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The transcription factor C/EBP-β and its role in ovarian function; evidence for direct involvement in the ovulatory process

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Gonadotropins are responsible for maturation of the ovarian follicle and the oocyte. Ovulation is the ultimate step in this process and involves disintegration of the follicular wall and subsequent release of an oocyte into the oviduct. These events are triggered by a surge of luteinizing hormone (LH). Genes expressed in the ovary, that respond to LH, are likely to be involved in the biochemical pathways that regulate ovulation. The transcription factor C/EBP-β is induced promptly in the ovary, as a response to an ovulatory dose of gonadotropins. We used an *ex vivo* perfusion system to demonstrate that a specific reduction in ovarian C/EBP-β expression inhibits ovulation. In such ovaries the oocytes appeared to be entrapped within the follicle. We have found a correlation between the expression level of the activating isoform of C/EBP-β and the number of oocytes ovulated in response to gonadotropins. Since a reduction in C/EBP-β expression does not affect the level of the ovulatory mediator prostaglandin endoperoxide synthase-2 (PGS-2), these findings support the view of C/EBP-β as an important factor in the ovulatory process and highlight a C/EBP-β-dependent and PGS-2-independent pathway that takes part in regulation of ovulation.

Keywords: C/EBP-β/gonadotropin/ovulation/PGS-2

Introduction

In the ovary, several gene products have been shown to be regulated by gonadotropins, among them: VEG/PF (vascular endothelial growth/permeability factor; Koos, 1995), α2-macroglobulin (a protease inhibitor; Gaddy-Kurten and Richards, 1991), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) receptors (Camp *et al*., 1991), PGS-2 (prostaglandin endoperoxide synthase 2; Wong *et al*., 1989), the proto-oncogene c-*mos* (Keshet *et al*., 1988), ovarian aromatase cytochrome P450 (Hickey *et al*., 1990) and cyclin D2 (Sicinski *et al*., 1996). Recently, the transcription factors C/EBP-α (Piontkewitz *et al*., 1993) and C/EBP-β (also known as NF-IL6; Sirois and Richards, 1993) were added to the family of gonadotropin-regulated genes expressed in the ovary. Among the gonadotropin-induced factors, C/EBP-β is rather unique as it is induced very promptly in response to an ovulatory dose of LH. Within 30 min after administration of an ovulatory dose of human chorionic gonadotrophin (hCG; an LH-like hormone), C/EBP-β mRNA can be detected in the ovary (Sirois and Richards, 1993). Furthermore, C/EBP-β is the only known transcription factor that is induced in such a rapid and distinct manner as a consequence of an ovulatory dose of gonadotropin. The promoter of the PGS-2 gene contains a *cis*-element that interacts with C/EBP-β. When this site is mutated so that C/EBP-β can no longer bind, a reporter gene construct containing an artificial hybrid PGS-2 promoter will lose ~50% of its gonadotropin inducibility (Sirois and Richards, 1993). Since PGS-2 is the rate-limiting step in the synthesis of the ovulatory mediators prostaglandins E and F2α, it has been suggested that C/EBP-β acts as a regulator of PGS-2 expression (Sirois and Richards, 1993). However, when an intact PGS-2 promoter is used, in a reporter gene construct, mutations within the C/EBP-β-binding site fail to abolish gonadotropin inducibility (Morris and Richards, 1996). The fact that the transcription regulator C/EBP-β is induced promptly by gonadotropins and that recent investigations suggest that its biological function is distinct from that of regulating PGS-2 expression prompted us to investigate C/EBP-β as a possible regulator of ovulation through a previously unknown pathway.

To test this hypothesis, we used an *ex vivo* perfusion system in which an ovary, with connecting blood vessels, from an equine chorionic gonadotropin (eCG)-treated female rat was surgically removed and connected to a perfusion system which has been described earlier (Koos *et al*., 1984). In this perfusion system, the entire ovary is perfused through its capillary bed with oxygenated and heated (37°C) culture medium maintaining the integrity of the ovary for up to 24 h. During this period of time, the ovary ovulates as a response to exogenously added LH (Koos *et al*., 1984; Brannstrom *et al*., 1987). Oocyte morphology and maturation are indistinguishable from those *in vivo* (Kobayashi *et al*., 1983b; Cajander *et al*., 1984; Brannstrom *et al*., 1987). Rabbit oocytes that have ovulated in an *ex vivo* perfusion system will establish normal pregnancies when transferred to a recipient (Kobayashi *et al*., 1983a). Thus, the biological pathways that induce ovulation in this *ex vivo* system closely mimic *in vivo* conditions. In this *ex vivo* system, we registered the ability of antisense oligonucleotides to specifically reduce C/EBP-β expression. The biological effects of such a reduction were monitored as the ability of ovaries to ovulate in response to exogenous gonadotropins. Gonadotropin-induced differences in ovarian gene expression have been used previously to identify genes of potential interest

