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Complete List of Authors:	Galardo, María; Centro de Investigaciones Endocrinológicas (CEDIE-CONICET), Riera, María; Centro de Investigaciones Endocrinológicas (CEDIE-CONICET), Regueira, Mariana; Centro de Investigaciones Endocrinológicas (CEDIE-CONICET), Pellizzari, Eliana; Centro de Investigaciones Endocrinológicas (CEDIE-CONICET), Cigorraga, Selva; Centro de Investigaciones Endocrinológicas (CEDIE-CONICET), Meroni, Silvina; Centro de Investigaciones Endocrinológicas (CEDIE-CONICET),
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Different signal transduction pathways elicited by bFGF and IL1β regulate CREB phosphorylation in Sertoli cells

MN Galardo[1], MF Riera[1], M Regueira[1], EH Pellizzari[1], SB Cigorraga[1] and SB Meroni[1].

[1]Centro de Investigaciones Endocrinológicas (CEDIE-CONICET), Hospital de Niños Ricardo Gutiérrez. Gallo 1330, C1425EDF-Buenos Aires, Argentina.

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CORRESPONDENCE TO: Silvina B. Meroni, PhD

Centro de Investigaciones Endocrinológicas (CEDIE-CONICET)

Hospital de Niños Ricardo Gutiérrez

Gallo 1330, (C1425EDF) Buenos Aires, Argentina

TEL: 5411-4963 5931

FAX: 5411-4963 5930

e-mail: smeroni@cedie.org.ar

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ABBREVIATIONS

fibroblast growth factor	bFGF
interleukin 1β	IL1β
protein kinase A	PKA
cAMP response element binding protein	CREB
phosphatidylinositol 3-kinase	PI3K
protein kinase B	PKB
c-Jun-NH2-terminal kinase	JNK
lactate dehydrogenase	LDH
N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate	dbcAMP
PD98059	PD
U0126	U
SB203580	SB

wortmannin	W
phosphorylated CREB	P-CREB
phosphorylated p38-MAPK)	P-p38-MAPK
total CREB	T-CREB
total p38-MAPK	T-p38-MAPK
radioimmunoassay	RIA

ABSTRACT

BACKGROUND AND AIM: bFGF and IL1β belong to the set of intratesticular regulators that provide for the fine-tuning of processes implicated in the maintenance of spermatogenesis. The aim of this study was to investigate if bFGF and IL1β activate CREB, what signaling pathways may be participating and the possible relationship between CREB activation and the regulation of Sertoli cell function. METHODS: Twenty-day-old rat Sertoli cell cultures were used. RESULTS: Cultures stimulated with bFGF and IL1β produced a time-dependent increment in phosphorylated CREB levels that reached maximal values in 5- and 15-minute incubations respectively. MEK inhibitors -PD98059 and U0126- blocked the effect of bFGF on phosphorylated CREB while a p38-MAPK inhibitor -SB203580- blocked the effect of IL1β on phosphorylated CREB. A possible correlation between CREB regulation and two Sertoli cell-differentiated functions, *Ldh A* and transferrin expression, was explored. PD98059 blocked the ability of bFGF to stimulate *Ldh A* expression and SB203580 blocked the ability of IL1β to stimulate *Ldh A* expression and LDH activity. Concerning transferrin, PD98059 and U0126 were able to inhibit the ability of bFGF to stimulate its secretion. On the contrary, SB203580 was unable to block IL1β induced increase in transferrin secretion suggesting that the p38-MAPK pathway does not participate in the mechanism of action of the cytokine to regulate transferrin. CONCLUSIONS: The results presented herein suggest that CREB is stimulated in response to bFGF and IL1β through p42/p44-MAPK and p38-MAPK pathways and that this transcription factor may be partially responsible for the regulation of Sertoli cell function.

INTRODUCTION

The Sertoli cell, somatic component of the seminiferous tubule, plays an essential role in spermatogenesis. Androgens and the pituitary gonadotropin follicle-stimulating hormone (FSH) control Sertoli cell function. A regulatory role of a large set of locally produced factors, which include growth factors and cytokines, has also been postulated (1). In this context, the effects of basic fibroblast growth factor (bFGF) and interleukin (IL)1β have been analyzed by several authors. FSH, bFGF and IL1β utilize different signal transduction pathways; however, these hormones ultimately regulate specific Sertoli cell biological responses in a similar way (2, 3).

bFGF produces its effects by binding to receptors that belong to the tyrosine kinase family. After ligand binding and receptor dimerization, the receptors become capable of phosphorylating specific residues on their own cytoplasmic tails and on each other's. Phosphorylated tyrosine residues, in turn, recruit other signaling molecules to the activated receptors and propagate the signal through several transduction pathways that may vary in different cell types. Riera et al. (2) have shown that in Sertoli cells, bFGF activates p42/p44-MAPK and PI3K/PKB pathways. Finally, activation by IL1β of several signal transduction pathways, which may also vary according to the cell type, has been observed. Particularly in Sertoli cells, Ishikawa et al. (4) demonstrated that the cytokine is able to activate c-Jun-NH2-terminal kinase (JNK) pathway and more recently it has been observed that IL1β activates PI3K/PKB and p42/p44-MAPK pathways (3).

The transcription factor CREB is activated by phosphorylation at serine residue 133. Phosphorylation of CREB promotes recruitment of transcriptional co-activator CBP/p300, thus allowing the interaction with the basal transcription machinery (5). CREB binds to the

palindromic CRE sequences at DNA as a dimer. Many genes, not having a classical CRE sequence, recognize CREB through CRE-like sequences (6). It is known that in Sertoli cells, CREB is activated under the stimulation of FSH (6), testosterone (7) and retinol (8) but not apparently by growth factors such as IGF-I (9).

