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—Original Article—

Role of endoplasmic reticulum stress in lipopolysaccharide-inhibited mouse granulosa cell estradiol production

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Abstract. The decrease in the level of estradiol (E_2) in granulosa cells caused by lipopolysaccharide (LPS) is one of the major causes of infertility underlying postpartum uterine infections; the precise molecular mechanism of which remains elusive. This study investigated the role of endoplasmic reticulum (ER) stress in LPS-induced E_2 decrease in mouse granulosa cells. Our results showed that LPS increased the pro-inflammatory cytokines [(interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α)], activated ER stress marker protein expression [(glucose-regulated protein 78 (GRP78) and CCAAT/enhancer-binding protein homologous protein (CHOP)], and decreased cytochrome P450 family 19 subfamily A member 1 (Cyp19a1) expression and E_2 production. Moreover, inhibition of ER stress by 4-phenylbutyrate (4-PBA) attenuated thapsigargin-(TG, ER stress agonist) or LPS-induced reduction of Cyp19a1 and E_2, pro-inflammatory cytokines expression (IL-1β, IL-6, IL-8, and TNF-α), and the expression of CHOP and GRP78. Additionally, inhibition of toll-like receptor 4 (TLR4) by resatorvid (TAK-242) reversed the inhibitory effects of LPS on Cyp19a1 expression and E_2 production, activation of GRP78 and CHOP, and expression of IL-1β, IL-6, IL-8, and TNF-α. In summary, our study suggests that ER stress is involved in LPS-inhibited E_2 production in mouse granulosa cells.

Key words: Endoplasmic reticulum stress, Estradiol, Granulosa cells, Lipopolysaccharide

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Microbial infections of the uterus or mammary glands are major causes of severe disease and infertility in humans and domestic animals [1, 2], and cause post-partum uterine infections, that disrupt the endometrium, hypothalamic-pituitary axis, and ovarian endocrine functions [3, 4]. Lipopolysaccharide (LPS), derived from gram-negative bacteria such as *Escherichia coli*, is found in ovarian follicular fluid [5, 6] and influences the granulosa cell functions by activating immune signaling pathways. In the bovine granulosa cells, LPS binds to toll-like receptor 4 (TLR4) promotes nuclear factor-kB (NF-κB) translocation from the cytoplasm to the nucleus, and increases the levels of pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, IL-8, and tumor necrosis factor (TNF)-α [7–10]. LPS also inhibits steroid production of bovine or porcine granulosa and theca cells [5, 11–13], decreases the expression of gonadotropin receptors and cytochrome P450 family 19 subfamily A member 1 (Cyp19a1) in bovine granulosa and theca cells [11, 14, 15] and perturbs bovine oocyte meiotic progression *in vitro* [8]. In the mouse liver, pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and LPS can induce endoplasmic reticulum (ER) stress and causes an acute phase response [16].

ER is essential for protein synthesis, folding, transport, and protein post-translational modifications [17, 18]. When the ER client protein load is in excess relative to the ER folding capacity, the accumulated proteins may cause ER stress [19, 20]. Under ER stress, ER-resident chaperone glucose-regulated protein 78 (GRP78) induced by unfolded proteins activates three ER membrane resident proteins: pancreatic ER kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) [21]. Under these conditions, ER load is ameliorated by following three methods: (1) a reduction in the entry of newly synthesized proteins into the ER through attenuating protein translation; (2) an increase in the protein-folding capacity by up-regulating ER gene expression; (3) the degradation of misfolded and unfolded proteins through ER-associated degradation (ERAD) and lysosome-mediated autophagy [18, 22, 23]. ER stress occurs widely in several physiological and pathological processes, including development, cell apoptosis, autophagy, and metabolic diseases [18, 24]. The role of ER stress in immune response [25, 26], inflammation [25], and female reproduction [27–31] have also been reported previously. Moreover, some studies have reported that LPS induces inflammation by regulating ER stress in acute lung injury models

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[32] and liver ischemia-reperfusion injury [33].

As mentioned above, LPS inhibits steroid production induced by the immune response, which further impairs female reproduction [8, 10, 14]. Moreover, ER stress participates in LPS-induced immune responses and hormones secretion in male and female reproductive systems [27–30]. Additionally, ER stress can regulate inflammation in some pathological models [32, 33]. However, whether ER stress is involved in LPS-inhibited estradiol (E_2) production, and the relationships between immune responses and ER stress caused by LPS in mouse granulosa cells are unclear. In this study, we aim to define the role of ER stress in LPS-induced immune factors and E_2 secretions in mouse granulosa cells.