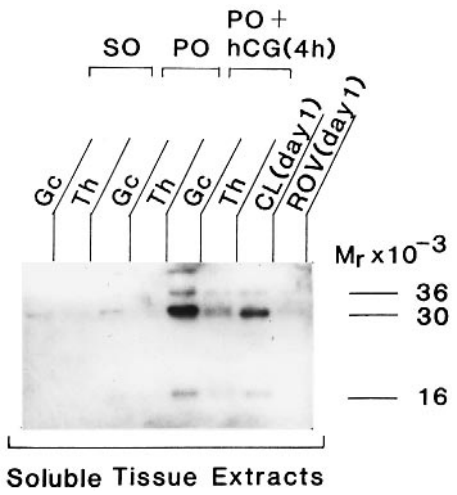

Fig. 1. Immunoblot demonstrating the induction of C/EBP-β in granulosa cells (Gc) and to a lesser extent in theca cells (Th) *in vivo* of pre-ovulatory follicles (PO) 4 h after an ovulatory dose of human chorionic gonadotropin (+hCG). Gc and Th cells were obtained from immature ovaries prior to pregnant mare's serum gonadotropin (PMSG) treatment (SO), 48 h after PMSG (PO) or 4 h after hCG treatment of PO ovaries [PO + hCG(4 h)]. Corpora lutea (CL) and residual ovarian tissues (ROV) were obtained at day 1 of pseudopregnancy. Shown is one out of three independent experiments showing very similar results with a clear induction of the activating isoform of C/EBP-β (LAP) of ~32 kDa and, to a variable degree, also a slight increase in the inhibitory isoform (LIP) of ~16 kDa. Thirty μg of total protein was loaded in each lane.

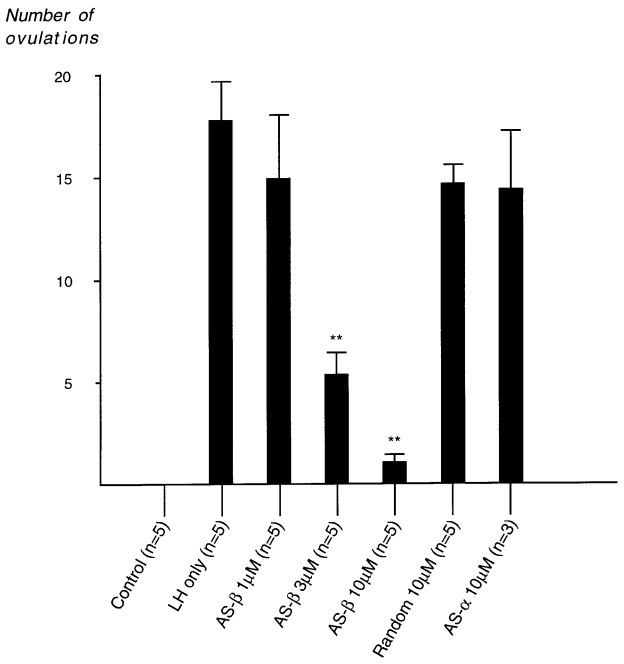

Fig. 2. Ovulation rate in *ex vivo* perfused ovaries in the presence of various concentrations of phosphorothioate antisense oligonucleotides directed against C/EBP-β (AS-β), C/EBP-α (AS-α) or a random sequence with no known similarities to any sequence in GenBank. Significantly lower number of oocytes ovulated ** ($P < 0.01$). Values are expressed as means ± SEM. The control group did not receive any LH, whereas the other groups were treated with an ovulatory dose of LH with or without various oligonucleotides as indicated in the figure.

as regulators of ovulation (Keshet *et al*., 1988; Wong *et al*., 1989; Hickey *et al*., 1990; Camp *et al*., 1991; Gaddy-Kurten and Richards, 1991; Koos, 1995). This *ex vivo* perfusion system enabled us to study the ovulation rate as a biological end point parameter. Thus, induced differences in gene expression can be monitored with regard to their effect on ovulation *per se*, not only as a gonadotropin-dependent difference in gene expression. Furthermore, this experimental approach has the advantage that it allowed us to study direct effects on ovarian function, induced by specific changes in its gene expression, eliminating secondary metabolic or hormonal effects originating from extra-ovarian sites.

