PRL receptors (Telleria *et al.* 1997). Thus, because PRL can no longer target the corpus luteum at the end of pregnancy, this may lead to a decrease in NFκB activity. In addition, because NFκB can inhibit 20αHSD gene expression and protect against apoptosis, the abrupt decrease in NFκB binding activity observed at the end of pregnancy may be permissive to apoptogenic and antisteroidogenic signals favoring luteal regression. Whereas 20αHSD gene is repressed by PRL, it is upregulated by prostaglandin F2α through the induction of transcription factor NUR77 (Stocco *et al.* 2000). The involvement of NFκB in the regulation of 20αHSD may indirectly affect the

intraluteal levels of prostaglandin F2α. The rabbit 20αHSD has prostaglandin E2 9-ketoreductase activity (Wintergalen *et al.* 1995) and may convert intraluteal prostaglandin E2 to prostaglandin F2α. Thus, lack of inhibition of 20αHSD during luteal regression by NFκB may lead, if the rat enzyme expresses the 9-ketoreductase activity, to conversion of prostaglandin E2 (potentially luteotropic and antiapoptotic) to prostaglandin F2α (luteolytic and proapoptotic), which may further stimulate the expression of 20αHSD.

It has been shown in hormonally sensitive cancer cells, such as prostate cancer cells, that androgen-induced resistance to apoptotic stimuli (e.g. radiation-induced apoptosis) involves activation of NFκB (Coffey *et al.* 2002). This protection was eliminated when prostate cancer cells over-expressed an IκB superrepressor, suggesting that activation of NFκB is required in the androgen survival signaling. Similarly in the corpus luteum of pregnancy we may assume that PRL, which is a survival factor according to recent evidence presented from our as well as another laboratory (Goyeneche *et al.* 2003b, Kawaminami *et al.* 2003), may elicit its effect by activating NFκB.

Our results also show that the binding of NFκB to DNA at mid-pregnancy could be further activated by the inflammatory endotoxin LPS. The induction of NFκB by LPS could mediate a survival or antiapoptotic signaling pathway in luteal cells. This speculation is based on the fact that by activating NFκB, LPS was capable of inducing resistance to apoptosis triggered by TNF-α, Taxol (a microtubule-binding diterpene), and okadaic acid (an inhibitor of serine/threonine protein phosphatase) in tumor cell lines (Manna & Aggarwal 1999). Our results showing that NFκB is capable of inhibiting 20αHSD gene expression and that LPS stimulates NFκB DNA binding are also consistent with our previous studies showing LPS-induced inhibition of 20αHSD mRNA expression in luteinized granulosa cells (Telleria *et al.* 2000). By stimulating NFκB, LPS may have a luteal cytoprotective effect inhibiting 20αHSD expression and opposing apoptosis. On the other hand, LPS has been shown to induce expression of inflammatory cytokines and prostaglandins in the pregnant rat uterus leading to preterm birth (Bennett *et al.* 2000). Thus, upon systemic LPS infusion, an initial LPS-induced cytoprotective effect in the corpus luteum mediated through

Figure 11 NF κ B/p65 inhibits 20 α HSD gene expression. (A) Luteal cells were co-transfected with 1 μ g/well of the 20 α HSD-Luc promoter, 3 μ g/well of a control β -galactosidase expression vector, and 0.5, 1, 2, or 5 μ g/well of an NF κ B/p65 expression vector. Relative light units were obtained by dividing the luciferase activity by the β -galactosidase activity. (B and C) Endogenous 20 α HSD mRNA levels in luteal cells transfected with none (-), or 5 μ g/well of the NF κ B/p65 expression vector. 20 α HSD mRNA was measured using semiquantitative RT-PCR. Experiments were repeated three times; results from a representative set are shown. Bars represent means $\pm$ S.E.M. ($n=3$).

NF κ B activation may be overridden by induction of prostaglandin production in the uterus, leading to uterine contraction, preterm delivery, and secondary luteal regression.

Another target gene whose expression has been shown to be regulated by NF κ B is that for manganese superoxide dismutase (Mn-SOD) (Jones *et al.* 1997). The enzyme encoded by this gene is expressed in the rat corpus luteum of pregnancy and protects the gland against toxic oxygen radicals by scavenging superoxide radicals (Sugino *et al.* 1993). We have previously shown that LPS stimulated Mn-SOD expression in pregnant rat corpora lutea within 2 h of treatment (Sugino *et al.* 1998b), which coincides with the induction of NF κ B/DNA binding induced by the endotoxin in our present study. Further studies will be needed, however, to determine whether NF κ B regulates Mn-SOD gene expression in the rat corpus luteum.

To the best of our knowledge, this is the first report showing that blockage of NF κ B/p65 binding to DNA in luteinized granulosa cells correlates with induction of cell death. Primary granulosa cells obtained from rats do not die if subjected to serum deprivation (C M Telleria, A A Goyeneche, C O Stocco and G Gibori, unpublished observations) or

to proapoptogenic compounds such as TNF- α (Xiao *et al.* 2001) or Fas ligand (Quirk *et al.* 1998) alone. Only if combined with the protein synthesis inhibitor cycloheximide, were TNF- α (Xiao *et al.* 2001) and Fas ligand (Quirk *et al.* 1998) capable of triggering granulosa cell death. TNF- α in these cells stimulated expression of inhibitor of apoptosis proteins (IAPs) through an NF κ B-dependent process (Xiao *et al.* 2001, 2002). Therefore, it is reasonable to speculate that NF κ B could play a central role in the prevention of cell death in the corpus luteum by stimulating the expression of IAPs, such as Xiap (Xiao *et al.* 2001) and/or FLIP (Xiao *et al.* 2002).

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