Among the nutritional factors that are necessary for the normal development of spermatogenesis lactate production and transferrin secretion by Sertoli cells are prominent. Transferrin provides iron and lactate is the energetic substrate for germ cells. Lactate is produced from pyruvate in a reaction that is catalyzed by lactate dehydrogenase (LDH). Previous studies have shown that in Sertoli cells, transferrin production and LDH activity are stimulated by FSH, bFGF and IL1β (10, 11). The gene promoter encoding for transferrin possesses a CRE like sequence and the one for *Ldh A* possesses a single copy of a consensus CRE-sequence (12, 13), consequently CREB activation may be involved in the regulation of expression of these genes.

Considering that we have previously observed that bFGF and IL1β were able to regulate tansferrin and *Ldh A* in a similar way to FSH, we pursued to investigate if bFGF and IL1β were able to activate CREB similarly to FSH, what signaling pathways were involved and if there was a relationship with the observed biological responses in Sertoli cells.

MATERIALS AND METHODS

Materials

Tissue culture media were purchased form GIBCO BRL (Life Technologies Ltd, Rockville, MD, USA). Ovine FSH (NIH-oFSH-S-16) was obtained from the National Hormone and Pituitary Program, NIDDK, Bethesda, MD, USA. Human recombinant bFGF was purchased from Invitrogen (Invitrogen, Argentina). N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate (dbcAMP) and rat IL1β were purchased from Sigma-Aldrich (St Louis, MO, USA). PD98059 (PD) and U0126 (U) (MEK inhibitors), SB203580 (SB) (p38-MAPK pathway inhibitor), wortmannin (W) (PI3K inhibitor) and H89 (PKA inhibitor) were purchased from Biomol (Plymouth Meeting, PA, USA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Sertoli cell isolation and culture

Twenty-day-old Sprague–Dawley rats were obtained from an animal care unit (Animal Care Laboratory, Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina). Animals were killed by CO_2 asphyxiation according to protocols for animal laboratory use following the principles and procedures outlined in the National Institute of Health Guide for Care and Use of Laboratory Animals.

Sertoli cells were isolated as previously described (14). Briefly, six rats were used, testes were removed, decapsulated and digested with 0.1% w/v collagenase and 0.006% w/v soybean trypsin inhibitor in Hanks' balanced salt solution for 5 minutes at room

temperature. Seminiferous tubules were saved, cut and submitted to 1 mol/l glycine–2 mmol/l EDTA (pH 7.4) treatment to remove peritubular cells. The washed tubular pellet was then digested again with collagenase for 10 minutes at room temperature to remove germinal cells. The Sertoli cell suspension, collected by sedimentation, was resuspended in culture medium which consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, supplemented with 20 mmol/l HEPES, 100 IU/ml penicillin, 2.5 µg/ml amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 µg/ml transferrin, 5 µg/ml insulin, 5 µg/ml vitamin E and 4 ng/ml hydrocortisone. Sertoli cells were cultured in 6- or 24-multiwell plates or in 25 cm^2 tissue culture flasks (5 µg DNA/cm^2) at 34◦C in a mixture of 5% CO_2: 95% air.

No myoid cell contamination was revealed in the cultures when an immunoperoxidase technique was applied to Sertoli cell cultures using a specific antiserum to smooth muscle α actin. Remaining cell contaminants were of germ cell origin and this contamination was below 5% after 48 h in culture as examined by phase contrast microscopy.

Culture conditions

Sertoli cells were allowed to attach for 48h in the culture medium mentioned above and then medium was replaced at this time (day3) by fresh medium without insulin. Treatments with bFGF or IL1β were performed on day 3 in the absence or presence of W, PD, U, SB as indicated in figure legends. In all cases the inhibitors were added 15 minutes prior to the addition of hormones. The 72-h conditioned media obtained on day 6 was used to evaluate transferrin levels. Cells treated for 48 h and harvested on day 5 were used to analyze *Ldh A* mRNA levels or LDH activity.

Cells harvested on day 6, pretreated as indicated in the figure legends, were used for Western blot analysis of phosphorylated CREB and p38-MAPK (P-CREB and P-p38-MAPK) levels. Viability of cells in culture was higher than 98% in all experimental conditions as evaluated by MTS assay.

Cell extracts and Western blot analysis

Cells harvested on day 6 and cultured on 6-well plates, treated as indicated in the figure legends, were used for Western blot analysis. Cells were washed once with phosphate-buffered saline (PBS) at room temperature. Then, 200 µl PBS containing 2 µl of a protease inhibitor cocktail (Sigma P-8340) and 2 mmol/l phenylmethylsulfonyl fluoride were added to the cells. Cells were then placed on ice and disrupted by ultrasonic irradiation. A 200 µl volume of 2X Laemmli buffer (4% w/v SDS, 20% v/v glycerol, 10% v/v 2-mercaptoethanol, 0.004% w/v bromophenol blue and 0.125 mol/l Tris-HCl, pH 6.8) was added and thoroughly mixed. Samples were immersed in a boiling water bath for 5 minutes and then immediately settled on ice. Proteins were resolved in 10% SDS-PAGE (10% acrylamide/bisacrylamide for the resolving gel and 4.3% acrylamide/bisacrylamide for the stacking gel) in a Mini Protean 3 Cell (Bio-Rad, Hercules, CA, USA). After SDS-PAGE, gels were equilibrated in transfer buffer for 10 minutes and electrotransferred at 100 V for 60 minutes onto polyvinylidene difluoride membranes (Hybond-P, Amersham Pharmacia Biotech, Amersham, Bucks, UK) using a Mini Trans-Blot Cell (Bio-Rad). Membranes were probed with commercial antibodies (Phospho-CREB (Ser133) Antibody, CREB Antibody, P-p38-MAPK (Thr180/Tyr182) Antibody and p38-MAPK Antibody; Cell Signaling

