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HSP70 inhibits pig pituitary gonadotrophin synthesis and secretion by regulating the corticotropin-releasing hormone signaling pathway and targeting SMAD3

G. Xu[a], J. Li[b], D. Zhang[c], T. Su[a], X. Li[a], S. Cui[a,c,d,*]

[a] State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing 100193, China
[b] Department of Reproductive Medicine and Genetics, The Seventh Medical Center of PLA General Hospital, Beijing 100700, China
[c] College of Veterinary Medicine, Yangzhou University, Yangzhou, 225009 Jiangsu, China
[d] Jiangsu Co-Innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, 225009 Jiangsu, China

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ABSTRACT

High levels or long periods of stress have been shown to negatively impact cell homeostasis, including with respect to abnormalities in domestic animal reproduction, which are typically activated through the hypothalamus–pituitary–adrenal axis, in which corticotropin-releasing hormone (CRH) and heat shock protein 70 (HSP70) are involved. In addition, CRH has been reported to inhibit pituitary gonadotrophin synthesis, and HSP70 is expressed in the pituitary gland. The aim of this study was to determine whether HSP70 was involved in regulating gonadotrophin synthesis and secretion by mediating the CRH pathway in the porcine pituitary gland. Our results showed that HSP70 was highly expressed in the porcine pituitary gland, with over 90% of gonadotrophic cells testing HSP70 positive. The results of functional studies demonstrated that the HSP70 inducer decreased FSH and LH levels in cultured porcine primary pituitary cells, whereas an HSP70 inhibitor blocked the negative effect of CRH on gonadotrophin synthesis and secretion. Furthermore, our results demonstrated that HSP70 inhibited gonadotrophin synthesis and secretion by blocking GnRH-induced SMAD3 phosphorylation, which acts as the targeting molecule of HSP70, while CRH upregulated HSP70 expression through the PKC and ERK pathways. Collectively, these data demonstrate that HSP70 inhibits pituitary gonadotrophin synthesis and secretion by regulating the CRH signaling pathway and inhibiting SMAD3 phosphorylation, which are important for our understanding the mechanisms of the stress affects domestic animal reproductive functions.

1. Introduction

The relationship between environmental stress and animal fertility has been widely studied. Stress from the external environment is considered to be one of the important factors that impair the reproduction of livestock including pig [1,2]. The activation of stress-coping mechanisms is believed to result from interactions among multiple pathways, with the activation of the hypothalamus–pituitary–adrenal (HPA) axis playing important role [3–5]. Corticotropin-releasing hormone (CRH) is a central neuropeptide of the HPA axis that is highly expressed under stress conditions and has been shown to affect porcine productivity [2,6]. Another important group of proteins involved in HPA axis and the stress response are heat shock proteins (HSPs), including the 70 kDa heat shock protein (HSP70), which has important roles in regulating

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* Corresponding author. Tel.: 86-010-62731283; fax: 86-10-62733443.
E-mail address: cuisheng@cau.edu.cn (S. Cui).

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reproductive functions [7,8]. However, the interactions and mechanisms by which CRH and HSP70 affect the reproductive system remain unclear.

It has been documented that the actions of the hypothalamic–pituitary–gonadal axis are suppressed by the HPA axis under stress, including the inhibiting effects of HPA axis on the synthesis and secretion of gonadotrophin LH and FSH. In pigs, it is reported that cortisol can act directly on pig pituitary to inhibit its responsiveness to gonadotrophin-releasing hormone (GnRH) and reduce the LH secretion [9,10]. Acting as a key neuroendocrine factor of the HPA axis, CRH has function to affect the synthesis and secretion of gonadotrophins in porcine pituitary [11]. In addition, HSP70 is highly expressed in the pituitary gland during stress [12], but it still remains to be elucidated about the regulating functions of HSP70 on the synthesis and secretion of pituitary gonadotrophins. These made us to hypothesize that HSP70 is involved in the CRH signaling pathway of regulating the synthesis and secretion of pituitary gonadotrophins.

In addition, a great deal of attention has been paid on the TGF-β signaling pathway, which is active under external pressure or stress and plays a role in regulating gonadotrophin secretion [13,14]. Moreover, HSP70 has been reported to be involved in the TGF-β signaling pathway by targeting SMAD3 [15,16], a downstream molecule of activin type I receptors and the GnRH receptor. The phosphorylation of SMAD3 plays a predominant role in regulating Fshβ promoter in mouse [17,18], porcine [19], and ovine [20–22]. Recent studies have shown that SMAD3 also regulates LHβ transcription and LH secretion [23]. However, whether HSP70 has a similar effect on SMAD3 in gonadotrophic cells remains unknown.

The aims of this study were to examine HSP70 expression and determine whether HSP70 was involved in the CRH signaling pathway to affect pituitary gonadotrophin synthesis and secretion in pigs.

2. Materials and methods

2.1. Cell culture and experimental design

All animal experiments were conducted in accordance with the Helsinki Declaration and the principles of the Chinese Association for Laboratory Animal Sciences. All experimental procedures were approved by the institutional ethics committee, and animals were treated humanely and painlessly in this study. Fresh pituitaries obtained from 60 mature female pigs, which were ovariectomized 1 mo before slaughter were washed in PBS 3 times. After washing, the pituitaries were cut into small fragments that were then digested with 1 mg/mL of collagenase II (Sigma, St. Louis, MO) for 30 min (min). Subsequently, 75-mm nylon filters (200 mesh) were used to filter the collected homogenate. To collect the cells, the filtered homogenate was centrifuged at 1,200 rpm for 5 min. The pelleted cells were then resuspended in DMEM/F12 (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Gibco). The cells were added to the wells of plates at a density of 1×10^6 cells/well and were then incubated in an incubator at 37°C under an atmosphere containing 5% CO_2. The porcine primary pituitary cells were cultured for 4 d in 10% fetal bovine serum-DMEM/F12 before further processing, with the culture medium being refreshed every 24 h (h). LβT2 cells, which were provided by Dr Mellon (University of California, San Diego, CA), were also cultured in DMEM/F12 supplemented with 10% fetal bovine serum and were subcultured every third day using a 1:2 split. Further treatments were added after at least 3 generations of cell passage.

To study the effects of HSP70 on gonadotrophin synthesis and secretion, porcine primary pituitary cells were cultured for 12 h in medium supplemented with culture medium (control), CRH (100 nmol/L; Sigma) [24], E2 (100 nmol/L; Sigma) [25], and GnRH (100 nmol/L; Sigma) [26]. Subsequently, the cultured cells were incubated for 1 h with culture medium (control), HSP70 inducer TRC051384 (25 μmol/L; HY-101712, MedChemExpress, Shanghai, China) or the HSP70 inhibitor VER-155008 (25 μmol/L; HY-10941, MedChemExpress) [27], after which CRH added, and the cells were incubated for an additional 12 h. All the treatments were performed in 4 separate experiments for real-time PCR and Western blot and 6 times for radioimmunoassay (RIA). The cells and medium were collected and stored at −80°C before assays.