Results

C/EBP-β is induced by gonadotropins in the ovary

In order to validate this *ex vivo* perfusion system further with regard to gonadotropin-induced changes in gene expression, we reproduced the previously described induction of C/EBP-β expression in pre-ovulatory ovaries after an ovulatory dose of gonadotropins *in vivo* (Sirois and Richards, 1993). After stimulation with an ovulatory dose of hCG, a hormone with LH-like effects, we could demonstrate an induction of the activating isoform of C/EBP-β. Small immature ovaries (SO; not treated with gonadotropin) and pre-ovulatory (PO) ovaries express almost no detectable C/EBP-β (Figure 1). The C/EBP-β gene is expressed as two isoforms, LAP (32 kDa), a transcriptional activator, and LIP (16 kDa), a transcriptional repressor (Descombes and Schibler, 1991). These isoforms are explained by differential use of two AUGs within the same transcript (Descombes and Schibler, 1991). In the ovary, the LAP isoform is the most abundant and is the dominating isoform during gonadotropin-induced C/EBP-β expression (Sirois and Richards, 1993). The data presented in Figure 1 agree well with this. In ovaries treated with an ovulatory dose of gonadotropins, granulosa cells are the major sites of C/EBP-β expression (Figure 1).

Specific inhibition of C/EBP-β expression induces a decrease in the number of ovulated oocytes

To study the effect of a decrease in gonadotropin-induced C/EBP-β expression on ovulation, we synthesized phosphorothioate oligonucleotides, AS-β, targeted against the C/EBP-β transcript. When these oligonucleotides were added, in various concentrations as indicated in Figure 2, to the perfusion medium ~30 min before addition of an ovulatory gonadotropin dose, a significant reduction in the number of ovulated oocytes could be detected. The oligonucleotides directed against C/EBP-β (AS-β, Figure 2) suppressed ovulation dose-dependenly to ~5% of that of the LH group (Figure 2). In order to assess the specificity of the oligonucleotide-induced reduction in number of oocytes ovulated, two control groups were included in the experiment. One group was treated with oligonucleotides directed against the C/EBP-α transcript (AS-α; Figure 2). [C/EBP-α is a member of the same family of transcription factors as C/EBP-β. Identical oligonucleotides have been used previously to reduce ovarian C/EBP-α expression (Piontkewitz *et al*., 1996).] In another group of experiments, ovaries were treated with a random oligonucleotide with no known sequence similarity to any gene. The small decrease of ovulated

