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IGF-1 attenuates LPS induced pro-inflammatory cytokines expression in buffalo (*Bubalus bubalis*) granulosa cells

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ABSTRACT

Interaction between immune and endocrine system is a diverse process influencing cellular function and homeostasis in animals. Negative energy balance (NEB) during postpartum period in dairy animals usually suppresses these systems resulting in reproductive tract infection and infertility. These negative effects could be due to competition among endocrine and immune signaling pathways for common signaling molecules. The present work studied the effect of IGF-1 (50 ng/ml) on LPS (1 μg/ml) mediated pro-inflammatory cytokine expression (*IL-1β*, *TNF-α*, *IL-6*) and aromatase (*CYP19A1*) genes' expressions as well as proliferation of buffalo granulosa cells. The crosstalk between LPS and IGF-1 was also demonstrated through studying the activities of downstream signaling molecules (ERK1/2, Akt, NF-κB) by western blot and immunostaining. Gene expression analysis showed that IGF-1 significantly reduced the LPS induced expression of *IL-1β*, *TNF-α* and *IL-6*. LPS alone inhibited the *CYP19A1* expression. However, co-treatment with IGF-1 reversed the inhibitory effect of LPS on *CYP19A1* expression. LPS alone did not affect granulosa cell proliferation, but co-treatment with IGF-1, and IGF-1 alone enhanced the proliferation. Western blot results demonstrated that LPS caused the nuclear translocation of the NF-κB and increased the phosphorylation of ERK1/2 and Akt maximum at 15 min and 60 min, respectively. Nonetheless, co-treatment with IGF-1 delayed LPS induced phosphorylation of ERK1/2 (peak at 120 min), while promoting early Akt phosphorylation (peak at 5 min) with no effect on NF-κB translocation. Overall, IGF-1 delayed and reversed the effects of LPS, suggesting that high IGF-1 levels may combat infection during critical periods like NEB in postpartum dairy animals.

1. Introduction

After parturition, high yielding animals undergo metabolic stress to meet their energy demands and produce high milk yield. Consequently, most animals during post parturient period undergo a condition called negative energy balance (NEB), which is indicated by changes in blood hormones and metabolites. For example, insulin-like growth factor (IGF-1), an endocrine hormone, production was lower in postpartum animals under NEB (Beam and Butler, 1999). In cows, low levels of IGF-1 may cause delay in resumption of estrous cyclicity (Butler, 2003). IGF-1 is also vital for the granulosa cell functions like proliferation, differentiation and steroidogenesis (Adashi and Roban, 1992; Giudice, 1992; Gutierrez et al., 1997; Armstrong and Webb, 1997) by activating the PI3K/Akt or MAPK signaling pathways (Butt et al., 1999; Poretsky et al., 1999). Additionally, IGF-1 facilitates the fundamental roles of granulosa cells, such as nourishing and releasing of oocytes from ovarian follicles by stimulating mRNA expression of *CYP19A1* (Cytochrome P450 aromatase A1) and *HSD3B1* (3 beta hydroxyl steroid dehydrogenase),

Abbreviations: NEB, negative energy balance; LPS, lipopolysaccharides; IGF-1, insulin-like growth factor-1; IL-1β, interleukin-1beta; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; CYP19A1, cytochrome P450 aromatase A1; ERK1/2, extracellular signal-regulated kinase 1/2; NF-κB, nuclear factor kappa B; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; HSD3B1, 3beta-hydroxysteroid dehydrogenase type 1; TLR4, toll-like receptor 4; CD14, cluster of differentiation 14; MD2, myeloid differentiation 2; IL-8, interleukin-8; NOS2, nitric oxide synthase 2; INS-1, insulin-1; PBS, phosphate buffered saline; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; FSH, follicular stimulating hormone; RNA, ribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction; cDNA, complementary deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS–TV, Tris-buffered saline Tween 20 and vanadate; ECL, enhanced chemiluminescence; DAPI, 4′,6-diamidino-2-phenylindole; MCF-7, Michigan cancer foundation-7; BT-20, breast tumor-20.

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which are essential for the production of estrogen and progesterone, respectively (Mani et al., 2010; Sharma and Singh, 2012). These steroids play a major role in female reproduction.

The chances of getting uterine infections were high during the postpartum period due to the suppression of the immune system (Cai et al., 1994; Mallard et al., 1998; Kehrli et al., 1999). The incidence rate of uterine infections in postpartum period was higher in buffaloes compared to cows (Jainudeen, 1986; Azawi et al., 2008). Uterine infection with pathogenic bacteria not only causes the endometrial malfunction, but also has negative impact on ovarian function by reducing the steroidogenesis from granulosa cells, growth of the dominant ovarian follicle and delaying the ovulation process (Sheldon et al., 2002, 2009). Lipopolysaccharides (LPS) from the gram-negative bacteria like *Escherichia coli* can enter in to ovarian follicular fluid and influence the granulosa cell function by activating immune signaling pathways. LPS binds to Toll-like receptor 4 (TLR4)/cluster of differentiation 14 (CD14)/myeloid differentiation (MD2) complex, which is present on the granulosa cells, activates the ERK1/2 and p38 MAPK signaling pathways, promotes nuclear factor κB (NF-κB) translocation from cytoplasm to nucleus, and lead to increased levels of pro-inflammatory cytokines such as IL-1β, IL-6 as well as chemokines like IL-8 (Bromfield and Sheldon, 2011; Price and Sheldon, 2013). The steroidogenesis of granulosa cells was negatively affected by the influence of LPS. Treatment of granulosa cells from growing and dominant follicles with LPS showed decreased *CYP19A1* expression and estradiol secretion (Price and Sheldon, 2013; Herath et al., 2007).