To assess the effects of CRH on gonadotrophin synthesis and secretion, CRH (100 nmol/L; Sigma) [24] was added to the culture medium of LβT2 cells, and the cells and medium were subsequently collected after 0 (control), 1, 3, 6, 12, and 24 h and stored at −80°C for further study. All the treatments were performed in 4 separate experiments for real-time PCR and Western blot and 6 times for RIA.

To assess the HSP70-mediated signaling pathway, LβT2 cells were separately treated with culture medium (control), ERK signaling pathway inhibitor PD (20 μmol/L; Sigma), PKA signaling pathway inhibitor H89 (20 μmol/L; Sigma), P38 signaling pathway inhibitor SB (20 μmol/L; Sigma), JNK signaling pathway inhibitor SP (20 μmol/L; Sigma), or PKC signaling pathway inhibitor CH (20 μmol/L; Sigma) for 1 h. Subsequently, CRH (100 nmol/L; Sigma) was added, and the cells were incubated for an additional 12 h [28]. The cells and medium were collected and stored at −80°C before assays. All the treatments were performed in 4 separate experiments.

To identify the downstream factor of HSP70, cells were incubated in culture medium containing VER-155008 (25 μmol/L; MedChemExpress), TRC051384 (25 μmol/L; MedChemExpress) or CRH (100 nmol/L; Sigma) [24] for 1 h (cells treated with culture medium as control), after which GnRH (100 nmol/L; Sigma) [26] was added, and the cells were incubated for an additional 12 h. All the treatments were performed in 4 separate experiments for real-time PCR and Western blot and 6 times for RIA. The cells and medium were collected and stored at −80°C before assays.

2.2. Real-time PCR

Real-time PCR was carried out according to previous reports [29,30]. RNAiso Plus reagent (Takara, Dalian, China) was used to extract total cellular RNA from fresh tissues and collected cells according to the manufacturer's instructions, the quantity and purity of which was assessed

spectrophotometrically by NanoDrop UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA) according to the manufacturer's procedure. Two micrograms of total mRNA was used to synthesize the first-strand complementary DNA (cDNA) as a template by using the M-MLV reverse transcription kit (Promega, Madison, WI) according to the manufacturer's procedure. The real-time PCR was performed by an ABI PRISM 7500 Real-time System (Applied Biosystems, Foster City, CA) using SYBR premix Ex Taq (TaKaRa) according to the manufacturer's procedure. The PCR conditions were as following: 5 min at 95°C, 40 cycles of 95°C for 10 s, and 60°C for 30 s. The single gene-specific peak was verified by the melt curve, and the primer set efficiency was determined by the standard curve with a 2-fold serial dilution. All target mRNA expression levels were normalized to the level of *GAPDH* and the $2^{-\Delta\Delta Ct}$ method was used to normalize data [31] using the expression in the control group as the calibrator (RQ = 1) and are presented as RQ ± SEM.

Primers for real-time PCR were designed by NCBI Genbank sequences and Primer-BLAST (http://www.ncbi.nlm.nih.gov/) and the sequences of primers are shown in Table 1.

2.3. Western blot

Radioimmunoprecipitation assay buffer (Cell Signaling, Danvers, MA) supplemented with 1% phenylmethylsulfonyl fluoride (Cell Signaling) was used to lyse cultured pig primary pituitary cells or LβT2 cells, and bicinchoninic acid assay reagent (Vigorous Biotechnology, Beijing, China) was used to determine total soluble protein levels. Approximately 25 μg of total protein was electrophoresed on a 12% SDS-PAGE, with the bands subsequently transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Richmond, CA). After being blocked with 5% (w/v) skim milk for 1 h, the membranes were incubated overnight at 4°C with one of the following antibodies: anti-HSP70 (1:1,000; GTX25439, GeneTex, Irvine, CA, USA), anti-ERK1/2 (1:1,000; ab17942, Abcam, Cambridge, MA), anti-phospho-ERK1/2 (1:1,000; ab50011, Abcam), anti-SMAD3 (1:1,000; ab40854, Abcam), anti-phospho-SMAD3 (1:1,000; ab52903, Abcam) or anti-GAPDH (1:1,000; AM4300, Ambion, Austin, TX). The membranes were then washed in Tris-Buffered Saline Tween-20 (TBST) 3 times and treated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:10,000; Zymed, CA), or HRP-conjugated goat anti-rabbit IgG (1:10,000; Zymed, CA) as secondary antibodies for 2 h at room temperature before being washed in TBST for 30 min. Chemiluminescence detection was performed using a SuperSignal West Pico Kit (Thermo Scientific, Waltham, MA) [28,32].

2.4. Immunofluorescence and cell calculation

The immunofluorescence analysis was performed according to our previous reports [28,29]. Pig pituitary glands were embedded in paraffin, cut into 5-μm thick sections, and then dewaxed using an alcohol gradient. After growing for 2 d on cover glass, LβT2 cells were treated with precooled methanol for 20 min. The sections and cells were then treated with PBS containing 10% normal goat serum for 1 h to prevent nonspecific binding of antibodies. Then, the sections and cells were incubated overnight at 4°C with primary antibody against HSP70 (1:50; GTX25439, GeneTex) and the primary rabbit against porcine GH, TSHβ, PRL, ACTH, FSHβ, and LHβ antibodies (1: 200, obtained from National Hormone and Peptide Program, NIDDK) [33]. Subsequently, the sections and cells were washed in PBS 3 times before being incubated for 2 h at room temperature with either CFL 555-conjuncted goat antirabbit IgG (1:150; sc-362272, Santa Cruz Biotechnology) and CFL 488-conjuncted goat antimouse IgG (1:150; sc-362257, Santa Cruz Biotechnology). Finally, the sections and cells were mounted and visualized using a fluorescence microscope (1X71; Olympus, Tokyo, Japan). The images were captured by Olympus Cellsens Standard imaging software (Olympus, Tokyo, Japan) at a magnification of 20× [28].

Table 1
Primer names and sequences.

