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Antimicrobial peptide expression in swine granulosa cells in response to lipopolysaccharide

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ABSTRACT

Antimicrobial peptides (AMP) are host defense peptides present in all species examined. The objective of the current study was to characterize the expression of a group of antimicrobial peptides in ovarian cells, and to investigate their expression response to pathogen ligands. It was found that while PG1 transcript was not detected in the ovary, the expression of BD2 is the highest in small follicle derived granulosa cells (SGC), and its expression decreases during follicular development to large follicle stage (LGC; $p < 0.05$). The expression of BD2 in cumulus cells also decreased from GV to MII stage of oocyte maturation. ANG4 expression increased in granulosa cells during follicular development from SGC to LGC stage ($p < 0.05$), although no significant difference was observed in cumulus cells from different stages of oocyte maturation. We further examined AMP expression in follicle cells treated with different toll-like receptor (TLR) ligands which mimic pathogen exposure in the ovary. Of the four TLR ligands examined, lipopolysaccharide (LPS) exposure resulted in a 11.5 fold increase of BD2 expression, and a significant decrease of LYZ in LGC. A similar response pattern in BD2 and LYZ expression was also observed in SGC. These responses of AMP expression to LPS are associated with increased TLR4 signaling pathway component in mRNA and protein level, such as MyD88 and NFkB, and pro-inflammatory cytokines/chemokines, such as IL-6, TNFα and IL-8 ($p < 0.05$). Our data suggest that AMPs may play a role in innate defense as well as other physiological functions during ovarian follicular development and oocyte maturation.

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1. Introduction

In the ovary, granulosa cells form a physical barrier around the developing oocyte within the follicle. In addition, it is known that transzonal projections (TZP) from granulosa cells form adherens junctions at the oocyte surface and/or gap junctions at the oocyte plasma membrane, providing the structural basis for more complex patterns of intercellular communication that emerge at later stages of follicle development [1]. With this intimate association, granulosa cells not only offer physical support, but also provide hormones and other growth factors and thereby support the growth and maturation of the oocytes [2,3]. Continued oocyte-granulosa communication is facilitated by cytoplasmic extensions on the granulosa/cumulus cells that extend through the zona pellucida and form gap junctions with the oocyte cell membrane [4]. Molecules known to be transferred across gap junctions include amino acids, glucose and ribonucleosides, which enable the oocyte and granulosa cells to influence each other's growth and differentiation through the flow of nutrients, metabolic precursors, and signaling molecules. The formation of gap junctions from oocyte to granulosa cell and between granulosa cells renders the developing follicle a functional syncytium (reviewed in Ref. [5]). While each cell carries out its own autonomous processes, the intimacy between the cells of the follicle coordinately supports the oocyte's development.

In addition to their supporting roles during oocyte maturation, accumulating studies have also demonstrated the innate immune

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capacity of granulosa cells [3,6–11]. As one of the first lines of defense against microbial pathogens in the ovary during oocyte maturation, granulosa cells express pattern recognition receptors (PRRs), among which Toll like receptors (TLRs) have been studied most extensively [6,10]. In an ex vivo culture system of cow follicles, granulosa cells were found to express TLR4, CD14 and MD-2 transcripts during follicle development [7]. Further studies show that granulosa cells are able to respond to the activation of TLR2 and TLR4 and their corresponding pathogen-associated molecular patterns (PAMPs; e.g. Pam(3)CysSK and LPS). The initial engagement then triggers intracellular signaling cascades that result in the expression of a variety of proinflammatory cytokines and chemokines [7–10]. IL-1β, IL-6, and IL-8 were all detected in granulosa cell culture supernatants and the expression of IL6, IL-1β, IL-10, TNF, IL-8, and CCL5 in bovine granulosa cell was shown to respond to LPS or Pam(3)CysSK stimulation [8]. The involvement of TLRs in the granulosa cell immune response was further demonstrated by knockdown or knockout of TLRs [10,12]. The engagement of TLR4 and LPS also altered hormone production in the ovary. For example, LPS inhibits granulosa cell estradiol production [7].

Antimicrobial peptides are host defense peptides and are found among all classes of life (reviewed in Ref. [13]). As a part of the innate immune response, they are important for the phagocytic and epithelial host defenses. However, knowledge pertaining to antimicrobial peptide expression and its regulation in the ovary is limited. We thus sought to characterize the expression of beta defensin 2 (BD2), beta defensin 3 (BD3), angiogenin 4 (ANG4), lysozyme (LYZ) and protegrin 1 (PG1) in granulosa cells and cumulus cells of the porcine ovary, and to investigate their expression response to pathogen membrane LPS.

2. Materials and methods

2.1. Granulosa cell isolation

Granulosa cells were isolated as described previously [14,15]. Briefly, porcine ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in PBS at room temperature within two hours of being slaughtered. Granulosa cells were aspirated from large-sized (4–6 mm in diameter), and small (SM; <3 mm in diameter) follicles using a 20-mL syringe fitted to an 18-gauge needle. Cells and follicular fluid were centrifuged at 500×g for 5 min and the cell pellet was washed with a large volume of DMEM/F12 (Gibco, Carlsbad, CA, USA) supplemented with 1X antibiotic/antimicotic (Gibco). Trypan blue staining was performed to determine viability which is >90%.