Technology, Inc., Danvers, MA, USA) that allow specific recognition of both phosphorylated (P-CREB and P-p38-MAPK) and total (T-CREB and T-p38-MAPK) CREB and p38-MAPK. The intensities of autoradiographic bands were estimated by densitometric scanning using NIH Image software (Scion Corporation, Frederick, MD, USA).

Analysis of *Ldh A* mRNA levels

Northern blot analysis was carried out in total RNA samples isolated from Sertoli cells cultured in 25 cm^2 tissue culture flasks by the guanidinium isothiocyanate method (15). The amount of RNA was estimated by spectrophotometry at 260 nm. About 20 µg total RNA was electrophoresed on a 1% agarose-10% formaldehyde gel. After migration, RNAs were transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech, Buenos Aires, Argentina) by capillary transfer with 10 x SSC (10 x stock solution: 1.5 mol/l NaCl and 0.15 mol/l sodium citrate, pH 7.4) and fixed with U.V. Stratalinker (Stratagene Cloning Systems, La Jolla, CA, USA). cDNA probes (rat *Ldh A* 3'UTR 0.4 Kb insert, Pst1-Bgl2; and 18S oligonucleotide) were labeled with [α-^{32}P]deoxy-CTP (NEN, Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA) using a random-primed labeling kit (Prime-a-Gene Labeling System, Promega Corporation, Madison, USA). Blots were prehybridized for 3 h at 42ºC in 50% w/v formamide, NaCl/phosphate/EDTA (0.75 mol/l NaCl, 20 mmol/l sodium phosphate (pH 7.5) and 1 mmol/l EDTA), 5 x Denhart's solution, 10% w/v dextran sulfate, 0.5% w/v SDS and 100 µg/ml herring sperm DNA. Hybridization was then performed overnight at 42ºC in the same hybridization buffer containing 1-4 x 10^6 c.p.m./ml ^{32}P-labeled probe. Membranes were washed twice in 2x SSC-0.5% w/v SDS (20

minutes, room temperature) followed by two washes in 1x SSC-0.1% w/v SDS (30 minutes, 65ºC). Membranes were exposed to Kodak X-Omat S films (Eastman Kodak, Rochester, NY, USA). The 18S signal was used to standardize *Ldh A* mRNA contents.

LDH activity measurement

After incubation of Sertoli cells in the absence or presence of the different stimuli, culture medium was discarded and cells were disrupted by ultrasonic irradiation in NaCl (0.9% w/v) and centrifuged (15800 ***g***, 10 min). The supernatant was used to measure total LDH activity. Total LDH activity was determined by a routinely used spectrophotometric method (Randox Laboratories, Crumlin, UK).

Transferrin determination

Rat transferrin was measured by radioimmunoassay (RIA) as described by Handelsman et al. (16). A polyclonal antibody raised against rat transferrin in rabbits was used (Cappel Laboratories, Cochranville, PA, USA). The cross-reactivity of human transferrin in this assay is less than 0.003%. This RIA has a sensitivity of 3 µg/tube and intra- and interassay coefficients of variation are 7% and 16% respectively.

Other assays

DNA was determined by the method of Labarca & Paigen (17).

Statistical analysis

All experiments were run in triplicates and repeated three to four times. One-way ANOVA with Tukey-Kramer post test was performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). Probabilities <0.05 were considered statistically significant.

RESULTS

bFGF and IL1β activate CREB in rat Sertoli cells

Sertoli cell cultures were stimulated for variable periods of time (5, 15 and 30 minutes) with 30 ng/ml bFGF or 50 ng/ml IL1β. Stimulation of the cultures with bFGF and IL1β showed time-dependent increments in P-CREB levels reaching maximal stimulus at 5 and 15 minutes respectively (Fig. 1A and 1B). Pooled data from three independent experiments performed in 5 minute incubations with 30 ng/ml bFGF revealed a 11.5±1.6-fold stimulation of P-CREB levels and in 15 minute incubations with 50 ng/ml IL1β a 9.7±1.5-fold stimulation of P-CREB levels was observed (mean ± SD).

A p42/p44-MAPK pathway participates in the activation of CREB by bFGF

The p42/p44-MAPK and PI3K/PKB pathways are activated upon interaction of bFGF with its receptor. We then decided to analyze whether these signaling pathways were involved in

CREB phosphorylation induced by bFGF. To achieve this goal Sertoli cell cultures were incubated for 15 minutes with W or with MEK inhibitors -PD or U-, then they were stimulated with bFGF for 5 minutes and the levels of P-CREB were determined. Figure 2A shows that W did not modify the effect of bFGF on P-CREB levels. Figure 2B shows that PD and U markedly reduced the levels of P-CREB.