Organism	Gene	Description	Genebank identification	Sequence (5′ to 3′)	Amplicon (bp)
Sus scrofa	*HSP70*	Heat shock protein family A (Hsp70)	NM_001123127.1	F: GCCCTGAATCCGCAGAATA R: TCCCCACGGTAGGAAACG	152
	FSHβ	Follicle-stimulating hormone subunit beta (FSHB)	NM_213875.1	F: ACCCCCATCTCCCAATCTGT R: GGGCCCATATCCCTGTCTTG	138
	LHβ	Luteinizing hormone subunit beta (LHB)	NM_214080.1	F: TGCTCCAGAGACTGCTGTTGT R: ATGCAGACAGGGCAAGCCTCA	133
	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_001206359.1	F: TTTTAACTCTGGCAAAGTGGAC R: TGGCCTTTCCATTGATGACA	155
Mus musculus	*Hsp70* (*Hspa1a*)	Heat shock protein 1A (Hspa1a)	NM_010479.2	F: GAAGGTGCTGGACAAGTGC R: GCCAGCAGAGGCCTCTAATC	237
	Fshβ	Follicle-stimulating hormonebeta (Fshb)	NM_008045.3	F: CTGAATGTCACTGTGGCAAGT R: GCAATGTCCATCGTCGTTTAT	115
	Lhβ	Luteinizing hormone beta (Lhb)	NM_008497.2	F: CTGCCCAGTCTGCATCACC R: AGGCACAGGAGGCAAAGC	94
	Gapdh	Glyceraldehyde-3-phosphate China	NM_001289726.1	F: GGTTGTCTCCTGCGACTTCA R: GGGTGGTCCAGGGTTTCTTA	186

F, forward primer; R, reverse primer. Q3

To calculate the number of cells coexpressing with HSP70 and pituitary hormones, 10 high-power fields (magnification: 20×) were selected and imaged using a fluorescence microscope (Leica Microsystems). Adobe Photoshop (Adobe Systems Inc, San Jose, CA) was used to merge the hormone-positive and HSP70-positive cells. The fluorescent-positive cells were counted using the counting tool in Photoshop. The rate of double-stained cells was calculated using the formula R = Bn/An, where An and Bn (n = 1 to 10) denote the number of hormone-positive and double-stained cells, respectively [29].

2.5. Immunohistochemistry

The sections were treated as described above for immunohistochemistry (IHC) analysis. The sections were incubated with an anti-HSP70 antibody (1:50; GTX25439, GeneTex) at 4°C overnight. After being washed with PBS 3 times, the sections were incubated at room temperature for 2 h with biotinylated goat antimouse IgG (1:150; sc-2039, Santa Cruz Biotechnology). Then, the sections were washed and incubated with an avidin–biotin complex (Vector Laboratories) for 1 h. Peroxidase activity was detected by staining with diaminobenzidine (DAB, D4293, Sigma) containing 0.1% H_2O_2 for 2 min, after which the sections were counterstained with hematoxylin and observed under a microscope (Leica Microsystems) and photographed [28].

2.6. Radioimmunoassay

FSH and LH concentrations were analyzed using RIA reagents obtained from Beijing North Institute Biological Technology (Beijing, China) following the manufacturer's protocols [29]. The limit of detection of FSH and LH were 2.5 mIU/mL and 5 mIU/mL, respectively. The sensitivity of both LH assay and FSH assay were less than 1.0 mIU/mL. The intra- and interassay CVs were less than 15% and 10%, respectively.

2.7. Statistical analysis

Real-time PCR was performed in 4 separate experiments, and the materials for each experiment were from pooled cells of pituitaries from several pigs. Each real-time PCR was run in duplicate and SPSS Statistics, version 25

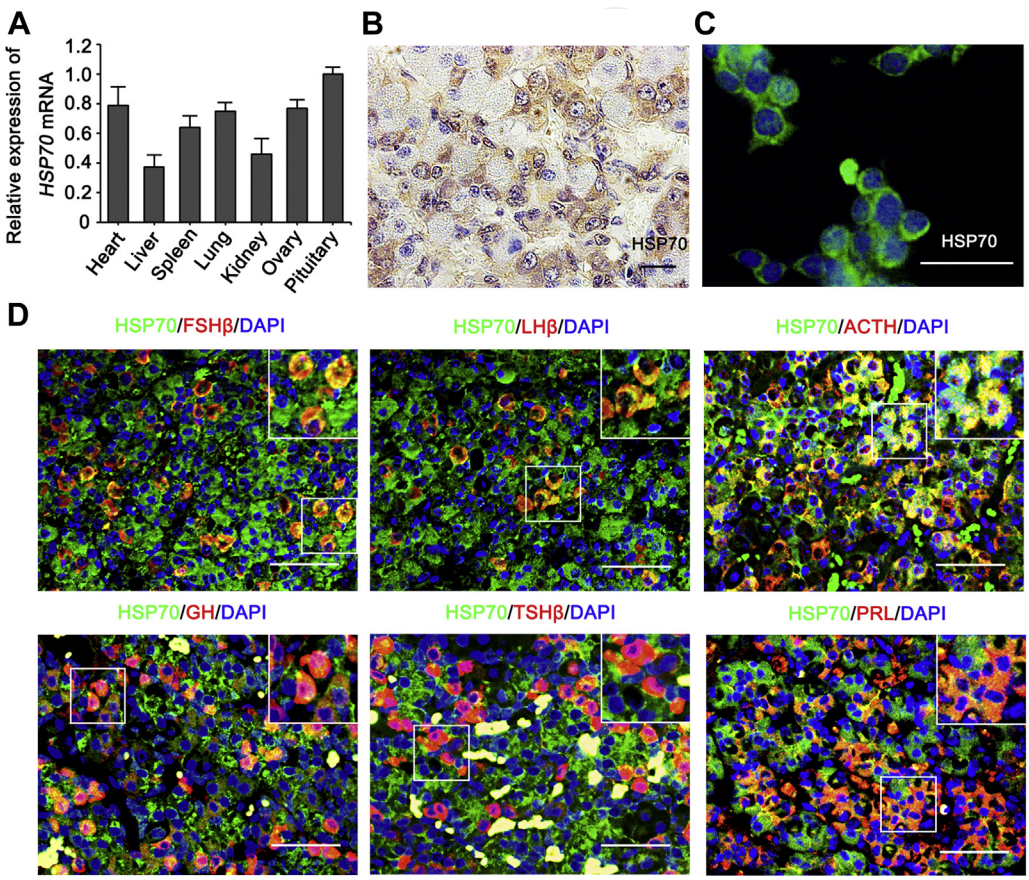


Fig. 1. HSP70 is expressed in the porcine pituitary gland. (A) *HSP70* mRNA levels in different porcine tissues normalized to *GAPDH* levels. The data are presented as the means ± SEM, n = 4. (B) Immunohistochemistry detection of HSP70 in the porcine pituitary gland. Scale bar: 20 μm. (C) Immunofluorescence detection of HSP70 in the LβT2 cells. Scale bar: 20 μm. (D) Immunofluorescence double-staining of HSP70 and GH, TSH, PRL, LHβ, FSHβ, and ACTH in the porcine pituitary gland. Green fluorescence: HSP70-positive cells. Red fluorescence: hormone-positive cells. Yellow fluorescence: cells with HSP70 and hormone colocalization. Scale bar: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(IBM SPSS Statistics, Armonk, NY) was used for statistical analysis. One-way ANOVA analysis followed by Tukey's multiple comparison test was used for results comparison. Western blot were performed in 4 separate experiments and the materials for each experiment were from pooled cells of pituitaries from several pigs. The results were analyzed by one-way ANOVA analysis followed by Tukey's multiple comparison test. For RIA, the experiments were performed in 6 separate experiments, and the results were analyzed by one-way ANOVA analysis followed by Tukey's multiple comparison test. The data are presented as the means ± SEM. Differences were considered significant at $P < 0.05$, with $P < 0.01$ considered to be extremely significant.