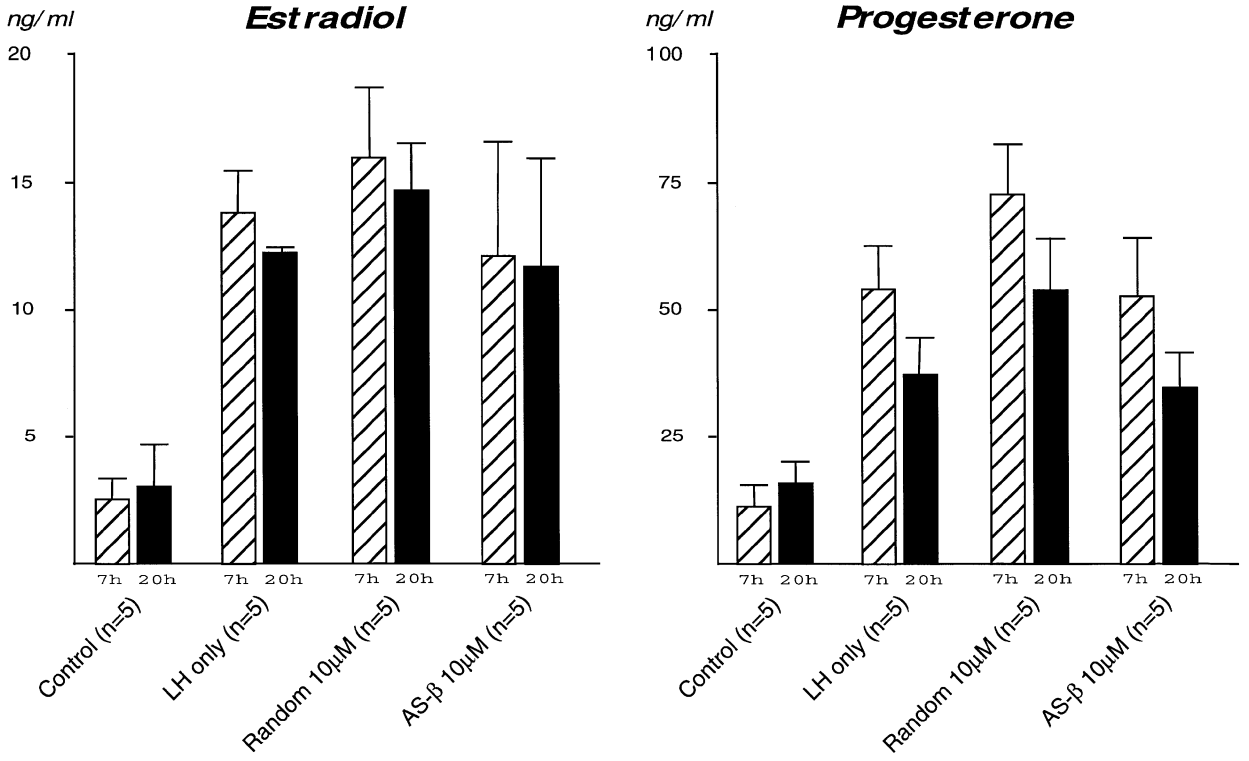

Fig. 3. Levels of progesterone and estradiol in the perfusion medium, measured by RIA (see Materials and methods) 7 h (7 h, hatched bars) and 20 h (20 h, solid bars) after LH administration. No significant differences between groups were found. Values for ovaries not treated with LH (Control) have been included to demonstrate the LH-dependent induction of ovarian hormone synthesis.

oocytes in these control groups was not significant, whereas the dose-dependent decrease in number of ovulated oocytes for AS-β at 3 and 10 μM was clearly significant ($P < 0.01$, Figure 2). Ovarian secretion of progesterone and estradiol was increased by LH, which also is the case *in vivo*. This pattern of secretion was not affected by the presence of any of the oligonucleotides used in this study (Figure 3).

Figure 4 demonstrates that ovaries perfused with 10 μM antisense oligonucleotides directed against C/EBP-β have lost their expression of the major activating isoform of C/EBP-β (LAP), whereas ovaries treated with either no antisense oligonucleotide, antisense oligonucleotide directed against C/EBP-α or a random oligonucleotide expressed unaffected levels of LAP. Thus, the dose-dependent decrease of ovulated oocytes in ovaries treated with antisense oligonucleotides directed against C/EBP-β (Figure 2) is specific and agrees well with the findings in Figure 4. Apart from the major isoforms of C/EBP-β, LAP (32 kDa) and LIP (16 kDa), in some experiments we observed immunoreactive forms migrating with an apparent M_r of 34–36 kDa (Figure 4). The presence of immunoreactive forms with a molecular mass greater than that of LAP (32 kDa), in the size range of 34–45 kDa, has been reported by several investigators (Descombes and Schibler, 1991; Doppler *et al*., 1995; Raught *et al*., 1995). Typically, in situations where LAP (32 kDa) and LIP (16 kDa) are regulated, these larger immunoforms remain unaffected (Raught *et al*., 1995). The origin of these immunoforms is presently unclear; alternative isoforms of C/EBP-β as well as post-translationally modified forms of LAP (32 kDa) and/or LIP (16 kDa) have been suggested (Doppler *et al*., 1995; Raught *et al*., 1995).

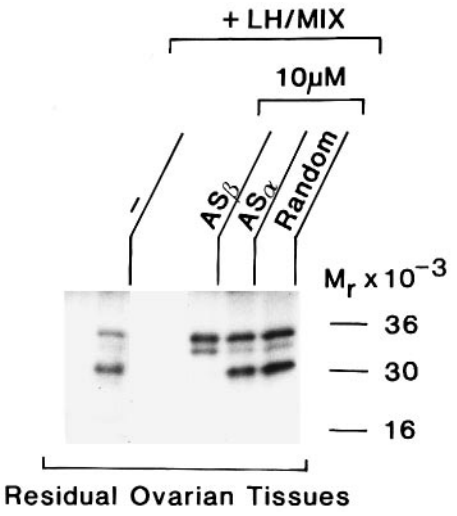

Fig. 4. Immunoblot demonstrating the contents of C/EBP-β in ovaries obtained at 4 h after addition of LH. Treatment with LH induced the expression of the major activating isoform (LAP) of C/EBP-β (32 kDa). Treatment with antisense oligonucleotides directed against C/EBP-β (AS-β) resulted in a complete loss of the 32 kDa form (LAP), whereas antisense oligonucleotides directed against C/EBP-α (AS-α) or a random oligonucleotide were without effect. This is one of three independent experiments showing very similar results; 30 μg of total protein was loaded in each lane.