Participation of p42/p44-MAPK signal transduction pathway in the regulation of Sertoli cell function by bFGF

The results presented above showed the participation of a p42/p44-MAPK signal transduction pathway in the increase of P-CREB levels produced by bFGF. As mentioned in the introduction, the gene encoding *Ldh A* and transferrin possess a single copy of a consensus CRE-sequence and a CRE-like sequence respectively in their promoters. We then decided to analyze a possible relationship between CREB activation and regulation of Sertoli cell function by bFGF. To achieve this goal *Ldh A* mRNA levels and transferrin secretion in the presence of MEK inhibitors, which reduced the levels of P-CREB, were analyzed. Figure 3A shows that PD inhibited the ability of bFGF to stimulate *Ldh A* expression. Figure 3B shows that, as previously shown (2), U and PD reduced the ability of bFGF to stimulate transferrin secretion.

A p38-MAPK signal transduction pathway participates in the activation of CREB by IL1β

Several signal transduction pathways may be stimulated in response to IL1β. Among them the PI3K/PKB and p42/p44-MAPK signaling pathways are present. We then decided to

analyze whether these signaling pathways were involved in the increase in CREB phosphorylation observed in the presence of IL1β. Sertoli cell cultures were incubated for 15 minutes with W, with PD or U, then stimulated with IL1β for 15 minutes and the levels of P-CREB determined. Figure 4 A and B shows that neither PI3K nor MEK inhibitors blocked IL1β stimulation of CREB phosphorylation.

The p38-MAPK signaling pathway has also been related to the mechanism of action of IL1β; however, its activation in Sertoli cells had not been previously demonstrated. We then decided to evaluate if IL1β was able to activate p38-MAPK pathway in Sertoli cells and if this pathway participated in CREB activation. Figure 4C shows that the cytokine increased P-p38-MAPK levels in a time dependent manner. Figure 4D shows that in cells treated for 15 minutes with SB–a p38-MAPK pathway inhibitor– and then for 15 minutes with IL1β, P-CREB levels decreased (Figure 4D).

Participation of p38-MAPK signal transduction pathway in the regulation of Sertoli cell function by IL1β

As shown before IL1β increased phosphorylation of CREB by a p38-MAPK signal transduction pathway but not by a PI3K/PKB and p42/p44-MAPK signaling pathway. We next analyzed whether p38-MAPK signal transduction pathway participated in the regulation of *Ldh A* and transferrin by ILβ1. Figure 5A shows that SB inhibited the ability of IL1β to stimulate *Ldh A* expression. As expected, SB also blocked IL1β stimulus of LDH activity (Table 1). Figure 5B shows that SB did not modify transferrin secretion. This unexpected result prompted us to analyze whether the other two signaling pathways, which

are not involved in CREB phosphorylation, participated in transferrin regulation by IL1β. Figure 5B also shows that W did not modify IL1β response while PD partially blocked transferrin up-regulation by the cytokine.

DISCUSSION

Sertoli cells provide nutrients and growth factors to the germinal epithelium. FSH and testosterone actions on these cells are essential for optimal production of sperm in mammals. The Gαs/cAMP/PKA pathway has been considered for more than 20 years as the key mechanism relaying FSH biological actions and CREB as the major mediator of FSH-induced changes in gene expression (6, 18, 19). In this context, Scobey et al. (20) have demonstrated that phosphorylated CREB in Sertoli cells is an essential factor that is required to support spermatogenesis.

Riera et al. (2) have demonstrated that bFGF activates PI3K/PKB and p42/p44-MAPK pathways in Sertoli cells. However, a possible role of bFGF in the phosphorylation of CREB had not been explored yet. The results presented herein show that bFGF increases P-CREB levels in Sertoli cells. Furthermore, by the ability of PD and U to reduce bFGF-induced phosphorylation of CREB and the inability of W to have the same effect, we suggest that bFGF utilizes a p42/p44-MAPK dependent pathway to regulate P-CREB levels and consequently the expression of CREB responsive genes in Sertoli cells. It is worth mentioning that CREB phosphorylation by bFGF with the participation of p42/p44-MAPK signaling pathway has been demonstrated in just a few cell types (21).

The presence of MEK inhibitors not only lowered the levels of phosphorylation of p42/p44-MAPK and CREB in response to bFGF, but of two well-known Sertoli cell functions that

rise in response to bFGF –*Ldh A* expression and transferrin production. Previous studies from our laboratory demonstrated that MEK inhibitors are able to block the ability of bFGF to stimulate LDH activity (2). As mentioned previously, *Ldh A* and transferrin gene promoters contain a CRE and a CRE-like sequence respectively (12, 13); therefore, it is tempting to speculate that increased levels of phosphorylated CREB in response to bFGF might be involved, at least in part, in *Ldh A* and transferrin regulation.

The present study also shows that IL1β augments P-CREB levels. Our laboratory has previously demonstrated that IL1β activates PI3K/PKB and p42/p44-MAPK pathways to regulate Sertoli cell function (3). In this study, we observed that neither W nor PD and U modified IL1β stimulation of P-CREB levels, which rules out the participation of PI3K/PKB and p42/p44-MAPK pathways in the activation of CREB by IL1β. Petersen et al. (22) had previously observed that IL1α activates p38-MAPK in immature Sertoli cells. However, the possibility that IL1β was also able to utilize the p38-MAPK pathway in Sertoli cells had not been previously analyzed. The results presented show that IL1β activates p38-MAPK pathway in Sertoli cells. On the other hand, it had been observed that CREB could be activated in a p38-MAPK-dependent manner in some cell types (23, 24, 25). In this respect, we were able to demonstrate that the p38-MAPK pathway participates in the regulation of P-CREB levels in Sertoli cells. In experiments performed in the presence of a p38-MAPK inhibitor, lower levels of phosphorylation of CREB in conjunction with lower *Ldh A* expression and LDH activity were observed suggesting a relationship between these two phenomena. Regulation of gene expression usually involves several transcription factors and consequently the results presented do not rule out the participation of other transcription factors. In this respect, our laboratory has previously shown that IL1β also increases *Ldh A* expression utilizing a p42/p44-MAPK dependent

pathway, which is not involved in CREB phosphorylation, probably involving other transcription factors (3).