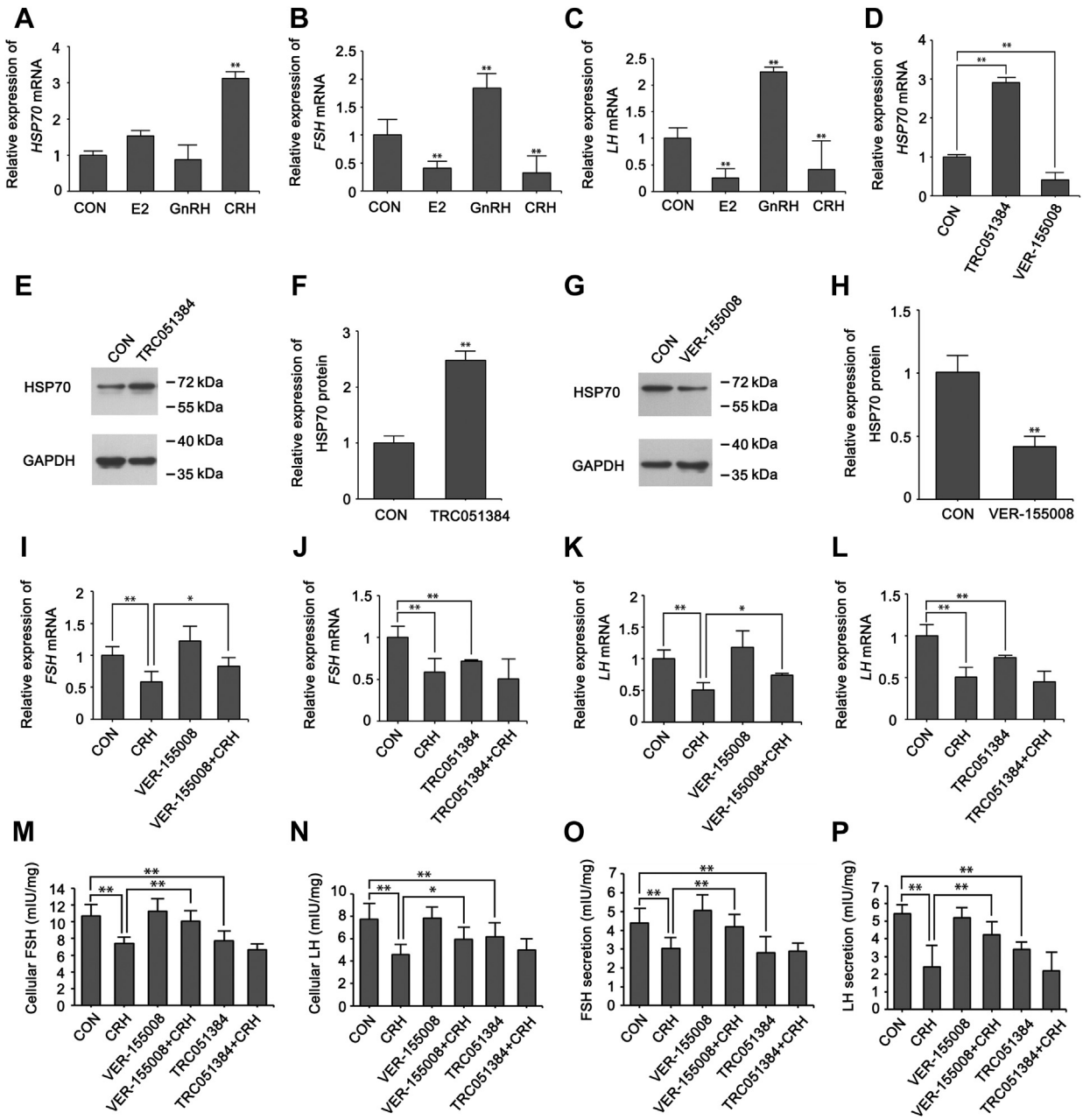


Fig. 2. HSP70 mediates the signal pathway affecting corticotrophin-releasing hormone (CRH) regulation of gonadotrophin synthesis. (A–C) Porcine primary pituitary cells were treated with E2, GnRH, and CRH (100 nmol/L) for 12 h. *HSP70*, *FSH*, and *LH* mRNA levels were assayed in each group and normalized to the level of *GAPDH*. (D–H) After treatment with TRC051384 (25 μmol/L) or VER-155008 (25 μmol/L) for 24 h, the relative expression of *HSP70* mRNA in porcine primary pituitary cells was assayed by real-time PCR and HSP70 protein levels were assayed by using Western blots. (I–L) The cultured porcine primary pituitary cells were incubated with TRC051384 or VER-155008 (25 μmol/L) for 1 h, after which CRH (100 nmol/L) was added, and the cells were cultured for an additional 12 h. *FSH* and *LH* mRNA levels were assayed and normalized to the level of *GAPDH*. (M–P) Cellular FSH and LH, FSH and LH secretion levels. The data are presented as the means ± SEM, for RIA: n = 6, for real-time PCR: n = 4. $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. HSP70 is highly expressed in the porcine pituitary gland

We first detected and compared HSP70 expression among different porcine tissues via real-time PCR, with the results indicating that the level of *HSP70* mRNA was the highest in the pituitary gland among all the tissues examined (Fig. 1A). We then identified the types of pituitary gland cell expressing HSP70 by IHC, and HSP70 was detected in the cytoplasm of cells in the anterior pituitary gland (Fig. 1B). HSP70 was then assessed for colocalization with GH, TSH, PRL, FSHβ, LHβ and proopiomelanocortin by immunofluorescence. The results (Fig. 1D) showed that approximately 90% of ACTH-positive cells, 28% of PRL-positive cells, 21% of GH-positive cells, and 18% of TSHβ-positive cells were dual stained with HSP70. As expected, approximately 90% of LHβ-positive cells and 92% of FSHβ-positive cells expressed HSP70. In addition, HSP70 expression in LβT2 cells was assessed by immunofluorescence, with the results showing that all LβT2 cells expressed HSP70 (Fig. 1C). These results suggest that HSP70 is involved in regulating gonadotrophin secretion.