Materials and Methods

Animals and chemicals

Immature female Kunming white mice (21–28 days old) were obtained from the Experimental Animal Center of Jiujiang University, China. All mice were fed chow and water daily and housed in a controlled temperature and humidity environment with a 12-h light-dark cycle at 25°C. All procedures were approved by the Committee for Ethical Animal Care and Experimentation of Jiujiang University, China [Approval No. SYXK(GAN)2017-0001].

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. LPS (*E. coli* (O55:B55)) was prepared as stock solutions in distilled phosphate buffer solution (PBS). ER stress inhibitor 4-phenylbutyrate (4-PBA), ER stress agonist thapsigargin (TG), and TLR4 inhibitor resatorvid (TAK-242) were dissolved in anhydrous dimethyl sulfoxide (DMSO) to prepare 7, 5, and 2.5 mM stock solutions, respectively. All stock solutions were stored at –20ºC. The final concentrations of 4-PBA, TG, and TAK-242 for culture were 700 nM, 500 nM, and 250 nM, respectively. Prior to use, they were diluted with the culture medium, and the final concentration of DMSO was 0.1%.

Isolation, culture, and treatment of granulosa cells

Mouse granulosa cells were collected and cultured based on previous reports with minor modifications [34, 35]. The immature female mice were intraperitoneally injected with 8 IU pregnant mare serum gonadotropin (PMSG, Ningbo Sansheng, Ningbo, China) to facilitate granulosa cells proliferation. After 44 h, the mice were sacrificed by decapitation, and their ovaries were quickly removed and placed in PBS containing 80 IU/ml penicillin and 100 mg/ml streptomycin. Granulosa cells were harvested by puncturing individual ovarian follicles with a 27-gauge needle attached to a 1 ml syringe, collected by centrifugation (1000 rpm, 5 min) and cultured in a humidified incubator at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium/ Ham's F 12 nutrient medium (DMEM/F12, Hyclone, USA) containing 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS, Corning, Corning, NY, USA).

After 48 h of initial establishment, the cells were treated with 0.01–10 µg/ml LPS for 24 h to analyze pro-inflammatory cytokine expression (including IL-1β, IL-6, IL-8, and TNF-α), ER stress, and Cyp19a1 expression or for 48 h to detect E_2 production. Meanwhile, an additional group of cells was exposed to 1 µg/ml LPS and 700 nM ER stress inhibitor 4-PBA or 250 nM TLR4 inhibitor TAK-242 for 24 h or 48 h. To investigate whether the ER stress pathway is responsible for LPS-induced E_2 reduction in mouse granulosa cells, the cells were also treated with 500 nM TG with or without 700 nM 4-PBA for 24 h or 48 h. All experiments were performed in triplicate.

Enzyme-linked immunosorbent assay

Cells were seeded in a 24-well plate at 1×10^5 cells/well. After culturing, the cells were counted, and the culture supernatant was centrifuged to remove debris. The concentration of IL-1β (cat. no. JYM0531Mo), IL-6 (cat. no. JYM0012Mo), IL-8 (cat. no. JYM0457Mo), and TNF-α (cat. no. JYM0218Mo) in the culture medium was analyzed at 24 h using commercial enzyme-linked immunosorbent assay kits (ELISA, Ji Yin Mei, Wuhan, China) according to the manufacturer's instructions. The concentration of E2 in the culture supernatant was measured at 48 h by an E_2 ELISA kit (cat. no. JYM0379Mo; Ji Yin Mei), according to the manufacturer's instructions. The differences in intra-assay and inter-assay of all the ELISA kits are less than 9% and 15%, respectively. The optical density at 450 nm of each well was determined using a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis

Mouse granulosa cells were rapidly washed with ice-cold PBS. The lysate was separated from cellular debris by centrifugation at 13,000 rpm for 10 min. After total protein was measured by a bicinchoninic acid (BCA) assay (Nanjing KeyGen, Nanjing, China), the samples were stored at –80°C for subsequent use. Before electrophoresis, the samples were heated to 100°C for 5 min, cooled on ice immediately, and then centrifuged at 12,000 × *g* for 5 min. Each sample was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Boston, MA, USA). After incubation in blocking buffer for 1 h at 37°C, the membrane was incubated with anti-β-actin (1:2000; cat. no. KM9001L, Tianjin Sanjian, Tianjin, China), anti-CHOP (1:1000; cat. no. ab10444, Abcam, Cambridge, UK), anti-GRP78 (1:1000; cat. no. ab32618, Abcam, Cambridge, UK), and anti-Cyp19a1 (1:500; cat. no. sc14245, Santa Cruz, CA, USA) overnight at 4°C. After washing, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase at 37°C for 30 min. Finally, immunoreactive bands were visualized using a Super Signal West Pico kit (Proteintech, Wuhan, China) by the Bio-Rad imaging system (Bio-Rad, CA, USA) according to the manufacturer's instructions. The protein band densities were semi-quantified by densitometric analysis using ImageJ software 1.48 (Bethesda, MD, USA).

Statistical analysis

Data were analyzed by one-way ANOVA, and the analysis was performed using SPSS software (Version 13.0; SPSS, Chicago, IL, USA) with Tukey's post hoc test. $P < 0.05$ was regarded as statistically significant. All data are represented as the mean ± SEM of at least three separate experiments.

Results

LPS induces pro-inflammatory cytokine expression in mouse granulosa cells

The results revealed that the concentrations of TNF-α, IL-1β, IL-6, and IL-8 in culture supernatants were increased in a dose-dependent manner in response to LPS (Fig. 1).

LPS inhibits E_2 production and Cyp19a1 protein expression

To assess the effects of LPS on steroid hormone levels, we measured the concentrations of E_2 in the culture medium after treatment with LPS for 24 h. The results showed that LPS inhibited E_2 production in a dose-dependent manner (Fig. 2A). Similar to E_2 production, LPS suppressed the expression of Cyp19a1 in mouse granulosa cells (Fig. 2B).

LPS induces ER stress in mouse granulosa cells

To assess the effects of LPS on ER stress, the expression levels of ER stress-related proteins were analyzed by western blotting. The results showed that LPS induced the expression of GRP78 and CHOP in a dose-dependent manner. Moreover, 0.01 μg/ml LPS was sufficient to activate GRP78, but the lowest concentration of

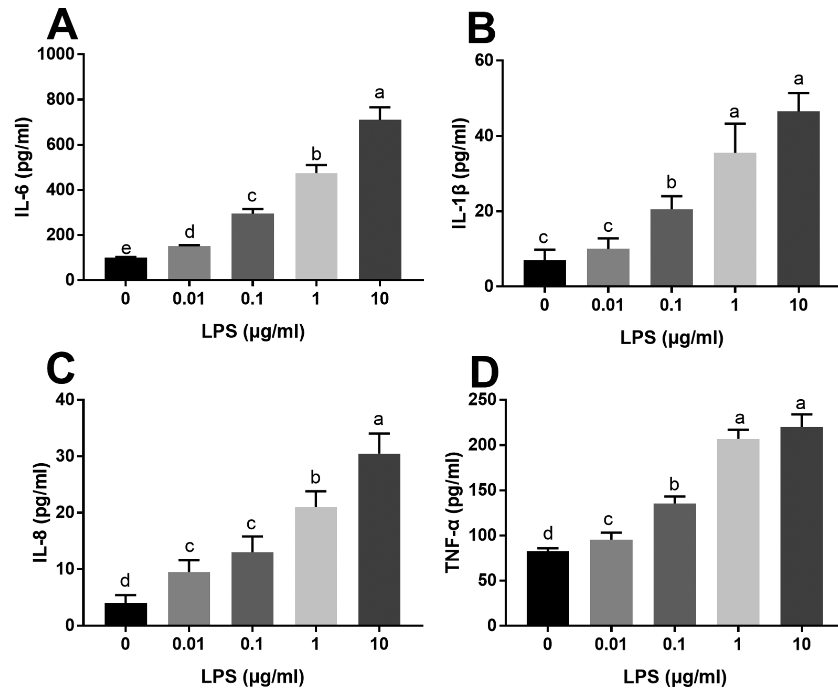

Fig. 1. Expression of pro-inflammatory cytokines (IL-6 (A), IL-1β (B), IL-8 (C), and TNF-α(D)) in mouse granulosa cells after treatment with different concentrations (0–10 μg/ml) of LPS for 24 h. Data are presented as the mean ± SEM of three independent experiments. Bars with different letters are significantly different ($P < 0.05$).