To our surprise, inhibition of the p38-MAPK pathway did not result in the impairment of IL1β effect on transferrin production. Additionally, inhibition of PI3K/PKB pathway did not result in a decreased IL1β effect on transferrin production either and just the inhibition of p42/p44-MAPK pathway resulted in a partial decrease in transferrin production. Regulation of gene transcription depends on the binding of a large set of transcription factors to the gene promoter; however, not all of them have to be bound at the same time to enhance transcription. For example, Chaudhary & Skinner (26) demonstrated that in rat Sertoli cells, the increase in transferrin secretion produced by a paracrine testicular factor (PModS) is not dependent on the transcription factor CREB but on those transcription factors that recognize E-box sequence (bHLH proteins) which are present in the transferrin promoter.

Altogether the results presented herein show that in Sertoli cells in addition to FSH, the transcription factor CREB may also be stimulated by bFGF and IL1β. Additionally, these results show that p42/p44-MAPK and p38-MAPK signaling pathways are involved in the regulation of CREB and of Sertoli cell functions by bFGF and IL1β.

FSH and testosterone actions on Sertoli cells are required for optimal production of sperm in mammals. CREB is a major mediator of the action of both hormones (7, 20). The importance of this transcription factor in male reproduction is additionally suggested in this study by the ability of locally produced factors such as bFGF and IL1β to regulate its activation.

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The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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FIGURE LEGENDS

Figure 1. *Effect of bFGF and IL1β on P-CREB levels in rat Sertoli cells.* Sertoli cells were stimulated for variable periods of time (5, 15 and 30 minutes) with 30 ng/ml bFGF (A) or 50 ng/ml IL1β (B). Cell extracts were prepared and utilized for Western blot analysis using antibodies specific for T-CREB or P-CREB. The upper panels show a representative experiment out of three. The lower panels show pooled data of three independent experiments indicating the fold increase in phosphorylation (ratio of P-CREB to T-CREB in each sample) relative to basal. Results are expressed as means±S.D.

Figure 2. *Effect of PI3K and MEK inhibitors on bFGF-stimulated levels of P-CREB in rat Sertoli cells.* Sertoli cells preincubated or not for 15 minutes with (A) wortmannin (W 0.01 and 0.1 μmol/l), (B) PD98059 (PD 30 μmol/l) or U0126 (U 1 μmol/l) were stimulated for 5 minutes with 30 ng/ml bFGF. Cell extracts were prepared and utilized for Western blot analysis using antibodies specific for T-CREB or P-CREB. The upper panels show a representative experiment out of three. The lower panels show pooled data of three

independent experiments indicating the fold increase in phosphorylation (ratio of P-CREB to T-CREB in each sample) relative to basal. Results are expressed as means±S.D.

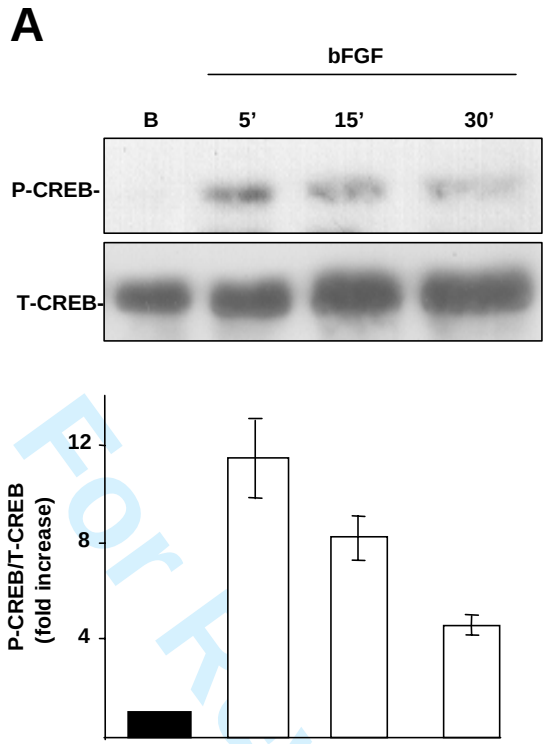
Figure 3. *Effect of MEK inhibitors on bFGF-stimulated Ldh A mRNA levels and transferrin secretion.* (A) Sertoli cells preincubated or not for 15 minutes with PD98059 (PD 10 or 30 µmol/l) were stimulated for 48 hours with 30 ng/ml bFGF. Total RNA was utilized to perform Northern blot analysis. The upper panel shows a representative experiment out of three. The lower panel shows pooled data of three independent experiments indicating the fold increase in *Ldh A* mRNA levels (ratio of *Ldh A* mRNA to 18S in each sample) relative to basal. Results are expressed as means±S.D. (B) Sertoli cells preincubated or not for 15 minutes with U0126 (U 1 µmol/l) or PD98059 (PD 30 µmol/l) were stimulated for 72 hours with 30 ng/ml bFGF. Transferrin was determined in the conditioned media recovered on day 6. Results are expressed as means±S.D. of triplicate incubations in one representative experiment out of three. Different letters situated above columns indicate statistically significant differences among treatment groups ($p<0.05$).

