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Inhibition of NF-κB promotes autophagy via JNK signaling pathway in porcine granulosa cells

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ABSTRACT

The transcription factor nuclear factor-κB (NF-κB) plays an important role in diverse processes, including cell proliferation and differentiation, apoptosis and inflammation. However, the role of NF-κB in porcine follicle development is not clearly elucidated. In this study, we demonstrated that follicle stimulating hormone (FSH) increased the level of inhibitor of NF-κB (IκB) protein and promoted the cytoplasmic localization of p65, indicating that FSH inhibits the activation of NF-κB in porcine granulosa cells. Moreover, inhibition of NF-κB by FSH or another specific inhibitor of NF-κB, pyrrolidine dithiocarbamate (PDTC), could activate JNK signaling and enhance autophagic activity in porcine granulosa cells. Knockdown of RelA (p65) Subunit of NF-κB by RNA interference abrogated the activation of JNK signaling pathway and the increase of autophagic protein expression by FSH. Meanwhile, the functional significance of FSH or PDTC-mediated autophagy were further investigated. Our results demonstrated that the increased autophagy promoted progesterone secretion in porcine granulosa cells. Blockage of autophagy by chloroquine obviated the FSH or PDTC-induced progesterone production. Taken together, these results indicate that inhibition of NF-κB increased autophagy via JNK signaling, and promote steroidogenesis in porcine granulosa cells. Our results provide new insights into the regulation and function of autophagy in mammalian follicle development.

1. Introduction

The transcription factor nuclear factor-κB (NF-κB) plays an important role in diverse cellular processes, including cell differentiation, apoptosis and inflammation. In mammals, five different isoforms of the NF-κB family have been identified, namely, RelA (p65), RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52) [1]. Under resting conditions, NF-κB dimerizes and binds to the inhibitor of NF-κB (IκB) protein, which generally restricts the shuttling of NF-κB dimers into the nucleus and leads to their sequestration in the cytoplasm [2]. Cellular stress induces the phosphorylation and proteasomal-dependent degradation of IκB, allowing NF-κB dimers to shuttle into the nucleus to induce or repress the transcription of an array of genes [3]. Besides acting as a transcription factor, NF-κB can also interact with other signaling pathways to modulate cell behavior. For example, the constitutive activation of the p44/p42 MAPK signaling pathway in THP-1 cells inhibits NF-κB activity [4]. In Ewing sarcoma cells, tumor necrosis factor alpha (TNFα)-induced NF-κB activation results in the rapid inactivation of JNK [5]. Due to its importance in cell biology, NF-κB is widely studied in cancer and immune diseases. However, only a few studies address the role of NF-κB in mammalian follicle development. For example, FSH increased X-linked inhibitor of apoptosis (XIAP) expression by activating the p13K/ATK/NF-κB signaling pathway in rat granulosa cells cultured in the absence of serum, thereby promoting their survival [6]. Furthermore, p65 and p50 overexpression affects progesterone production in gilt granulosa cells [7].

Mammalian follicle development is a dynamic process that is tightly regulated by gonadotropins and growth factors [8]. Pituitary-secreted follicle stimulating hormone (FSH), which binds to the G protein-coupled receptor (GPCR), can activate several signaling molecules such as cAMP-dependent protein kinase (PKA), protein kinase B (PKB), p38MAPK (MAPK14), ERK1/2 (MAPK3/1), c-Jun N-terminal kinase (JNK) and its downstream substrate c-Jun, leading to increased granulosa cell proliferation, survival and steroidogenesis [9–11]. Recently, FSH has also been shown to regulate autophagy in rat granulosa cells [12].

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Autophagy is a process of degradation that recycles intracellular proteins and organelles [13]. It is well established that the mammalian ortholog of the autophagy related gene 6, beclin1, regulates autophagosome formation and autophagy flux [14]. Several transcription factors can control beclin1 expression such as p65, FoxO1, FoxO3, c-Jun and E2F1 [15–18]. In addition to its classical roles in autophagy, *beclin1* also functions in steroidogenesis, and ablation of beclin1 in the rat ovary suppresses progesterone production [19].

Unfortunately, the regulation of NF-κB and its crosstalk with other signaling molecules during follicle development are not yet clear. The objective of this study was to investigate the function of NF-κB in porcine granulosa cells.

2. Materials and methods

2.1. Chemicals and antibodies

Unless otherwise specified, all chemicals used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA). Primary antibodies were purchased from Cell Signaling Technology (Boston, MA, USA).