Ovaries with decreased C/EBP-β expression contain entrapped oocytes

The decrease in the number of ovulated oocytes and amount of immunostainable C/EBP-β in the ovaries treated with antisense oligonucleotides directed against C/EBP-β (Figures 2 and 4) made us interested in the morphology

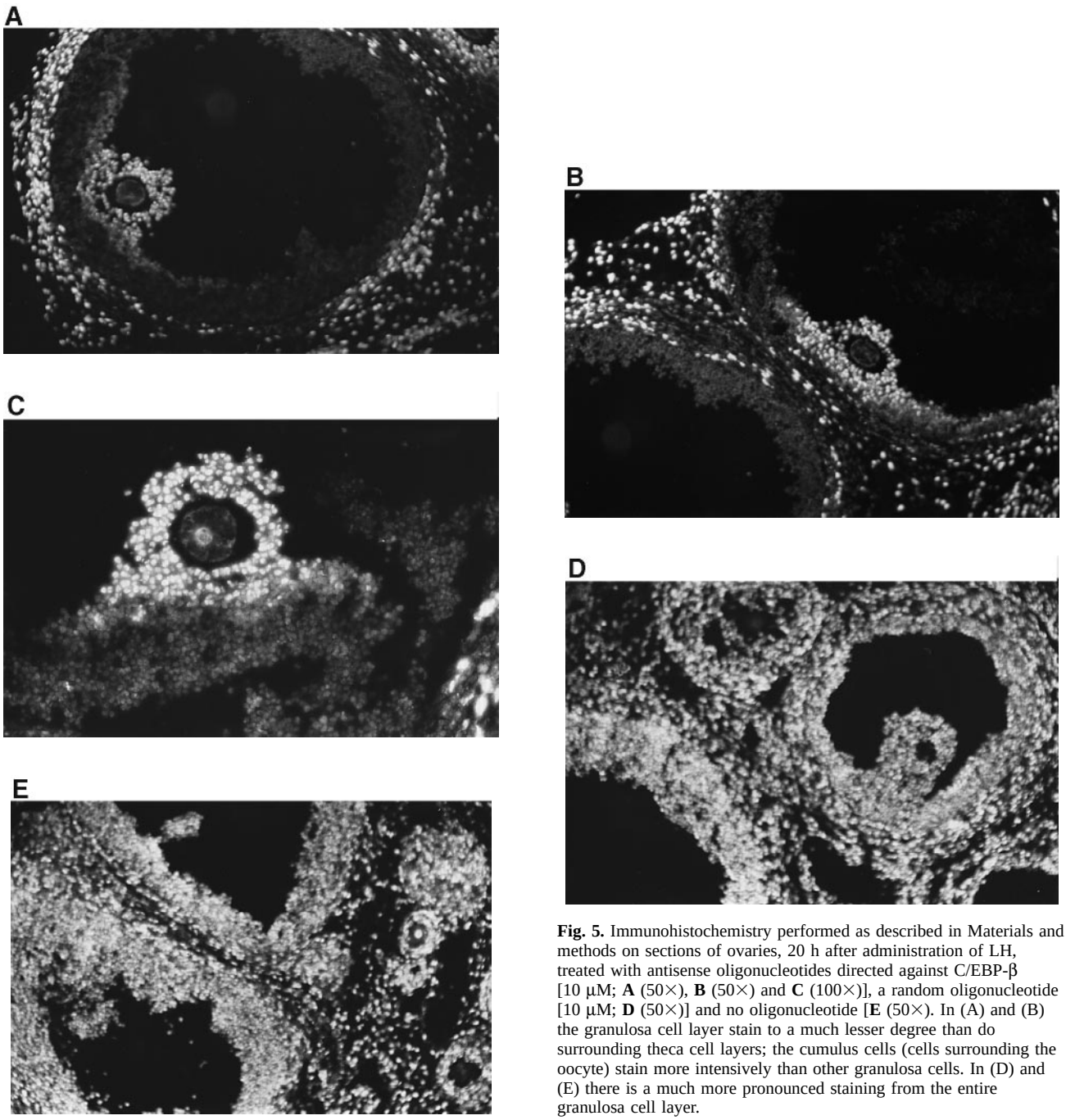

Fig. 5. Immunohistochemistry performed as described in Materials and methods on sections of ovaries, 20 h after administration of LH, treated with antisense oligonucleotides directed against C/EBP-β [10 μM; **A** (50×), **B** (50×) and **C** (100×)], a random oligonucleotide [10 μM; **D** (50×)] and no oligonucleotide [**E** (50×). In (A) and (B) the granulosa cell layer stain to a much lesser degree than do surrounding theca cell layers; the cumulus cells (cells surrounding the oocyte) stain more intensively than other granulosa cells. In (D) and (E) there is a much more pronounced staining from the entire granulosa cell layer.