Figure 4. *Effect of PI3K, MEK and p38-MAPK pathway inhibitors on IL1β-stimulated levels of P-CREB in rat Sertoli cells.* Sertoli cells preincubated or not for 15 minutes with either (A) wortmannin (W 0.01 and 0.1 µmol/l) or (B) PD98059 (PD 30 µmol/l) or U0126 (U 1 µmol/l) were stimulated for 15 minutes with 50 ng/ml IL1β. (C) Sertoli cells were stimulated for variable periods of time (5, 15 and 30 minutes) with 50 ng/ml IL1β. (D) Sertoli cells preincubated or not for 15 minutes with SB203580 (1 and 2 µmol/l) were stimulated for 15 minutes with 50 ng/ml IL1β. Cell extracts were prepared and utilized for Western blot analysis using antibodies specific for T-CREB or P-CREB (A, B and D) or T-

p38-MAPK and P-p38-MAPK (C). The upper panels show a representative experiment out of three. The lower panels show pooled data of three independent experiments indicating the fold increase in phosphorylation (ratio of P-CREB to T-CREB or P-p38-MAPK to T-p38-MAPK in each sample) relative to basal. Results are expressed as means±S.D.

Figure 5. *Effect of p38-MAPK pathway inhibitor on IL1β-stimulated Ldh A mRNA levels and transferrin secretion.* (A) Sertoli cells preincubated or not for 15 minutes with SB203580 (SB 1 or 2 µmol/l) were stimulated for 48 hours with 50 ng/ml IL1β. Total RNA was utilized to perform Northern blot analysis. The upper panel shows a representative experiment out of three. The lower panel shows pooled data of three independent experiments indicating the fold increase in *Ldh A* mRNA levels (ratio of *Ldh A* mRNA to 18S in each sample) relative to basal. Results are expressed as means±S.D. (B) Sertoli cells preincubated or not for 15 minutes with SB203580 (SB 2 µmol/l), wortmannin (W 0.1 µmol/l) or PD98059 (PD 30 µmol/l) were stimulated for 72 hours with 50 ng/ml IL1β. Transferrin was determined in the conditioned media recovered on day 6. Results are expressed as means±S.D. of triplicate incubations in one representative experiment out of three. Different letters situated above columns indicate statistically significant differences among treatment groups ($p<0.05$).

Figure 1



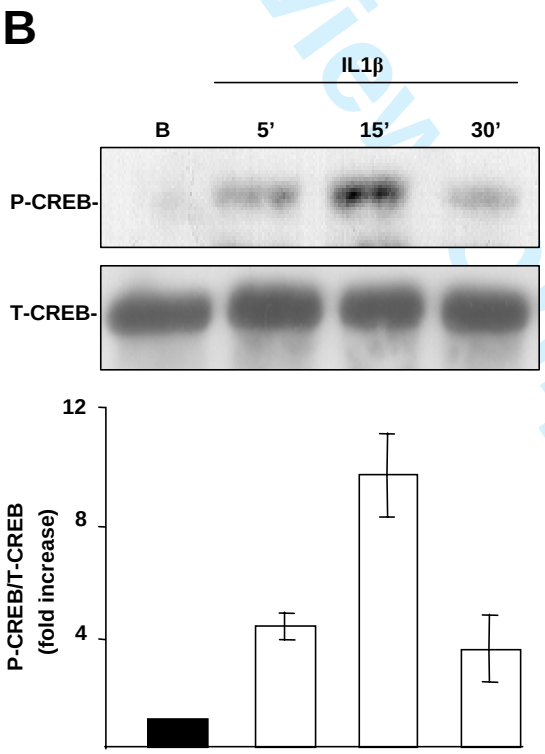
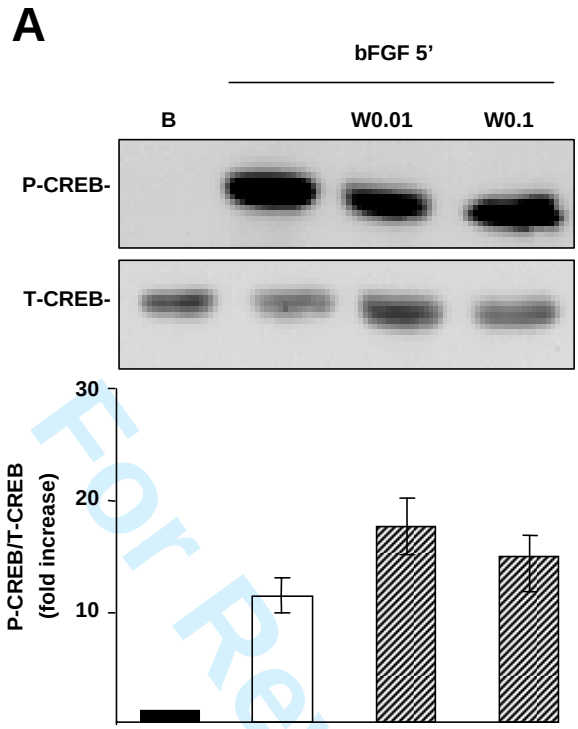