The interaction between immune and endocrine systems is essential for cellular function and fertility in animals. Involvement of common signaling pathways (MAPK, PI3K/Akt pathways) between IGF-1 and LPS may lead to crosstalk between endocrine and immune signaling. It has been shown that administration of IGF-1 inhibited the LPS induced sickness behavior in mice and pro-inflammatory cytokine expression in rat brain (Dantzer et al., 1999; Pang et al., 2010). Activation of PI3K/Akt signaling pathway by IGF-1 inhibited LPS induced expression of NOS2 (nitric oxide synthase 2), which is responsible for the production of nitric oxide in response to LPS, in INS-1 cells (insulin secreting beta cell derived line) (Castrillo et al., 2000) and the production of pro-inflammatory cytokines in human monocytic cells (Guha and Mackman, 2002). Based on these studies, we hypothesized that IGF-1 may influence the effects of LPS on buffalo granulosa cells function by competing with LPS induced signaling pathways.

2. Materials and methods

2.1. Collection of ovaries

A total of 200 buffalo ovaries were collected in normal saline (0.9% NaCl) containing antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) from slaughter house, Delhi, within 30 min after slaughtering of the animals. Collected ovaries were brought to laboratory at 4 °C within 4 h.

2.2. Granulosa cells isolation and culture

Ovaries were initially washed with normal saline for four times, then once with 70% ethanol for 30 s, and finally washed with normal saline again prior to further processing. Healthy developing follicles were assessed by the presence of vascularized theca externa and clear amber follicular fluid with no debris. Aspiration of follicular fluid from small and medium follicles (<8 mm) was done by using 18 gauge needle attached with sterile, nontoxic, nonpyrogenic and monoinjected brand syringes (Dispovan, Faridabad, Haryana, India). The follicular fluid was collected in PBS containing penicillin (100 U/ml) and streptomycin (100 mg/ml) under sterile conditions while continuously maintaining the cells on ice. The granulosa cells were pelleted at 1500 rpm for 5 min. The number and viability of cells were estimated by hemocytometer using trypan blue exclusion method.

All the reagents required for the granulosa cells culture were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA) unless otherwise stated. Cells at a density of 2×10^5 viable cells/ml of DMEM serum free culture medium were seeded in 6-well plates for protein, 24-well plates for RNA, and 96-well plates for proliferation assays (Nunc, Roskilde, Denmark). The cells were maintained at 37 °C under atmosphere of 5% CO_2 and 95% air for 48 h for initial establishment of the culture (Monga et al., 2011). The DMEM serum free culture medium contained the following additional components: protease-free BSA (1 mg/ml), L-glutamine (3 mM), sodium selenite (4 ng/ml), transferrin (2.5 mg/ml), androstenedione (2 μM), bovine insulin (10 ng/ml), nonessential amino acid mix (1.1 mM), ovine FSH (1 ng/ml), human rIGF-1 (1 ng/ml), penicillin (100 U/ml), and streptomycin (100 mg/ml) (Monga et al., 2011).

2.3. Experimental design

After initial establishment of the cultures for 48 h, the granulosa cells were used for further treatments. To identify the effect of LPS and IGF-1 on pro-inflammatory cytokines and *CYP19A1* genes expression of granulosa cells, the cells were treated with or without LPS (1 μg/ml), IGF-1 (50 ng/ml) or both by replacing the old media with fresh media (no FSH and IGF-1). Medium without LPS (1 μg/ml), IGF-1 (50 ng/ml) was included as a control. The culture was incubated for 2 h as well as for 24 h. The working concentrations of LPS and IGF-1 were earlier standardized (unpublished data and (Mani et al., 2010)). After each treatment period, the media were removed and stored at −20 °C till further use, and the cells were harvested in TRIzol reagent (Invitrogen, CA, USA) and were processed for RNA isolation. Similarly, granulosa cells were plated in 96 well plates and treated for 24 and 48 h for proliferation assay.

In order to study the effect of LPS and IGF-1 on phosphorylation of ERK1/2 and Akt, granulosa cells were treated in a time dependent manner (5, 15, 30, 60 and 120 min). After initial establishment of the culture, old media were replaced with fresh media (no FSH, IGF-1) with or without LPS (1 μg/ml), IGF-1 (50 ng/ml) or both, and incubated for 5, 15, 30, 60 and 120 min. Medium without LPS (1 μg/ml) and IGF-1 (50 ng/ml) was included as a control (0 min treatment). Immediately after treatment period, the media were removed and the cells were collected for protein isolation. Similarly, cells were treated with LPS (1 μg/ml), IGF-1 (50 ng/ml) or both for 1 h to understand the effect of LPS and IGF-1 on nuclear translocation of NF-κB.

2.4. RNA isolation and cDNA preparation

Total RNA was isolated by using TRIzol (Sigma–Aldrich Co.) reagent and its quantity and integrity were evaluated by nanophotometer (Implen, Schatzbogen, Munchen, Germany) and denaturing agarose gel electrophoresis, respectively. The RNA was used immediately for RT-PCR or stored at −80 °C until further use. By using the first-strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) and random hexamers, cDNA was synthesized from a total of 100 ng RNA for each set of the experiments. The reaction mixture contained 100 ng total RNA, 1 μl random hexamer (0.2 μg/μl), and nuclease free H_2O to a final volume of 11 μl. The contents were incubated for 10 min at 65 °C followed by 2 min at 25 °C. The reagents added further were 4 μl of 5× reaction buffer (250 mM Tris–HCl, pH 8.3; 250 mM KCl, 20 mM $MgCl_2$, and 50 mM dithiothreitol), 1 μl of RNase inhibitor (20 IU), 2 μl dNTP