as well as the tissue distribution of C/EBP-β expression. For this purpose, ovaries from three groups, treated with LH only, LH + a random oligonucleotide (10 μM) and LH + C/EBP-β antisense oligonucleotides (10 μM), were fixed, sectioned and stained 20 h after an ovulatory LH dose had been given. There was a clear difference in morphology. The control groups (LH only and LH + a random oligonucleotide), i.e. the groups that ovulated some 15–20 oocytes (Figure 2), had a wide range of different sized follicles. In the periphery of these ovaries, large follicles were present without visible oocytes, with the appearance of follicles that had recently ovulated. These ovaries displayed normal morphology (not shown). The ovaries that had been treated with LH + C/EBP-β antisense oligonucleotides, which ovulated 0–2 oocytes, had several large follicles, in the periphery of the ovary, in which the oocytes still were enclosed (not shown). Apart from this finding, there was no obvious difference in the morphology when compared with the control groups.

In order to evaluate further the tissue distribution of C/EBP-β expression in these three groups of ovaries, we used immunohistochemistry (Figure 5). In the ovaries treated with LH + C/EBP-β antisense oligonucleotides, a clear reduction of immunostainable C/EBP-β was evident in the granulosa cell layers (Figure 5A–C). Sparsely distributed positive cells within the theca cell layer were also present (Figure 5A and B). Furthermore, the cumulus cells, surrounding the entrapped oocyte, exhibited a relatively high level of C/EBP-β expression compared with other granulosa cells (Figure 5A–C). In sections

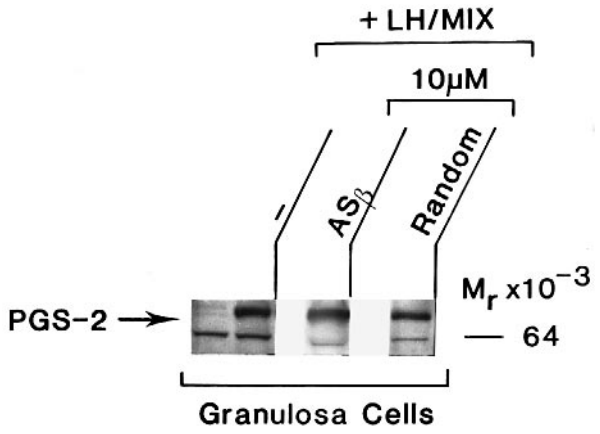

Fig. 6. Immunoblot demonstrating the content of PGS-2 in granulosa cells of ovaries obtained 4 h after LH administration. Control ovaries contained minute amounts of the protein. The addition of LH resulted in an increase of PGS-2 (72 kDa). Treatment with AS-β or random oligonucleotides did not affect the expression of PGS-2. This is one of three independent experiments showing very similar results; 15 μg of total protein was loaded in each lane.

containing the nucleolus, it was evident that this structure also stained positive (Figure 5C). In the control groups (LH only and LH + a random oligonucleotide), very few, if any, entrapped oocytes were present. In these groups, the staining pattern was strong and uniformly distributed in the entire granulosa cell population (Figure 5D and E). The cells contained within the theca cell layers were stained to a lesser degree when compared with granulosa cells, and the signal was similar to that seen in theca cell layers from the antisense C/EBP-β-treated ovaries; i.e. an unevenly distributed signal (Figure 5D and E).

PGS-2 expression is not altered in response to specific inhibition of C/EBP-β

In Figure 6 we show that no major alteration in PGS-2 expression could be detected in any of the groups. This suggests that the inhibitory effect on ovulation, specifically mediated by the C/EBP-β antisense oligonucleotides, is independent of gonadotropin-induced PGS-2 expression. This is in accordance with the finding that an intact PGS-2 promoter, in a reporter gene construct, is not dependent on C/EBP-β for its gonadotropin inducibility (Morris and Richards, 1996). These results suggest that a C/EBP-β-dependent pathway, capable of regulating ovulation, is independent of PGS-2.