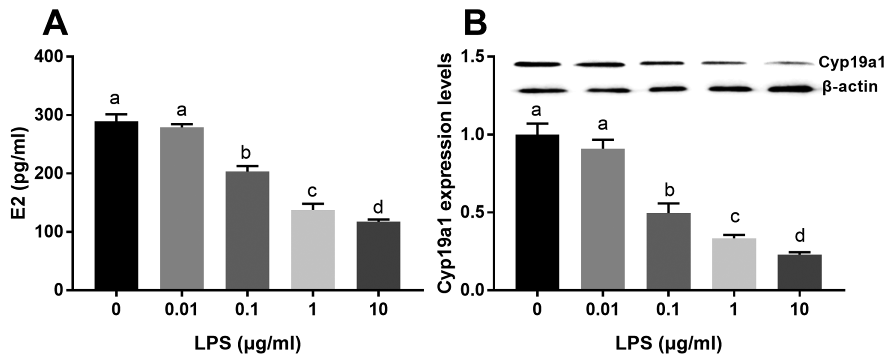

Fig. 2. The effects of LPS on E_2 production (A) and Cyp19a1 expression (B). Data are presented as the mean ± SEM of three independent experiments. Bars with different letters are significantly different ($P < 0.05$). β-actin acted as an internal control.

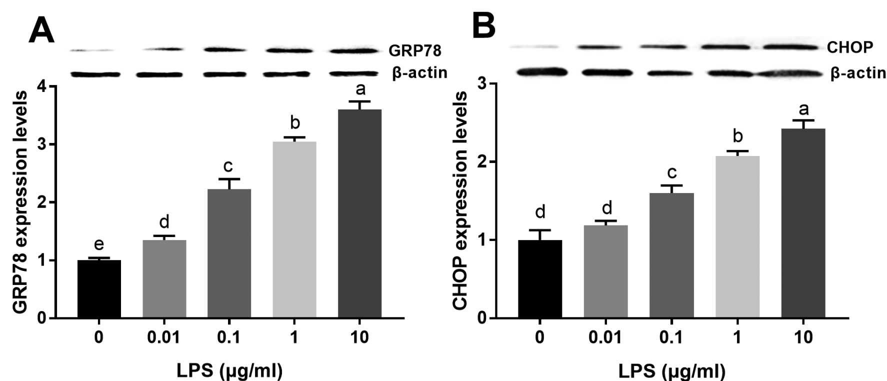

Fig. 3. The effects of LPS on the GRP78 (A) and CHOP (B) protein expression. Data are presented as the mean ± SEM of three independent experiments. Bars with different letters are significantly different ($P < 0.05$). β-actin acted as an internal control.

LPS required for the induction of CHOP expression was 0.1 μg/ml (Figs. 3A and 3B).

ER stress is involved in the LPS-induced E_2 decrease

After mouse granulosa cells were cultured and treated with 500 nM TG or 0.1 μg/ml LPS, ER stress-related markers (GRP78 and CHOP) were activated (Figs. 4A–4C), and E_2 and Cyp19a1 were decreased (Figs. 4A, 4D, and 4E). Moreover, 700 nM 4-PBA inhibited the TG- or LPS- induced GRP78 and CHOP expression (Figs. 4A, 4B, and 4C) and partly rescued the TG- or LPS -induced decrease of Cyp19a1 and E_2 (Figs. 4A, 4D, and 4E). In addition, LPS and TG increased the expression levels of cytokine factors (IL-6, IL-β, IL-8, and TNF-α) and 4-PBA treatment decreased the levels of cytokine factors caused by LPS or TG (Figs. 4F–I).

TLR4 is involved in the LPS-induced E_2 decrease and ER stress

LPS was added to the medium containing 250 nM TLR4 inhibitor TAK-242, and the LPS-induced pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) were decreased by the presence of TAK-242 after culture for 24 h (Figs. 5A–5D). Moreover, TAK-242 reduced the protein levels of GRP78 and CHOP (Figs. 5F and 5G); and the LPS-induced decreases in E_2 and Cyp19a1 were partly inhibited by TAK-242 (Figs. 5H and 5I).