Figure 2



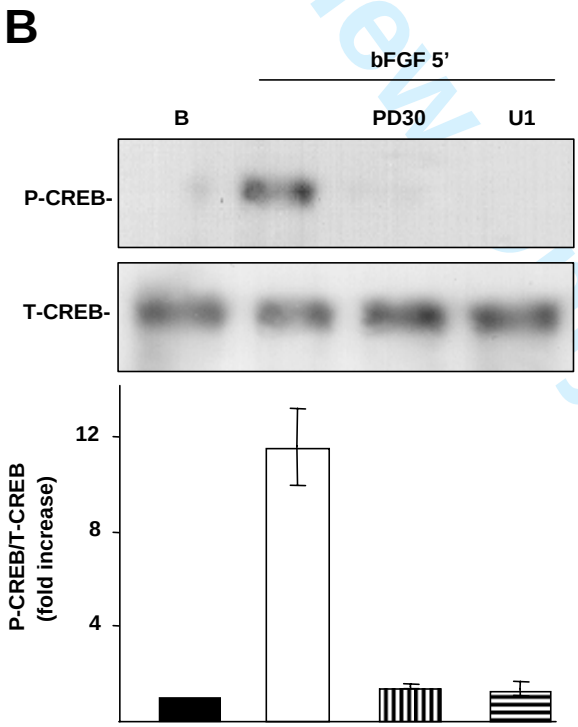
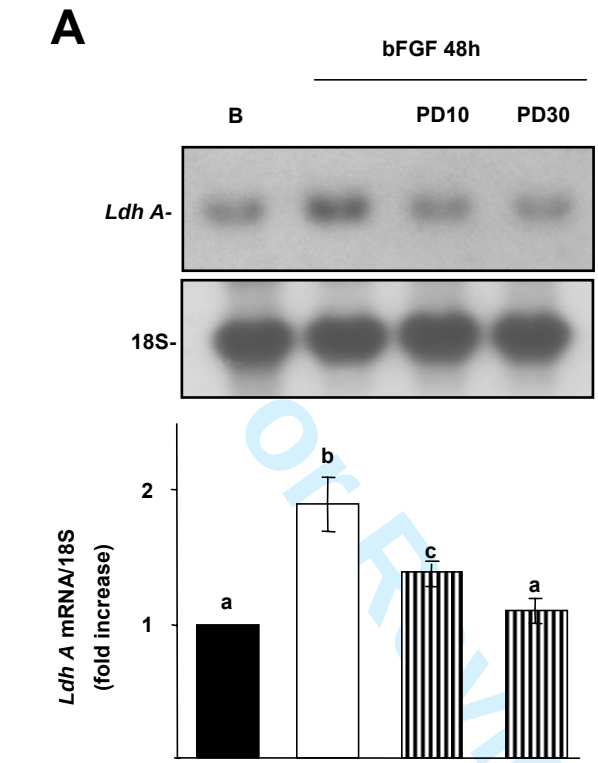