2.2. Cell culture and treatments

Viable cells of 2.5 mL and 0.5 mL at the density of 0.6×10^6/mL were seeded respectively in 6- or 24-well tissue culture-treated plates in DMEM/F12 with 10% FBS (Gibco) and 1X antibiotic/antimicotic (Gibco). To explore the effects of TLR ligands on the expression of BD3 and ANG4 in LGC, SGC and cumulus, cells were cultured at 37 °C in the absence and presence of LPS, lipoteichoic acid (LTA) and peptidoglycan (PGN) or Pam3CSK4 (Sigma, St. Louis, MO, USA) at the concentration of 1 μg/mL for 24 h. At the end of the culture, cells were immediately lysed, collected, stored at −80 °C for further RNA analysis. For all other experiments, cells were treated with different doses of LPS (0.1, 1 and 10 μg/mL) for 24 h or were pretreated with 20 μM ST2825 (MyD88 inhibitor) for 3 h follwed with different doses of LPS stimulation for 24 h. At the end of the culture, supernantants were collected for cytokine determination and cells were immediately lysed, stored at −80 °C for further RNA analysis.

2.3. In vitro maturation (IVM) of pig cumulus oocyte complex (COC)

The COC isolation and maturation were described previously [16]. Briefly, COCs were isolated from large-sized follicles (4–6 mm in diameter). The COCs surrounded by a compact cumulus mass and having evenly granulated cytoplasm were picked out by mouth pipette under a microscope. TCM-199 (Gibco BRL, Burlington, ON), buffered with 10 mM HEPES and 26 mM bicarbonate, was used for washing COCs. Pools of 45–50 COCs were then matured in vitro in 0.5 mL TCM199 (Invitrogen, Hercules, CA), supplemented with 0.1 mg/mL cysteine (Sigma), 10 ng/mL epidermal growth factor (EGF; Sigma), 5 IU/mL follicle-stimulating hormone (FSH; Sioux Biochemicals) and 5 IU/mL luteinizing hormone (LH; Sioux Biochemicals) in 4-well plates (NUNC, Denmark) at 38.5 °C in a 5% CO2 in a humidified air atmosphere [17]. At 44 h of IVM, each COC was pipetted into a well of 96-well plate and 150 IU hyaluronidase was added and incubated for 3 min at 37 °C. Then 90% oocytes were observed the extrusion of the first polar body under microscope. The oocytes with extrusion of the first polar body were regarded as MII oocytes and were picked out using mouth pipette. The surrounding MII cumulus cells were collected by centrifugation at 1000×g for 3 min at room temperature. Cumulus cells, oocytes and spent media samples were stored at −80 °C until further analysis.

2.4. Quantitative RT-PCR

Total RNA isolation kit (Norgen Biotek, Mississauga, ON, Canada) was used to isolate RNA from granulosa and cumulus cells and oocytes. For each sample, 500 ng of total RNA was treated with DNase I (Invitrogen), and reverse transcription was performed using random hexamers (Invitrogen) in a 15 μL reaction volume. Quantitative RT-PCR was performed using Perfecta SYBR Green SuperMix (Quanta Biosciences, Gaithersburg, USA) as described [18]. Briefly, reactions contained 300 nM of each primer and 10% cDNA, and were run on a Mx3005P System (Stratagene, La Jolla, USA) for 40 total cycles. A two-step amplification protocol was used with a denaturing temperature of 95 °C and an annealing temperature of 60 °C. Primers and expected product sizes are shown in Table 1. One reaction in which cDNA was replaced by water was included to detect potential contamination and one reaction using an RT negative control ensured the efficacy of the DNase digestion. Product sizes were verified by agarose gel electrophoresis and all products were sequenced to confirm identity. To improve the reliability of quantitative RT-PCR, 5 different reference genes which have previously been investigated for their expression stability in a wide range of porcine tissues were tested. They are they are β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peptidyl prolyl isomerase A (PPIA), ribosomal protein L4 (RPL4), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWH) [19,20]. A geometric mean of their Ct value was used as an internal control [21] to verify the presence of cDNA and to calculate the relative level of target gene expression using the ΔΔCt method [22]. The amplification efficiencies of all assays were confirmed to be 90–100% using a cDNA standard curve.

2.5. Protein isolation and Western Blot analysis

Protein was isolated using a previously described method [23]. Approximately 2.5×10^6 cells were homogenized in 250 μl ice-cold RIPA buffer (R0020, Solarbio, Beijing, China) using a homogenizer. Protein was quantified using BioRad protein quantification reagent, and approximately 30 μg of protein was subjected to 10% SDS-PAGE. Protein was transferred to a PVDF membrane (Millipore), which was blocked overnight in 5% skim milk at 4 °C. The membranes

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