3.2. HSP70 mediates the corticotrophin-releasing hormone signaling pathway in the regulation of gonadotrophin synthesis and secretion

To determine whether HSP70 mediates the CRH regulation of FSH and LH, cultured porcine primary pituitary cells were treated with CRH (100 nmol/L), E2 (100 nmol/L), or GnRH (100 nmol/L) for 12 h, after which the levels of *HSP70*, *FSH*, and *LH* mRNA were assayed by real-time PCR. The results showed that a 12-h CRH treatment increased the *HSP70* mRNA levels by over 3-fold compared with that observed in the control ($P < 0.01$), whereas E2 ($P = 0.15$) and GnRH ($P = 0.31$) had no significant effects on *HSP70* mRNA expression (Fig. 2A). In addition, as expected, the CRH ($P < 0.01$) and E2 ($P < 0.01$) treatments significantly decreased *FSH* and *LH* mRNA levels, whereas GnRH ($P < 0.01$) notably increased *FSH* and *LH* mRNA levels (Fig. 2B and C).

Subsequently, to confirm our hypothesis that HSP70 is involved in regulating gonadotrophin secretion, cultured porcine primary pituitary cells were incubated with either the HSP70 inducer TRC051384 (25 μmol/L) or the HSP70 inhibitor VER-155008 (25 μmol/L) for 24 h. The PCR results showed that the 24-h TRC051384 treatment increased

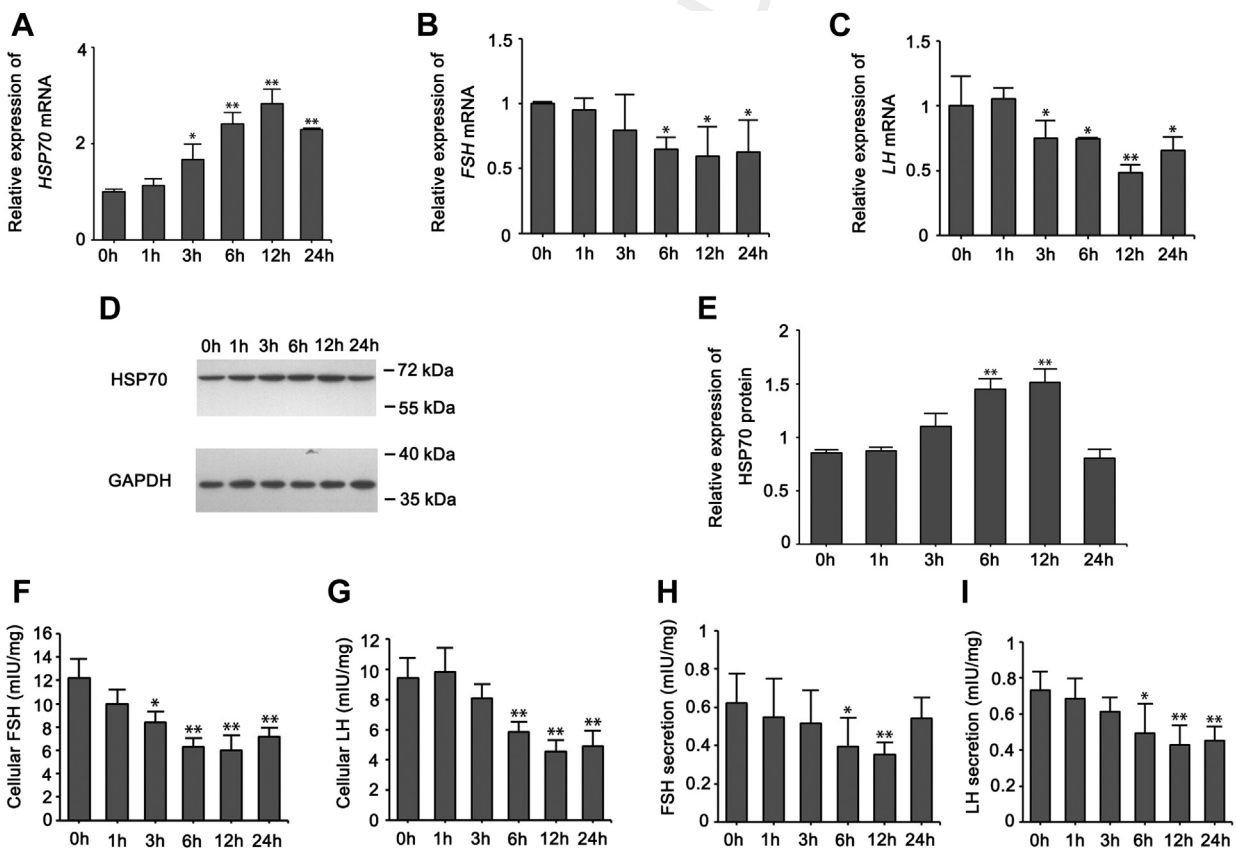


Fig. 3. Corticotrophin-releasing hormone (CRH) enhances HSP70 expression in LβT2 cells. (A–C) Cultured LβT2 cells were treated with CRH (100 nmol/L) for 0, 1, 3, 6, 12, and 24 h. *HSP70, FSH*, and *LH* mRNA levels were assayed and normalized to the level of *GAPDH*. (D, E) HSP70 protein levels in cells were assayed by Western blots. (F–I) Cellular FSH and LH, FSH and LH secretion levels. The data are presented as the means ± SEM for RIA: n = 6, for real-time PCR: n = 4. $^{*}P < 0.05$, $^{**}P < 0.01$.

HSP70 mRNA levels approximately by 3-fold, whereas the levels of *HSP70* mRNA decreased by 60% after VER-155008 treatment ($P < 0.01$; Fig. 2D). The HSP70 protein levels also significantly increased ($P < 0.01$; Fig. 2E and F) after TRC051384 treatment and decreased ($P < 0.01$; Fig. 2G and H) after VER-155008 treatment.

To determine whether HSP70 is involved in the CRH signaling pathway to regulate gonadotrophin synthesis, cultured porcine primary pituitary cells were treated with TRC051384 or VER-155008 (25 μmol/L) for 1 h. Then, we treated the cells with CRH (100 nmol/L) for 12 h, after which the effects of these compounds on the synthesis and secretion of FSH and LH were assayed. The results demonstrated that the HSP70 inducer inhibited *FSH* ($P < 0.01$) and *LH* ($P < 0.01$) expression by 28% and 26%, respectively. In addition, the CRH treatment inhibited *FSH* ($P < 0.01$) and *LH* ($P < 0.01$) expression by 41% and 49% (Fig. 2J and L). In contrast, the HSP70 inhibitor blocked the inhibitory effects of CRH on *FSH* ($P < 0.05$) and *LH* ($P < 0.05$) gene expression in porcine primary pituitary cells by 24% and 23%, respectively (Fig. 2I and K). The levels of FSH and LH in the cultured cells (Fig. 2M and N) and in the culture supernatant (Fig. 2O and P) were detected by RIA, with the results showing that the levels of FSH and LH in the cells and medium showed the same tendency as the expression of *FSH* and *LH* mRNA. These data demonstrate that HSP70 is crucial for the CRH-mediated inhibition of gonadotrophin synthesis in porcine pituitary cells.