Discussion

Bacterial infections of the uterus or mammary glands perturb reduced ovarian follicle growth and functions, and these effects are mediated directly by bacterial LPS [12, 36–38]. Moreover, LPS activates the pro-inflammatory immune response through TLR4 signaling to decrease Cyp19a1 expression and E_2 production [9, 39]. It is well known that pro-inflammatory cytokines disrupt steroidogenesis in granulosa cells [12, 40, 41]. However, in granulosa cells, a potential role of ER stress in impaired steroidogenesis in response to LPS has received little attention. In this study, we first examined the LPS on pro-inflammatory cytokine expression in mouse granulosa cells. Our study showed that LPS activated pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8, which is in agreement with previous studies showing induction of pro-inflammatory cytokine expression by LPS in bovine granulosa cells [8, 9, 39, 42]. Previous studies have demonstrated that LPS significantly decreases the Cyp19a1 [39] and E_2 production [9, 39] in bovine. A similar decrease in Cyp19a1 expression and E_2 production, upon treating mouse granulosa cells with LPS was observed in this study as well. However, a study from bovine granulosa cells reported that LPS did not affect the E_2 production in granulosa cells isolated from small follicles, but down-regulated the aromatase expression in granulosa cells or large follicles [15].

Previous studies have reported that ER stress has broader physiological roles in the various reproductive processes of female mammals [18], including embryo implantation [43], preimplantation embryonic development [44], follicle atresia [28, 45], and hormone secretion [35, 43, 46]. Related studies confirmed that the long isoform of the ER stress signaling pathway protein CREBZF is closely related to murine implantation and can be up-regulated by E_2 [43]. Another study reported that TLR4-mediated ER stress is involved in LPS induced mouse intestinal stem cell apoptosis [47]. Therefore, we hypothesized that ER stress signaling might be activated by LPS in mouse granulosa cells. To study the effects of LPS on ER stress, the ER stress marker molecules, GRP78 and CHOP, were examined after treatment with various concentrations of LPS. The GRP78 and CHOP were increased in a dose-dependent manner after induction by LPS at 24 h. This result was similar to a previous study that LPS can induce ER stress in intestinal stem cells [47]. Moreover, some studies have reported that ER stress is related to hormone secretion [35, 43, 46]. Next, we assessed the involvement of ER stress in the LPS-induced E_2 decrease. The results showed that LPS inhibited the expression of Cyp19a1 and $E_{2,}$ and this effect was reversed by ER stress inhibitor 4-PBA, suggesting that the ER stress was involved in LPS-induced E_2 down-regulation. There are three ER-localized protein sensors for ER stress: IRE1α, PERK, ATF6. Previous studies have reported that ATF6 is related to both the E_2 production [48] and the inflammatory response [33]. Therefore, we hypothesized that ATF6 might be responsible for the LPS-induced E_2 decrease in the granulosa cells. However, further studies are also necessary