Figure 3



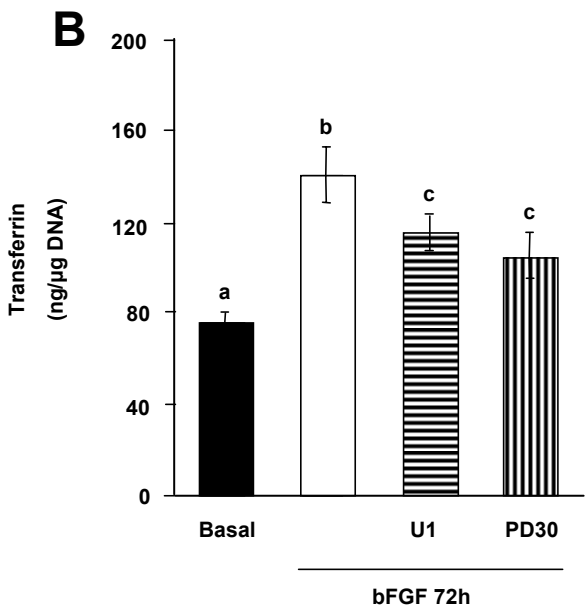


Figure 4

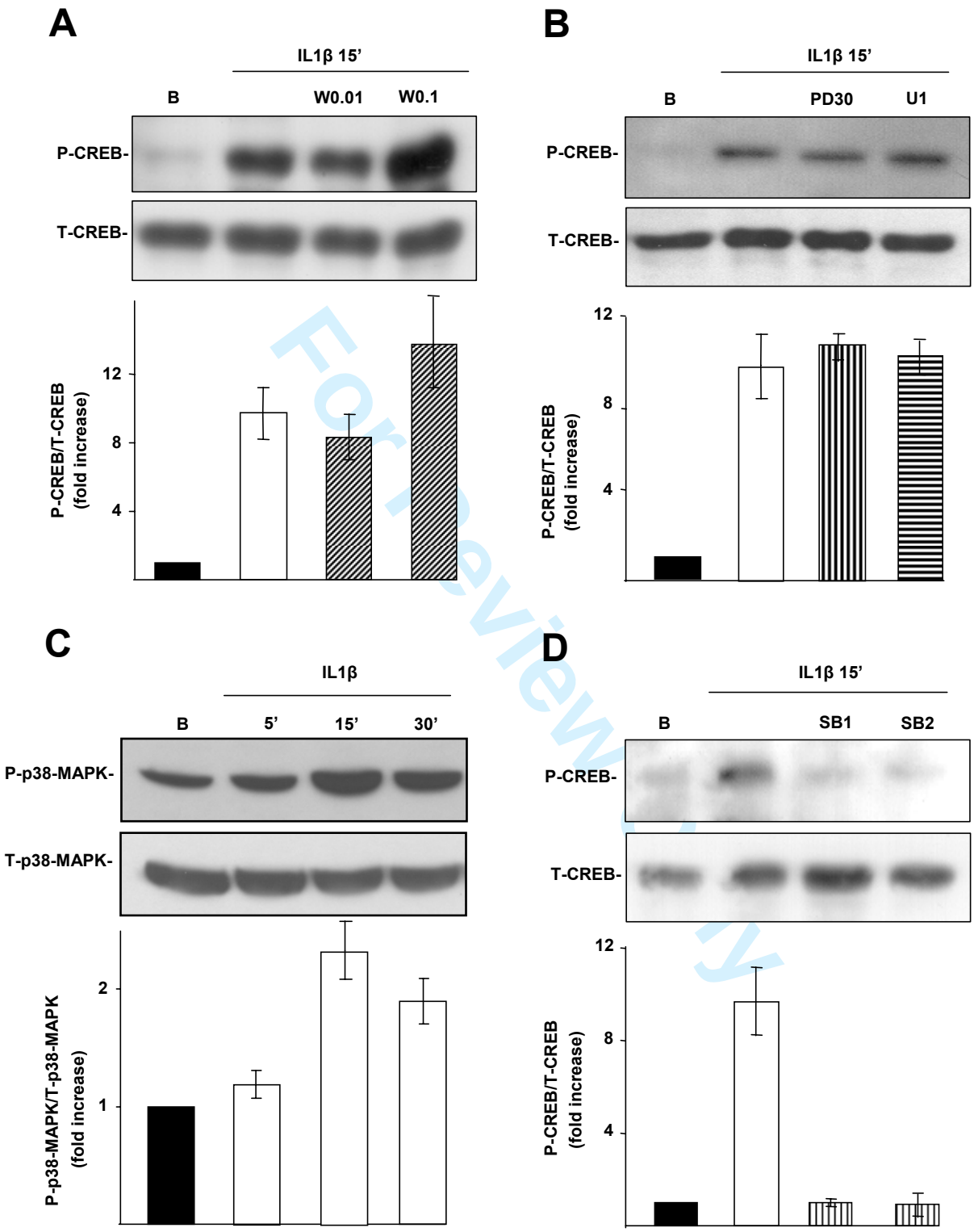


Figure 5

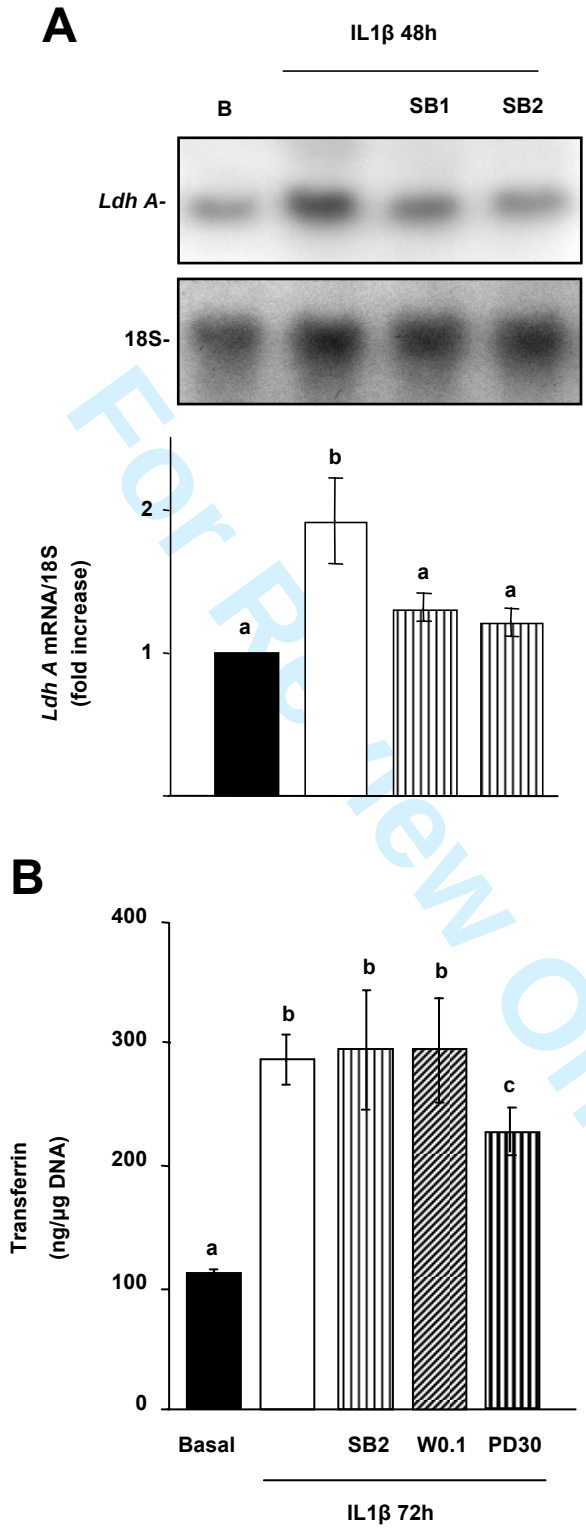


Table 1. Effect of SB on IL1β stimulus of LDH activity.

	LDH activity (mIU/μg DNA)
Basal	12.3±0.1[a]
IL1β 48h	16.4±0.7[b]
IL1β + SB1	13.0±0.6[a]
IL1β + SB2	12.4±0.5[a]

Sertoli cells were maintained under basal conditions or stimulated with 50 ng/ml IL1β in the absence or presence of SB203580 1 μmol/l (SB1) or 2 μmol/l (SB2). LDH activity was determined in the cellular monolayer. Values are expressed as mean±S.D. of triplicate incubations in one representative experiment out of three. Different letters indicate statistically significant differences ($p<0.05$).