2.2. Immunofluorescent staining

Porcine ovaries were collected at a local abattoir and transported to the laboratory within 2–3 h of collection. The specimens were processed as previously described [11]. In brief, ovaries were fixed and then embedded in paraffin. Thereafter, randomly selected 5 μm-thick sections were used for immunofluorescence and the TUNEL assay. To localize p65, cross-sections were deparaffinized with xylene, rehydrated in a decreasing ethanol series, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and then blocked with heat-inactivated goat serum. Cross-sections were incubated overnight with an anti-p65 antibody at a dilution of 1:100 (cat. no. sc-372; Santa Cruz, CA, USA) at 4 °C in a humidified chamber. Thereafter, a goat anti-rabbit fluorescein isothiocyanate-conjugated antibody was applied for 45 min at room temperature in the dark. Nuclei were counterstained with 1 μg/ml propidium iodide (PI) for 5 min. Immunofluorescence was observed under a Leica DMLB microscope, and images were acquired with a Leica DC 200 digital camera (Leica, Wetzlar, Germany) and Leica QWin © software (Leica Microsystems Image Solutions, Rijswijk, the Netherlands).

2.3. Detection of apoptotic cells by the TUNEL assay

TUNEL was performed using the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer instructions. In brief, cross-sections were digested with 20 μg/ml proteinase K, washed with PBS, treated with equilibration buffer, and then incubated with fluorescein-conjugated dUTP and terminal deoxynucleotide transferase for 1 h at 37 °C in the dark in a humidified chamber. Nuclei were counterstained with PI as previously indicated. Immunofluorescence was observed under a Leica DMLB microscope and images were acquired as previously stated.

2.4. Culture and treatment of primary porcine granulosa cells and follicles

Porcine ovaries were collected and washed as previously indicated. Granulosa cells were isolated by puncturing follicles with a 25-gauge hypodermic needle and washing three times with DMEM/F12 containing 1% fetal bovine serum, 100 IU/L penicillin and 100 mg/L streptomycin. Primary granulosa cells were selected and cultured in DMEM/F12 containing 3 mg/ml bovine serum albumin (BSA) in the presence or absence of FSH (Sioux Biochemical Inc., Sioux Center, IA, USA) or PDTC for the indicated times according to the experimental design. The concentration of FSH used in this study was identical to that of our previous study [11]. For the treatment of cells with LY294002, SP600125 and chloroquine, primary granulosa cells were pretreated for 1 h before the addition of FSH or PDTC. The stock concentration (10 mM) of these inhibitors were prepared in dimethylsulfoxide and stored at −20 °C until use. The working concentrations (10 μM) of these inhibitors were obtained by diluting 1:1000 with culture medium. Follicles were classified as healthy or atretic according to previously established morphological criteria [11]. In brief, healthy follicles had vascularized theca interna and clear amber follicular fluid with no debris. The slightly atretic and atretic follicles had gray theca interna and flocculent follicular fluid of different degrees.

2.5. Western blotting

The cells were lysed in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12% acrylamide gel), and the proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature, the membranes were incubated overnight with the indicated primary antibodies at 4 °C. After washing with TBS-T three times, the membranes were incubated for 1 h with the appropriate secondary antibodies conjugated to horseradish peroxidase at a dilution of 1:3000. The protein bands were detected using an enhanced chemiluminescence detection system (Applygen Technologies Inc., Beijing, China). Western blot images were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.6. Extraction of cytoplasmic and nuclear proteins

Cytoplasmic and nuclear extracts were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer instructions.

2.7. Knockdown of p65 by RNA interference

A control siRNA (si CTR), 5′–UUCUCCGAACGUGUCACGUTT–3′, and three validated siRNAs against porcine p65 (si p65 no. 1, 5′-UCCCUAUCCCUUUAGGCCTT-3’; si p65 no. 2, 5′- GUGCCUTTUGCUACCCGAATT-3′; si p65 no. 3, 5′- GCUUCGAUGGATTCGACGUCA-3′) were designed and synthesized by GenePharma (GenePharma, Shanghai, China). Primary porcine granulosa cells were washed with phenol red-free Opti-mem buffer (Gibco Invitrogen, Grand Island, NY, USA) three times, and centrifuged for 5 min at 270 × *g*. The cells were resuspended in electroporation buffer, transferred to a 4-mm gene pulsar cuvette (Bio-Rad) with siRNAs and maintained for 5 min at 4 °C. Granulosa cells were electroporated at 120 V, 8 pluses using the BTX ECM 830 electroporator (BTX, San Diego, CA, USA). Cells were maintained in the cuvette for 10 min and then resuspended in 2 ml of pre-warmed DMEM/F12 containing 3 mg/ml BSA. Cells were transferred to 35 mm culture dish (Nunc, Naperville, IL, USA) and incubated at 37 °C for 20 h before further use.