3.3. Corticotrophin-releasing hormone enhances HSP70 expression in LβT2 cells

Furthermore, to test the effect of CRH on HSP70 in LβT2 cell lines, we treated the cultured LβT2 cells with CRH (100 nmol/L) for 0 (control), 1, 3, 6, 12, and 24 h. Real-time PCR was performed to assay the levels of *HSP70*, *FSH*, and *LH* mRNA. The results showed that the 1-h ($P = 0.18$) CRH treatment did not have a significant effect on the expression of HSP70, whereas the 3-h ($P < 0.05$), 6-h ($P < 0.01$), 12-h ($P < 0.01$), and 24-h ($P < 0.01$) CRH treatments significantly increased HSP70 expression, with the maximum HSP70 expression observed after 12 h at both the gene and protein levels (Fig. 3A, D, and E). In contrast, the 6-h, 12-h, and 24-h CRH treatments significantly decreased *FSH* ($P < 0.05$; Fig. 3B) and *LH* ($P < 0.05$; Fig. 3C) mRNA levels. The concentrations of FSH and LH in the cultured cells ($P < 0.01$; Fig. 3F and G) and medium ($P < 0.05$; Fig. 3H and

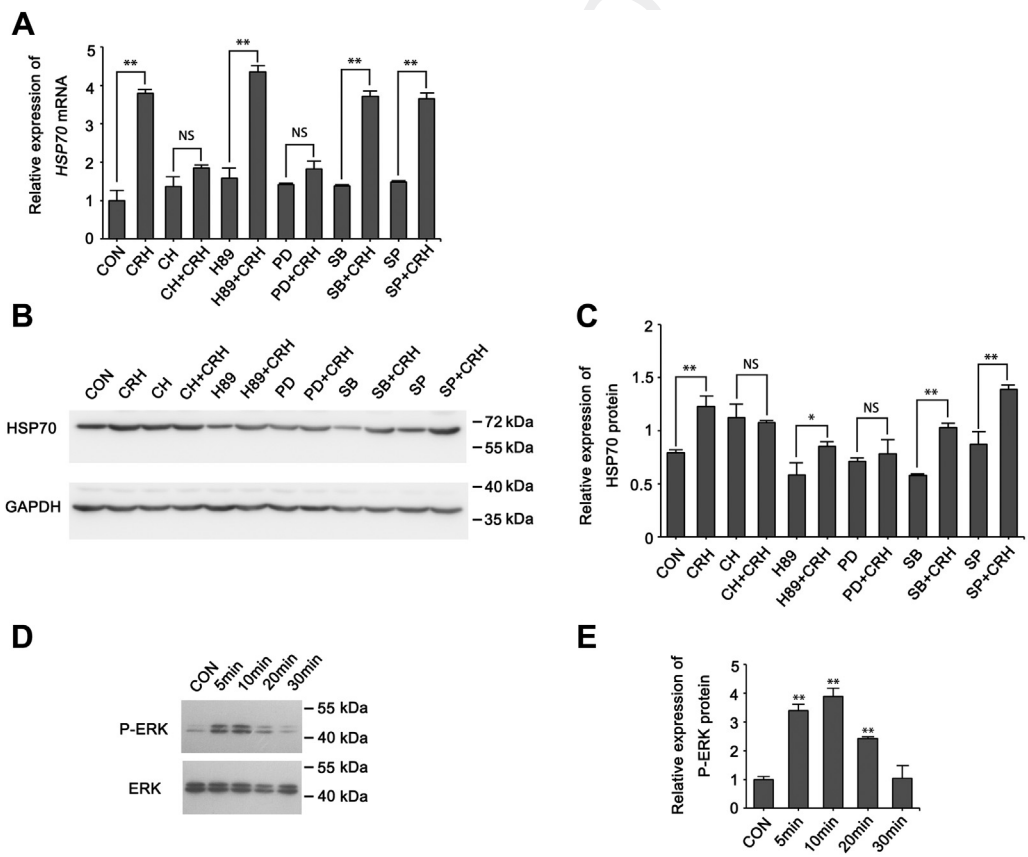
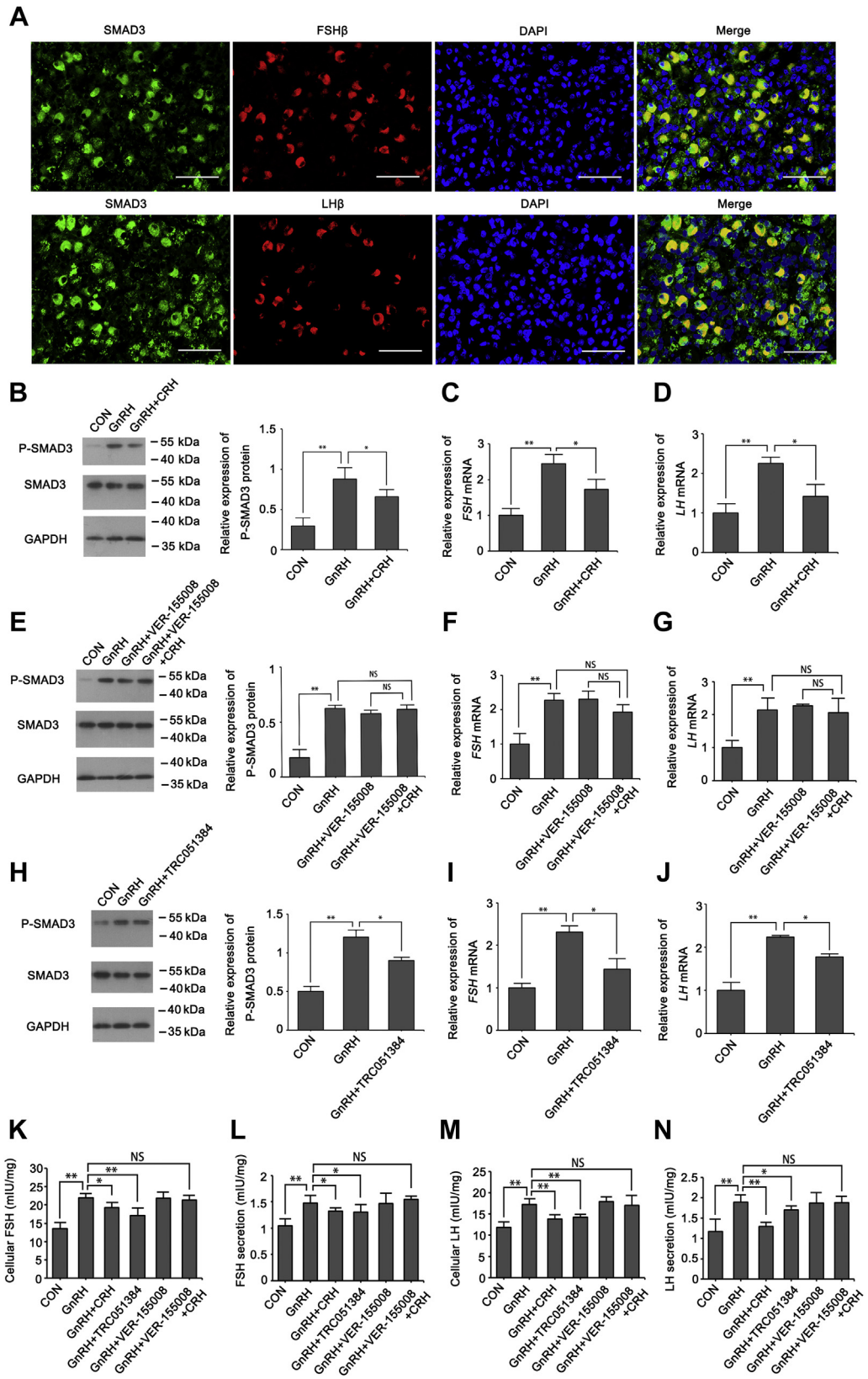


