—Original Article—

MAPK3/1 is conducive to luteinizing hormone-mediated C-type natriuretic peptide decrease in bovine granulosa cells

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Abstract. C-type natriuretic peptide (CNP) plays a role as an oocyte maturation inhibitor (OMI) in many species, including the bovine. However, the effects of luteinizing hormone (LH) on CNP expression and its potential mechanisms have not reported in the bovine. In the present study, we aimed to study the effects of LH on CNP expression and to illuminate the potential molecular mechanism in this process. Our results showed that LH induced epidermal growth factor receptor (EGFR) phosphorylation, mitogen-activated protein kinase3/1 (MAPK3/1) activation and CNP mRNA decrease in cultured bovine granulosa cells. Further study revealed that LH suppressed CNP expression via the MAPK3/1 signaling pathway, which was activated by the EGFR pathway. In conclusion, our research suggested that MAPK3/1 is involved in LH-mediated decrease of CNP and that this process is related to the EGFR and MAPK3/1 signal pathways.
Key words: C-type natriuretic peptide (CNP), Epidermal growth factor receptor (EGFR), Luteinizing hormone (LH), Mitogen-activated protein kinase3/1 (MAPK3/1)

The natriuretic peptides are a family of peptides that include atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and the C-type natriuretic peptide (CNP) [1]. These peptides show structural homology and similar pharmacological properties. ANP, BNP and CNP constitute a family of small (22–53 amino acid) polypeptides, each of which contains a highly conserved 17-member ring structure [1–3]. ANP, discovered in the rat atrium in 1981 by de Bold *et al.*, is now known to play an important role in maintaining cardiovascular and fluid homeostasis [4]. In fact, ANP regulates a variety of physiological functions including blood pressure, progesterone secretion, renin release, vasopressin release and endothelin release by interacting with receptors on the plasma membrane either to alter the levels of second messengers such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) or to affect ion channels [5]. BNP, synthesized primarily in the heart, not the brain, has similar actions. However, circulating levels of this peptide are quite low in the normal state and are only elevated in chronic disease states [3, 6]. CNP, unlike ANP and BNP, performs its major functions in an autocrine or paracrine manner. These functions include regulation of endochondral ossification, reproduction, nervous system development and maintenance of cardiovascular health [5].

Previous researchers in developmental biology reported that CNP was likely an oocyte maturation inhibitor (OMI) [7]. A recent study further suggested that CNP and its receptor (Natriuretic peptide receptor 2, NPR2) played a role as OMI in many species [8–14]. The combination of CNP and NPR2 promotes cGMP production in cumulus cells. Then cGMP from surrounding somatic cells diffuses into oocytes and regulates the cAMP concentration through inhibition of phosphodiesterase 3A (PDE3A) activity, thus preventing oocyte cAMP degradation and meiotic resumption [15, 16]. In response to the preovulatory luteinizing hormone (LH) surge, meiosis resumes as a result of a decrease in the expression of CNP and NPR2 and decrease in the guanylyl cyclase activity of NPR2 [17, 18]. Conversely, lower cGMP levels in somatic cells decreases oocyte cAMP levels during LH-induced oocyte meiotic resumption [15, 16]. These results strongly indicate the importance of CNP/NPR2 signaling in the somatic cell-dependent mechanism regulating oocyte meiotic arrest and resumption.

Recent work has suggested that LH signaling decreases the ovarian content of the NPR2 agonist C-type natriuretic peptide, thus decreasing cGMP in the cumulus-oocyte complex [17, 19]. LH signaling is also amplified by promoting the production of epidermal growth factor (EGF)-like peptides, including amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) [20, 21], and the epidermal growth factor receptor (EGFR) signaling pathway is involved in the activation of mitogen-activated protein kinase 3/1 (MAPK3/1) [22]. In addition, there is also report indicating that that LH induces MAPK3/1 phosphorylation [23]. Although previous studies have shown that EGFR and MAPK3/1 signaling have participated in LH-induced transmission of signaling pathway, the involvement of EGFR and MAPK3/1 in LH-induced CNP downregulation in the bovine remains unknown. The aim of the present study was to determine the molecular mechanisms of LH-induced decrease of CNP, especially the possible correlations among EGFR, MAPK3/1 and CNP, and to elucidate the signal transduction pathways involved in this process.