Discussion

In ovaries treated with antisense oligonucleotides directed against the transcription factor C/EBP-β we can demonstrate: (i) a decrease in gonadotropin-induced ovulation (Figure 2); (ii) a specific decrease in C/EBP-β expression assayed both with immunoblots (Figure 4) and by immunohistochemistry (Figure 5); and (iii) an altered morphology with seemingly entrapped oocytes (Figure 5). This is true only for ovaries with reduced C/EBP-β expression, whereas in ovaries with normal C/EBP-β expression none or very few entrapped oocytes can be demonstrated (Figure 5). In order to confirm a specific decrease in C/EBP-β expression, we have included several controls. Neither a specific control, i.e. an antisense oligonucleotide proven to be able specifically to reduce ovarian expression of C/EBP-α (Piontkewitz *et al.*, 1996), nor a non-specific random oligonucleotide, with no known sequence similarity to any gene, were able to affect the expression of C/EBP-β significantly (Figure 4). As one would expect, in the case of a specific and biologically significant decrease in gene expression, only in ovaries with a reduced level of C/EBP-β could we demonstrate these morphological and functional alterations, whereas ovaries derived from the control experiments did not, in any significant respect, differ from ovaries not treated with antisense oligonucleotides. Thus, in this study, we demonstrate that gonadotropin-induced expression of the activating isoform (LAP) of C/EBP-β, is an important factor for ovulation to take place in an *ex vivo* perfused ovary; since this system faithfully resembles *in vivo* conditions (Kobayashi *et al*., 1983a,b; Cajander *et al*., 1984; Koos *et al*., 1984; Brannstrom *et al*., 1987), this *ex vivo* study implicates C/EBP-β as a key regulator of ovulation *in vivo*. To our knowledge, this is the only demonstration of a transcription factor which exhibits a dose dependency between expression level and number of oocytes ovulated in response to gonadotropins.

After an ovulatory dose of gonadotropin, C/EBP-β is induced promptly in granulosa cells (Sirois and Richards, 1993) whereas C/EBP-α, another member of the C/EBP family of transcription factors, decreases in granulosa cells exposed to LH (Sirois and Richards, 1993; Piontkewitz *et al*., 1996). Based on these findings, we speculate that the role of C/EBP-α is to support follicular differentiation so that ovulation will occur in response to gonadotropins; on the other hand, C/EBP-β expression seems to be necessary for the ovulation process *per se*. The reduction in C/EBP-β expression does not seem to affect the expression of PGS-2 (Figure 6). This is in accordance with the fact that mutations within the C/EBP-β-binding *cis*-element in the PGS-2 promoter do not alter this gene's gonadotropin inducibility (Morris and Richards, 1996). We speculate that an increase in cAMP, due to a surge in LH concentration, would induce both C/EBP-β (Sirois and Richards, 1993) and PGS-2 (Sirois *et al*., 1993) in pre-ovulatory granulosa cells and that these events are parallel rather than dependent on each other in a linear way. Since a decrease in C/EBP-β expression initiates several morphological as well as functional events, without affecting PGS-2 expression, it is possible that there are several parallel cAMP-dependent pathways regulating ovulation.

The morphological appearance of ovaries with a reduced level of C/EBP-β resembles that of mice that lack cyclin D2, due to a targeted disruption of this locus (Sicinski *et al*., 1996). These mice fail to ovulate in response to gonadotropins and their oocytes are not released from the follicles (Sicinski *et al*., 1996). Both cyclin D2 and C/EBP-β are expressed in granulosa cells and they are also induced by cAMP (Metz and Ziff, 1991; Sicinski *et al*., 1996) and gonadotropins (Sirois and Richards, 1993; Sicinski *et al*., 1996). Furthermore, both cyclin D2 and C/EBP-β are known to interact directly and form complexes with the gene product of the granulosa cell expressed retinoblastoma tumor suppressor gene (pRb; Kato *et al*., 1993; Bukovsky *et al*., 1995; Chen *et al*., 1996). An intriguing possibility is that several parallel

cAMP-dependent ovulatory signaling pathways would converge with pRb as their target. pRb is a key cell cycle regulator and it is conceivable that cell cycle events regulating ovulation are dependent on cAMP-induced expression of both C/EBP-β and cyclin D2. Clearly more research is needed to see if any of these coincidences are relevant to ovulation control.