Fig. 4. Corticotrophin-releasing hormone (CRH) enhances HSP70 expression by stimulating the PKC/ERK signaling pathway. (A–C) Cultured LβT2 cells were treated with different signaling pathway inhibitors (20 μmol/L) for 1 h, after which the cells were incubated with CRH (100 nmol/L) or without CRH for 12 h. *HSP70* mRNA levels were assayed and normalized to the level of *GAPDH*, and HSP70 protein levels were assayed by Western blots. (D, E) Cultured LβT2 cells were incubated with CRH (100 nmol/L) and collected at different times. P-ERK and ERK levels were assayed by Western blots. The data are the means ± SEM, n = 4. $^{*}P < 0.05$, $^{**}P < 0.01$.


A
SMAD3
FSHβ
DAPI
Merge
SMAD3
LHβ
DAPI
Merge
B
CON
GnRH
GnRH+CRH
P-SMAD3
SMAD3
GAPDH
55 kDa
40 kDa
35 kDa
Relative expression of P-SMAD3 protein
C
Relative expression of FSH mRNA
D
Relative expression of LH mRNA
E
GnRH+VER-155008
GnRH+VER-155008 +CRH
F
G
H
GnRH+TRC051384
I
J
K
Cellular FSH (mIU/mg)
L
FSH secretion (mIU/mg)
M
Cellular LH (mIU/mg)
N
LH secretion (mIU/mg)
NS

I) after the 6-, 12-, and 24-h CRH treatments also significantly decreased. These results were in agreement with those obtained for the porcine primary pituitary cells, suggesting that HSP70 participates in the CRH-mediated regulation of gonadotrophin synthesis in porcine pituitary cells.

3.4. Corticotrophin-releasing hormone upregulates HSP70 expression via the PKC/ERK signaling pathway

To elucidate the signaling pathway by which CRH upregulates *HSP70* expression, cultured LβT2 cells were incubated with different signaling pathway inhibitors (CH [PKC], H89 [PKA], SP [JNK], SB [P38], and PD [ERK]; 20 μmol/L) for 1 h, after which the cells were incubated with CRH for 12 h. The results showed that only the PKC inhibitor CH and the ERK inhibitor PD blocked the CRH-mediated stimulation of *HSP70* mRNA expression (Fig. 4A) and protein level (Fig. 4B and C). Then, the cells were treated with CRH (100 nmol/L) for 0, 5, 10, 20, and 30 min. Western blot analyses were performed to assay the levels of phosphorylated ERK (P-ERK) 1/2. The results showed that the CRH treatments significantly increased P-ERK levels in cultured LβT2 cells, especially after 10 min ($P < 0.01$; Fig. 4D and E). These results indicate that CRH regulates *HSP70* expression via the PKC/ERK signaling pathway.

3.5. HSP70 suppresses gonadotrophin synthesis by inhibiting SMAD3 phosphorylation

Previous studies have shown that SMAD3 and SMAD3 phosphorylation plays important roles in regulating the Fshβ promoter and LH secretion [20,23,34] and that HSP70 has an inhibitory effect the activity of SMAD3 [16,35]. These led us to speculate as to whether SMAD3 is a downstream factor of HSP70 in porcine pituitary gonadotrophin cells. First, we performed dual immunofluorescence double-staining of SMAD3 with FSHβ and LHβ, with the results showing that almost all the gonadotrophic cells expressed SMAD3 (Fig. 5A). Furthermore, cultured LβT2 cells were treated with both GnRH and CRH (100 nmol/L) and then assessed for the levels of phosphorylated SMAD3 (P-SMAD3) by Western blot analysis. Real-time PCR analysis was performed to assess the expression of *FSH* and *LH* mRNA. As expected, SMAD3 phosphorylation and gonadotrophin expression were promoted by GnRH ($P < 0.01$) and impaired by the addition of CRH ($P < 0.05$) (Fig. 5B–D).

To determine whether SMAD3 is the downstream factor of HSP70 in regulating gonadotrophin synthesis, VER-155008 (25 μmol/L) was added to CRH (100 nmol/L) and GnRH (100 nmol/L) co-cultured cells. Western blot was used to assess the levels of P-SMAD3 and the expression of *FSH* and *LH* mRNA were assessed by real-time PCR. The results showed that when the VER-155008 was added, the SMAD3 phosphorylation ($P = 0.36$) and the expression of *FSH* ($P = 0.18$) and *LH* ($P = 0.45$) in the CRH and GnRH co-cultured cells were not significantly different from those in the GnRH group. These results suggest that VER-155008 blocked the CRH inhibition on SMAD3 phosphorylation and gonadotrophin expression promoted by GnRH (Fig. 5E–G). Furthermore, when we added TRC051384 into the cell culture medium, the previously observed increase in P-SMAD3 levels and *FSH* and *LH* expression caused by GnRH was inhibited ($P < 0.05$; Fig. 5H–J). In addition, the FSH and LH levels in the cultured cells and culture supernatant showed the same tendency (Fig. 5K–N). These results suggest that SMAD3 and its phosphorylation are involved in mediating the effect of HSP70 on gonadotrophin synthesis and secretion.