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Materials and Methods

Chemicals and hormones

All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. LH and EGF were prepared as stock solutions in distilled PBS containing 0.1% BSA. EGFR inhibitor AG1478 and MAPK3/1 inhibitor U0126 were dissolved in anhydrous dimethyl sulfoxide (DMSO) to form 10 mM and 5 mM stock solutions. All stock solutions were kept at –20ºC. The final concentrations of EGF, AG1478, and U0126 for culture were 100 ng/ml, 10 μM and 5 μM, respectively. Prior to use, they were diluted with culture medium, and the final concentration of DMSO was 0.1%.

Isolation and culture of bovine granulosa cells

Bovine ovaries from a local slaughterhouse were excised in physiological saline containing penicillin (100 IU/ml) and streptomycin (100 μg/ml) pre-warmed to 37ºC and transported and processed immediately. Follicles were manually separated from unnecessary underlying tissues. Medium-sized follicles (2–6 mm) were selected for isolation on the basis of their apparent appearance. The granulosa cells were isolated by the nonenzymatic needle puncture method. Briefly, they were centrifuged and washed with PBS three times. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) (Hyclone Laboratories, Logan, UT, USA) supplemented with penicillin (50 IU/ml), streptomycin (50 μg/ml) and 10% fetal bovine serum (FBS; Hyclone Laboratories) in cell culture dishes (Corning, Corning, NY, USA) at a density of 7×10^4 cells/ml [24, 25]. All cultures were carried out at 37ºC in a humidified atmosphere of 5% CO_2.

RNA isolation and quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. The cDNA was reverse-transcribed using a PrimeScipt RT reagent kit (TaKaRa) according to the manufacturer's instructions. The real-time quantitative polymerase chain reaction (RT-qPCR) procedure was carried out using a Bio-Rad IQ5 and the Bio-Rad IQ5 Optical System Software (Bio-Rad). Detection of RT-qPCR products was done with SYBR Premix Ex Taq II (TaKaRa) according to the manufacturer's instructions. The PCR cycling conditions were as follows: 1 cycle at 95ºC for 30 sec, followed by 40 cycles at 95ºC for 5 sec and at 60ºC for 30 sec. Experiments were performed in triplicate for each data point; the mean was used for the final analysis. Gene mRNA quantifications were performed by the $2^{-\Delta\Delta}$ct method, and the amount of transcripts in each sample was normalized using GAPDH levels as the internal control gene to correct for differences in the cDNA used. Furthermore, relative expression was calculated with respect to time zero [26]. The primers for CNP, GAPDH (internal control) [27], AREG, EREG [28] and BTC [29] were reported previously.

Western blot analysis

Whole-cell extracts from granulosa cells were prepared using RIPA buffer. Protein determination was performed by the BCA assay. Fifty micrograms of total protein per sample were separated by 12% SDS-PAGE and electrotransferred to a to PVDF membrane. After incubation in blocking buffer for 1 h at room temperature, the membrane was incubated overnight at 4ºC with the primary antibodies. All the primary antibodies for p-MAPK3/1, MAPK3/1, p-EGFR and EGFR were from Cell Signaling Technology. After washing, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase at room temperature for 1 h. Finally, immunoreactive bands were visualized using a SuperSignal West Pico kit according to the manufacturer's instructions.

Statistical analyses

All experiments were replicated at least three times for each group, and the data are presented as the mean ± SEM. The data were analyzed by ANOVA, followed by Fisher's least significant different test and independent samples Student's *t* test, with the SPSS software, version 13.0 (SPSS, Chicago, IL, USA). Differences were considered significant at P < 0.05.

Results

LH induces the expression of CNP mRNA in cultured granulosa cells

To test the possibility that LH-induced decrease of CNP in bovine granulosa cells, we examined the effects of LH on CNP expression levels after cultured for different times (0, 1, 2, 4, 8, 16, 24 h) and at different concentrations (0, 0.1, 0.5, 1, 2 IU/ml). We found that the expression of CNP in granulosa cells significantly decreased after 2 h of culture and reached the lowest levels at 16 h after stimulation with 1 IU/ml LH. In addition, the CNP expression levels still remained at low levels until the end of the experiment at 24 h (Fig. 1A). After the granulosa cells were cultured with different concentrations LH for 4 h, the results showed that the CNP expression was inhibited by LH in a dose-dependent manner. The minimum concentrations necessary to obtain a significant inhibitory effect was 0.5 IU/ml LH (Fig. 1B).