Materials and methods

Immunoblotting

Soluble tissue extracts were prepared as previously described (Piontkewitz *et al*., 1993) with minor modifications. Briefly, the tissues were homogenized by a polytron (Kinematica, Switzerland) in PE buffer (10 mM potassium phosphate buffer, pH 6.8, and 1 mM EDTA) containing 10 mM 3-[(3-cholamidopropyl)dimethyl-ammonio]1-propan sulfonate (CHAPS; Boehringer), aprotinin (1 μg/ml; Boehringer), Pefabloc (1 mg/ml, Boehringer), leupeptin (1 mg/ml, Boehringer) and pepstatin (1 mg/ml, Boehringer). The homogenate was sonicated (twice, 10 s each time) and centrifuged (12 000 *g*; 5 min); supernatants were stored at –70°C until analysis. The samples were diluted in SDS sample buffer before loading on a one-dimensional SDS–polyacrylamide gel (4.5% stacking gel, 12% separating gel; Novex system, San Diego, CA). The amount of total protein is indicated in the figure legends. The proteins were transferred to a polyvinyldifluoride membrane (Millipore, Bedford, MA) by electroblotting (Novex system, San Diego, CA). The membranes were then incubated with blocking buffers (Bronstein *et al*., 1992) containing rabbit polyclonal antibodies against C/EBP-β (Santa Cruz Biotechnology, Santa Cruz, CA) and PGS-2 (Cayman Chemicals, Ann Arbor, MI). Immunoreactive proteins were visualized by chemiluminescense using an alkaline phosphatase-conjugated second antibody and CSPD (Tropix, Bedford, MA) as substrate. The filters were exposed to ECL film (Amersham, UK) at room temperature for times of 30 s to 2 min, and the films subsequently were developed. Some ovaries were dissected so that theca cells and granulosa cells could be prepared as separate fractions (Richards *et al*., 1986)

Ex vivo perfusion system

Immature Sprague–Dawley rats were primed with eCG (15 IU) to obtain a first generation of pre-ovulatory follicles 48 h later. At this time, a laparatomy was performed with cannulations of the aorta and vena cava caudally to the branching of the ovarian vessels. All vessels branching from the aorta and vena cava segment, except the right ovarian artery and vein, were then ligated. The specimen was mounted in a recirculating perfusion system and kept viable by continuous perfusion with oxygenated medium M 199 with Earle's salts. Ovine LH, 0.1 μg/ml NIH o-25, was used in combination with 0.2 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (Kobayashi *et al*., 1983a; Cajander *et al*., 1984; Koos *et al*., 1984; Brannstrom *et al*., 1987).

Oligonucleotides

Phosphorothioate-substituted oligonucleotides were synthesized on a 3080A DNA synthesizer (Applied Biosystems). AS-β (in Figure 2) designates an equimolar mixture of AS-β-18 (GCG GCT CGG AGT CGG CTC) and AS-β-20 (CCA GCA GGC GGT GCA TGA AC) directed against the extreme 5′-end and the first in-frame AUG of the rat C/EBP-β transcript. AS-α (in Figure 2) designates an equimolar mixture of AS-α1 (CTT TGG GTC GCG AAT GGC) and AS-α2 (CAT GGG GGA GTT AGA GTT) directed against the extreme 5′-end and the first in-frame AUG of the rat C/EBP-α transcript. The random oligonucleotide (GTA AAA CGA CGG CCA GTG) has no known sequence similarity to any gene in the GenBank database.

Histology and immunohistochemistry

Ovaries were incubated in 4% paraformaldehyde–phosphate-buffered saline (PBS) for 4 h. For histology, these ovaries were routinely fixed and paraffin embedded. The sections (10–15 μm) were stained with hematoxylin and examined in a light microscope. For immunohistochemistry, the ovaries were frozen, cryosectioned and fixed in cold acetone at – 20°C for 10 min, then dried at room temperature. The slides were then hydrated with cold PBS and blocked with 5% non-fat milk for 30 min, before the addition of the primary antibodies directed against C/EBP-β (a rabbit polyclonal antiserum, Santa Cruz Biotechnology, Santa Cruz, CA) in a buffer solution (PBS with 1% bovine serum albumin, 0.2% Triton X-100, 0.1% NaN_3) overnight at room temperature. Bound antibodies were visualized with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies (Kemila, Sweden). In control sections, which showed only negligible signals, the first antibody was replaced by either rabbit serum or buffer alone (not shown). The sections were viewed and photographed with a Nikon microphot FX fluorescence microscope (Nikon, Japan).

Steroid measurements

Progesterone and estradiol were measured in the perfusion medium by radioimmunoassay (RIA) using specific antisera (Immuno-Chemicals, Sweden; Hedin *et al*., 1983)

Statistical analysis

Values are expressed as means ± SEM. Comparisons between groups were made by one- or two-way analysis of variance (ANOVA) followed by the Student–Newman–Kreuls multiple range test.

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