4. Discussion

The expression of HSP70 has been previously assessed in the pituitary glands of mice [36], zebrafish [37], and humans [12], and the results of the present study demonstrated that HSP70 was highly expressed in the porcine pituitary gland, in agreement with the results observed in other species. Moreover, for the first time, the results of this study identified the cell types expressing HSP70 and revealed its expression in gonadotrophic cells. Additionally, our results demonstrated that HSP70 has a negative role in regulating gonadotrophin synthesis and secretion in porcine pituitary gland cells and is involved in the CRH signaling pathway, although additional in vivo experiments are required to reveal how HSP70 affects reproductive functions.

HSP70 has been reported to have important roles in the reproductive system, and most of which addressed on the gonad. In rat luteal cells, the induction of HSP70 blocks the hormone-sensitive steroidogenesis by interrupting the translocation of cholesterol to the mitochondria [38]. In addition, HSP70 is abundant in naturally degraded rat corpus luteum and mediates luteal regression. However, the restriction of HSP70 in luteal cells can reverse the inhibition of prostaglandin F2α (PGF2α) on steroidogenesis and restore progesterone biosynthesis [39]. In pig granulosa cells (GCs), HSP70 is critically involved in the regulation of FSH receptor and reduced GCs functions [40]. The present study provided the evidence that HSP70 negatively affected pig reproduction by regulating gonadotrophin synthesis and secretion. In support, HSP70 is colocalized with FSHβ and LHβ, and HSP70 inducer significantly inhibited the synthesis and secretion of gonadotrophins in

Fig. 5. HSP70 regulates gonadotrophin synthesis and secretion by targeting SMAD3. (A) Immunofluorescence double-staining of SMAD3 and FSHβ or LHβ in porcine pituitary glands. Scale bar: 50 μm. (B–D) Cultured LβT2 cells were treated with CRH (100 nmol/L) for 1 h, followed by a 12-h treatment with GnRH (100 nmol/L). SMAD3 and P-SMAD3 protein levels and the expression of gonadotrophin were assayed. (E–G) Cultured LβT2 cells were incubated with VER-155008 (25 μmol/L) or CRH (100 nmol/L) for 1 h, after which GnRH (100 nmol/L) was added to the medium. After culturing for 12 h, SMAD3 and P-SMAD3 protein levels and the expression of gonadotrophin in cells were assayed. (H–J) The cultured cells were incubated with TRC051384 (25 μmol/L) for 1 h, followed by a 12-h treatment with GnRH (100 nmol/L). SMAD3 and P-SMAD3 protein levels and the expression of gonadotrophin were assayed. (K, M) Cellular FSH and LH levels in LβT2 cells. (L, N) FSH and LH secretion levels in LβT2 cells. The results are presented as the means ± SEM, for RIA: n = 6, for real-time PCR: n = 4. $^{*}P < 0.05$, $^{**}P < 0.01$.

cultured porcine primary pituitary cells, whereas HSP70 inhibitor blocked the inhibitory effects of CRH on gonadotrophin synthesis and secretion.

It has long been concerned about the effects of HPA axis, including CRH, on gonadotrophin synthesis and secretion. Our recent studies have showed that CRH activates the synthesis of the catecholamines [41], and norepinephrine of which affects the synthesis of FSH in pig [42]. In addition, there are reports that cortisol can directly act on the hypothalamus to block the serum concentrations of LH without affecting the pituitary response to GnRH [43], and similar results were confirmed by Estienne et al in 1991 [44]. However, the results presented here demonstrated that CRH can directly act on pig pituitary gonadotrophic cells to inhibit gonadotrophin synthesis and secretion, which is in agreement with the reports in rat [45] and rhesus macaques [46], although the conflicting results have been reported in sheep [47,48] and in pig [49], which may result from the different treatments on the animals or special physiological states responding differently to CRH.

In addition, our study has proved that HSP70 served as an intermediary molecule of CRH signaling pathway affecting gonadotrophin synthesis and secretion in the pig pituitary. This is supported by our results that the incubation of both porcine primary pituitary cells and LβT2 cells with CRH significantly increased HSP70 expression and decreased gonadotrophin synthesis. In addition, the PKC and ERK signaling pathway inhibitors blocked the effects of CRH on HSP70 expression, demonstrating that CRH enhances HSP70 expression through the PKC/ERK pathway, which have been reported other organs and cells [50,51].

Furthermore, the presented results of this study suggested that HSP70 played its roles in pig pituitary gland cells by targeting SMAD3. Firstly, SMAD3 was colocalized with LHβ and FSHβ in porcine pituitary gland by the dual immunofluorescence double-staining. In addition, CRH significantly inhibited the phosphorylation of SMAD3 induced by GnRH in LβT2 cells, and this effect of CRH was blocked when the inhibitor of HSP70 was added. Moreover, the HSP70 inducer also significantly inhibited the phosphorylation of SMAD3 caused by GnRH in LβT2 cells. These results indicated that SMAD3 was the downstream molecule of HSP70. In support, there are reports that SMAD3 is involved in regulating the functions of pituitary gonadotrophic cells [23,34] and the GnRH signaling pathway [23,52] and the transcriptional activation of GnRH receptor is also associated with SMAD3 levels [53,54].

It has been well documented that both CRH and GnRH play their functions through G protein coupled receptors, PKC/ERK, and SMAD3 pathway, but CRH and GnRH have different regulating acts on the synthesis and secretion of pituitary gonadotrophins. It is thus important to elucidate the intracellular interactions and related mechanisms among the signaling molecules of CRH and GnRH pathways in the future studies. In addition, the present study proposes that SMAD3 is the downstream molecule of HSP70 only by using the related inhibitors in the cultured porcine primary pituitary cells and LβT2 cell lines, and more precise experiments are required to prove how CRH and HSP70 affect the transcription activation of the gonadotrophin synthesis and secretion.

5. Conclusions

In summary, our results show that HSP70 is highly expressed in porcine gonadotrophic cells and plays a negative role in the CRH-mediated regulation of gonadotrophin synthesis and secretion. The signaling activity of CRH is transduced through the PKC/ERK pathway and subsequently enhances HSP70 expression. The enhanced HSP70 expression was shown to act as a link between CRH and GnRH by inhibiting SMAD3 phosphorylation in the porcine pituitary gland. These findings provide a new mechanism of how CRH affects gonadotrophin and may help us to understand the mechanisms of stress affecting porcine reproductive functions.

CRediT authorship contribution statement

G. Xu: Methodology, Writing - original draft, Writing - review & editing. **J. Li:** Resources, Writing - review & editing. **D. Zhang:** Writing - review & editing. **T. Su:** Methodology, Data curation, Writing - review & editing. **X. Li:** Data curation, Writing - review & editing. **S. Cui:** Conceptualization, Resources, Writing - review & editing.

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