LH promotes EGF-like factor mRNA expression and stimulates EGFR and MAPK3/1 signaling pathway activation

In order to investigate the effects of LH on the EGFR and MAPK3/1 signaling pathways, we examined the mRNA expression of EGF-like factors and the phosphorylation of EGFR and MAPK3/1 induced by 1 IU/ml LH. After treatment with LH, the results showed that EGF-like factor mRNA expression was increased at 1 h to 4 h and was the highest at 2 h (Fig. 2A). At same time, phosphorylation of EGFR and MAPK3/1 was detected. EGFR activity was detected after 1 h of LH stimulation and increased gradually until 4 h of LH treatment, and it then decreased and was diminished after 8 h of LH action (Fig. 2B). Meanwhile, MAPK3/1 was activated and had a pattern of phosphorylation similar to that of EGFR; however, its activity was very low after 8 h and a little higher after 16 h (Fig. 2C). In addition, quantitative RT-PCR and Western blot analyses also revealed that LH induced the mRNA levels of EGF-like factors and that the protein levels of p-EGFR and p-MAPK3/1 in bovine granulosa cells were increased in a dose-dependent manner after stimulation with LH for 4 h (Fig. 2D, E and F).

EGF induces MAPK3/1 activation and decrease of CNP

To confirm the relation of EGFR and MAPK3/1 with LH-induced decrease of CNP, bovine granulosa cells were treated with 100 ng/
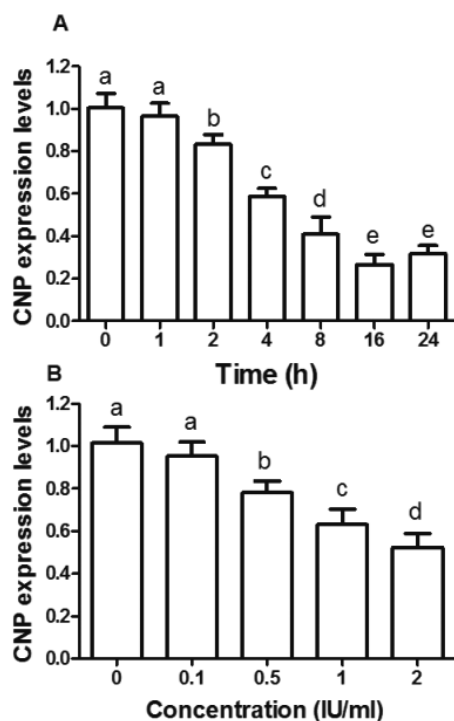
Fig. 1. The effects of LH on CNP mRNA expression in cultured bovine granulosa cells. A: Kinetics of CNP mRNA expression levels after LH induction in bovine granulosa cells. B: The effects of different concentrations of LH on CNP mRNA expression levels in cultured bovine granulosa cells after 4 h. The results are means ± SEM and representative of three independent experiments. Different letters represent significant differences (P < 0.05).

ml of EGFR agonist EGF in the presence or absence of the EGF inhibitor AG1478. Treatment with 10 μM AG1478 significantly suppressed MAPK3/1 activation and prevented decrease of CNP caused by EGF at 4 h after treatment, respectively (Fig. 3).

U0126 inhibits MAPK3/1 activation and the decrease of CNP induced by LH and EGF

To investigate the requirement for MAPK3/1 activity in LH-induced decrease of CNP, the MAPK3/1-specific inhibitor U0126 was added to the culture medium along with LH and EGF. The results showed that MAPK3/1 remained unphosphorylated in bovine granulosa cells at 4 h after culture with LH plus U0126 or EGF plus U0126 (Fig. 4B). The downregulation of CNP induced by EGF or LH was also inhibited at 4 h after U0126 treatment (Fig. 4A). In addition, we also detected the effects of MAPK3/1 singling on EGF- or LH-induced EGFR activation. The results showed that there were no obvious changes in the EGFR phosphorylation levels among the different treatments, including the EGF, EGF plus U0126, LH and LH plus U0126 treatments (Fig. 4C).

EGFR signaling is involved in LH-induced decrease of CNP and MAPK3/1 activation

Since both LH and EGF stimulated decrease of CNP and MAPK3/1 activation in bovine granulosa cells, we designed the following experiment to test if ecrease of CNP and MAPK3/1 phosphorylation induced by LH is mediated by EGFR. LH was added to the medium containing the EGFR-specific inhibitors AG1478 at the start of maturation culture. The LH-induced downregulation of CNP was reversed by the presence of AG1478 in the medium (Fig. 5A). The EGFR inhibitor AG1478 also blocked also block the inductive effect of LH on MAPK3/1 activation (Fig. 5B).

Discussion

The EGFR signaling pathway is essential for peripheral LH signaling to decreases the ovarian content of CNP [17, 19]. Moreover, it is involved in the activation of MAPK3/1 [22]. Here, we demonstrate that LH-mediated decrease of CNP occurs via the EGFR signaling pathway, which further activated the MAPK3/1 pathway to inhibit CNP expression in bovine granulosa cells. Our study is the first to demonstrate the suppression of CNP expression by LH stimulation in bovine granulosa cells and antral follicles (Supplementary Fig. 1), thus underscoring the role of CNP as an oocyte maturation inhibitor. Expanding a previous study indicating that CNP treatment inhibited spontaneous germinal vesicle breakdown of oocytes in cultured bovine COCs [11], the present findings showing a decline in CNP levels caused by LH stimulation provide further evidence for this intraovarian mechanism underlying meiotic resumption. Our data showing predominant expression of CNP transcripts in bovine granulosa cells are consistent with previous studies in mice [17, 19, 30]. Further study reveled that LH induced EGFR phosphorylation at 1 h after stimulation. Although small compared with the large increase in EGFR phosphorylation that occurs after 2–4 h exposure to LH, this early increase is significant. We also detected the transactivation of EGF-like factors by RT-PCR under the same culture conditions, and this occurred at the same time as EGFR phosphorylation at 1 h after stimulation. Our study is consistent with previous reports of LH-induced EGF-like factor expression and EGFR phosphorylation [28, 31, 32]. To study the role of MAPK3/1 in LH-suppressed CNP expression, we examined the effect of LH-induced phosphorylation of MAPK3/1 by Western blot analysis. LH increased phosphorylation of MAPK3/1 levels at 1 to 4 h after challenge, which is similar to the FSH and LH-inducible increases phosphorylation of MAPK3/1 abundance in porcine and mice granulosa cells [33, 34].

Previous studies have indicated that there is a gonadotropin-responsive ovarian paracrine pathway that leads to cell differentiation and modulation of gene expression. This pathway involves LH-dependent intraovarian expression of EGF-like factors processed and released from the cell surface to activate EGFR in a paracrine fashion, further leading to activation of MAPK3/1 cascade, which regulates various cellular processes through activation of additional kinases or transcription factors for modulation of gene expression [35, 36]. So we decided to test for the involvement of the MAPK3/1 cascade in the LH and EGFR regulation of CNP expression in bovine granulosa cells. We showed that LH and EGF provoke increases in MAPK3/1 phosphorylation and this increase of MAPK3/1 phosphorylation
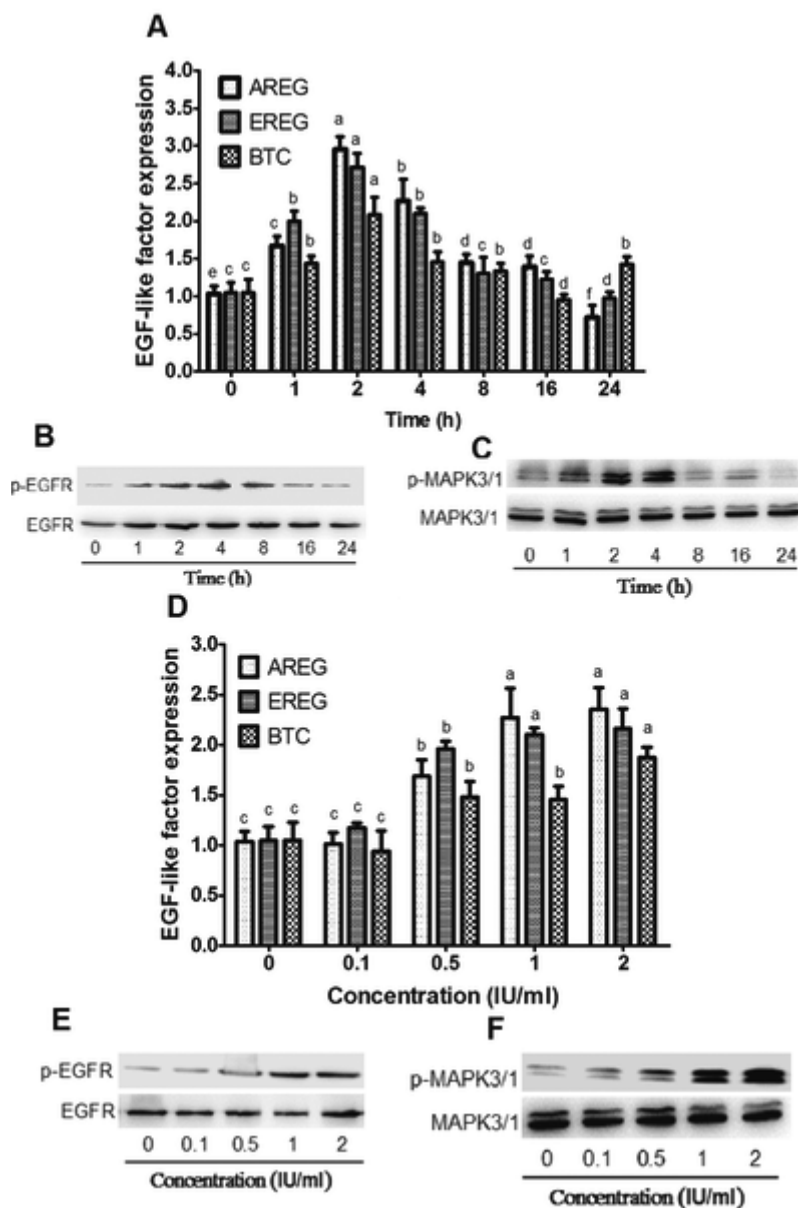
Fig. 2. The effects of LH on EGF-like factor mRNA expression and EGFR and MAPK3/1 activation in cultured bovine granulosa cells. A: The mRNA expression levels of EGF-like factors after stimulation with LH at different times. B: The phosphorylation levels of EGFR after stimulation with LH at different times. C: The phosphorylation levels of MAPK3/1 after stimulation with LH at different times. D: The effects of different concentrations of LH on EGF-like factor mRNA expression levels in cultured bovine granulosa cells after 4 h. E: The effects of different concentrations of LH on EGFR phosphorylation levels in cultured bovine granulosa cells at 4 h. F: The effects of different concentrations of LH on MAPK3/1 phosphorylation levels in cultured bovine granulosa cells after 4 h. The results are means ± SEM and representative of three independent experiments. Different letters represent significant differences ($P < 0.05$).

Fig. 3. The effects of EGF on CNP mRNA expression and MAPK3/1 activation in bovine granulosa cells after culture for 4 h. A: The mRNA expression levels of CNP after stimulation with EGF or EGF plus AG1478 in bovine granulosa cells for 4 h. B: The phosphorylation levels of MAPK3/1 after stimulation with EGF or EGF plus AG1478 in bovine granulosa cells for 4 h. The results are means ± SEM and representative of three independent experiments. C, control; E, EGF; A, AG1478. Different letters represent significant differences ($P < 0.05$).

induced by EGF or LH is dependent on the EGFR pathway. These results suggest that MAPK3/1 is involved in LH-mediated decrease of CNP in bovine granulosa cells and that this process occurs via the EGFR pathway. Our st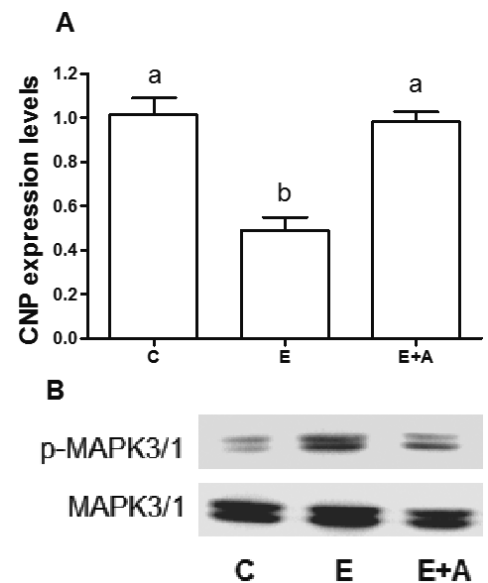udy is the first to demonstrate that inhibition of EGFR blocks LH-induced CNP expression in bovine granulosa cells. This part of our result further confirmed the fact that the LH-mediated decrease of CNP observed in mouse granulosa cells, which is dependent on EGFR, is also observed in bovine granulosa cells
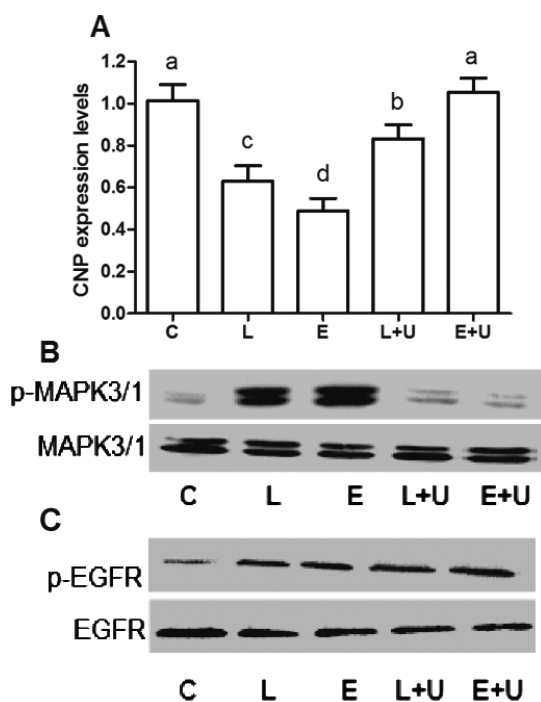
Fig. 4. The role of MAPK3/1 on LH- and EGF-induced CNP mRNA expression in bovine granulosa cells. A: The mRNA expression levels of CNP after stimulation with LH, EGF, LH plus U0126 or EGF plus U0126 in bovine granulosa cells. B: The phosphorylation levels of MAPK3/1 after stimulation with LH, EGF, LH plus U0126 or LH plus U0126 in bovine granulosa cells. C: The phosphorylation levels of EGFR after stimulation with LH, EGF, LH plus U0126 or LH plus U0126 in bovine granulosa cells. The results are means ± SEM and representative of three independent experiments. C, control; E, EGF; U, U0126; L, LH. Different letters represent significant differences (P < 0.05).

Fig. 5. The role of EGFR on LH-induced CNP mRNA expression and MAPK3/1 activation in bovine granulosa cells after cultured for 4 h. A: The mRNA expression levels of CNP after stimulation with LH or LH plus AG1478 in bovine granulosa cells. B: The phosphorylation levels of MAPK3/1 after stimulation with LH or LH plus AG1478 in bovine granulosa cells. The results are means ± SEM and representative of three independent experiments. C, control; E, EGF; A, AG1478; L, LH. Different letters represent significant differences (P < 0.05).

[37]. In addition, our study revealed for the first time that MAPK3/1 plays a key role in EGF- or LH-induced decrease of CNP. This was revealed by the fact that the MAPK3/1 inhibitor U0126 significantly inhibited the decrease of CNP induced by LH and EGF.

In conclusion, we revealed for the first time that LH-induced CNP downregulation is also present in the bovine and that this process depend on the EGFR and MAPK3/1 signaling pathways. Our results may provide a better understanding of mechanism regulating the CNP expression process and underscore the role of CNP as an oocyte maturation inhibitor in oocyte maturation.

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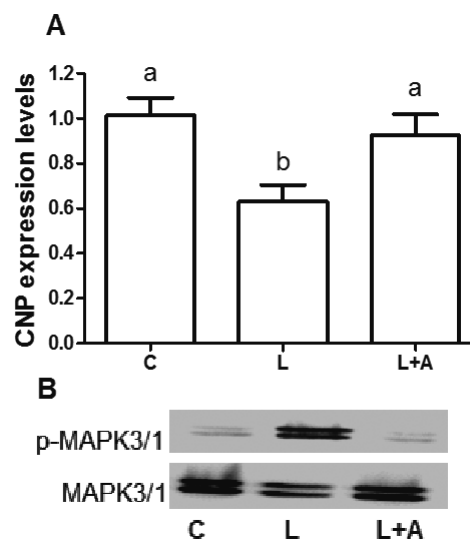
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