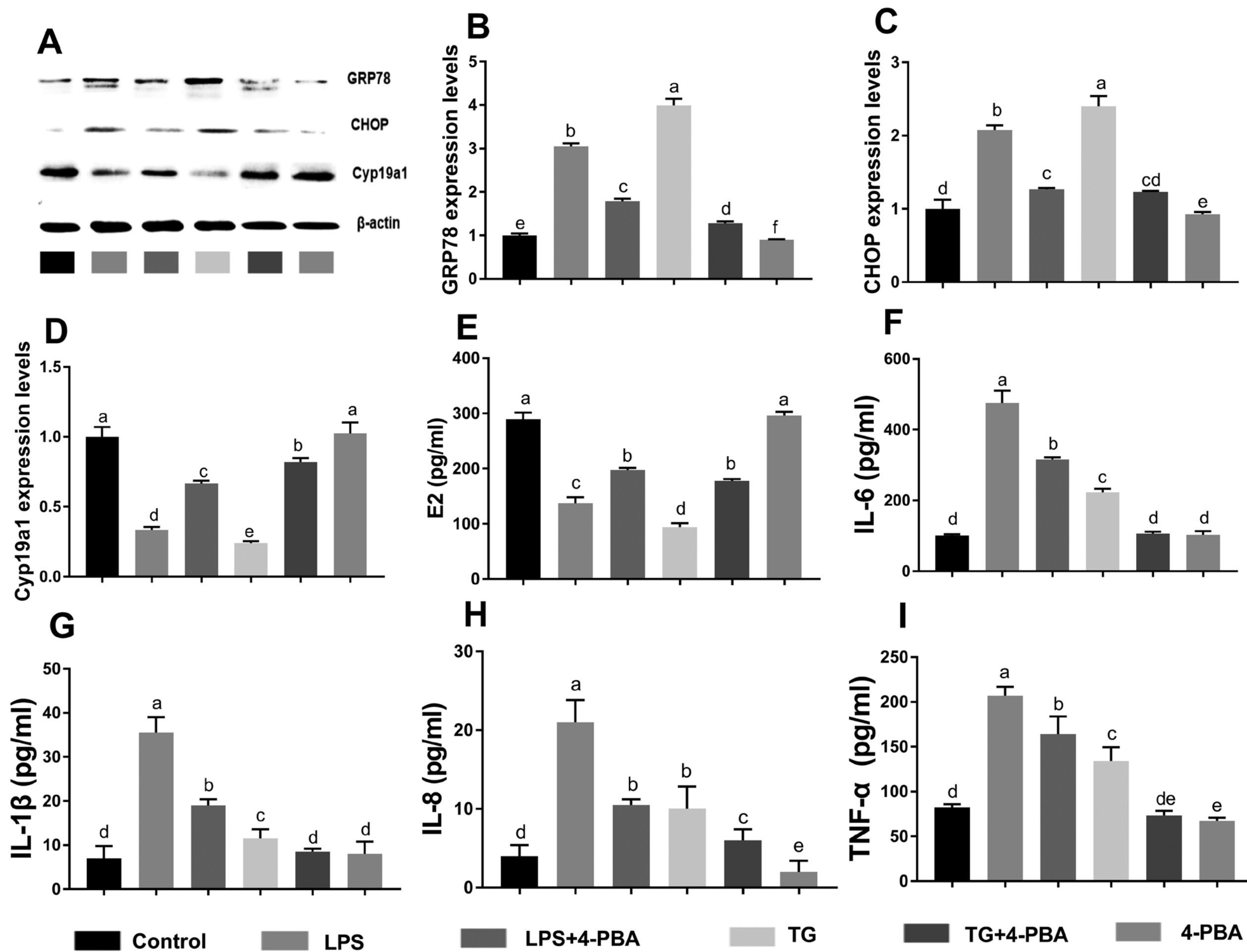

Fig. 4. Relationship between ER stress and LPS-induced E_2 reduction. (A–D) The protein expression levels of GPR78, CHOP, and Cyp19a1 in different groups; (E) E_2 levels of supernatant in different groups; (F–H) The expression of pro-inflammatory cytokines (IL-6 (F), IL-1β (G), IL-8 (H), and TNF-α (I)) in different groups. Different colors are used to represent different groups, TG: Thapsigargin. Data are presented as the mean ± SEM of three independent experiments. Bars with different letters are significantly different ($P < 0.05$). β-actin acted as an internal control.

to determine to test this hypothesis. ER stress, induced by TG, also decreased Cyp19a1 expression and E_2 production. Furthermore, LPS significantly increased the levels of cytokines (IL-6, IL-β, IL-8, and TNF-α), and the increased expressions of cytokine were inhibited by 4-PBA. This result coincides with a previous report showing that LPS decreases the IL-1β level in CHOP-/-mice, indicating that suppression of ER stress inhibits inflammation. The related mechanism in CHOP-/-mice was the low expression of caspase-11 after LPS treatment [49].

Previous studies have reported LPS induces pro-inflammatory cytokines expression in granulosa cells through TLR4 [8, 50], and the inflammatory response is related to ER stress and an E_2 decrease [25]. Therefore, we assumed that TLR4 might contribute to suppression of E_2 production and ER stress in mouse granulosa cells caused by LPS. Our data demonstrated that LPS stimulated pro-inflammatory cytokines as well as expression of GRP78 and CHOP, and reduced E_2. As expected, inhibition of TLR4 by TAK-242 significantly inhibited the LPS-induced inflammatory response, ER stress, and E_2 reduction. These results supported the previous studies in which LPS bound to TLR4 and promoted the expression of pro-inflammatory cytokine expressions in bovine granulosa cells [7–10]. Subsequently, pro-inflammatory cytokines induced ER stress to decrease Cyp19a1 expression and E2 production. As our results showed, blocking TLR4 reduced ER stress, suggesting that the inflammatory response was one of the factors that decreased E_2 production during LPS treatment. Conversely, inhibition of ER stress by 4-PBA attenuated pro-inflammatory cytokines expression, indicating that the inflammatory response and ER stresses interact with each other during the LPS-induced E_2 decrease in mouse granulosa cells. The possible mechanism might be an inflammatory response mediated disruption of ER homeostasis, causing ER stress; the inhibition of the inflammatory response, therefore, suppresses ER stress during LPS treatment, and a persistent ER stress might cause cell dysfunctions that further induces the inflammatory response. Thus, inhibition of ER stress also inhibits the inflammatory response; however, the exact underlying mechanism between them requires further investigation.

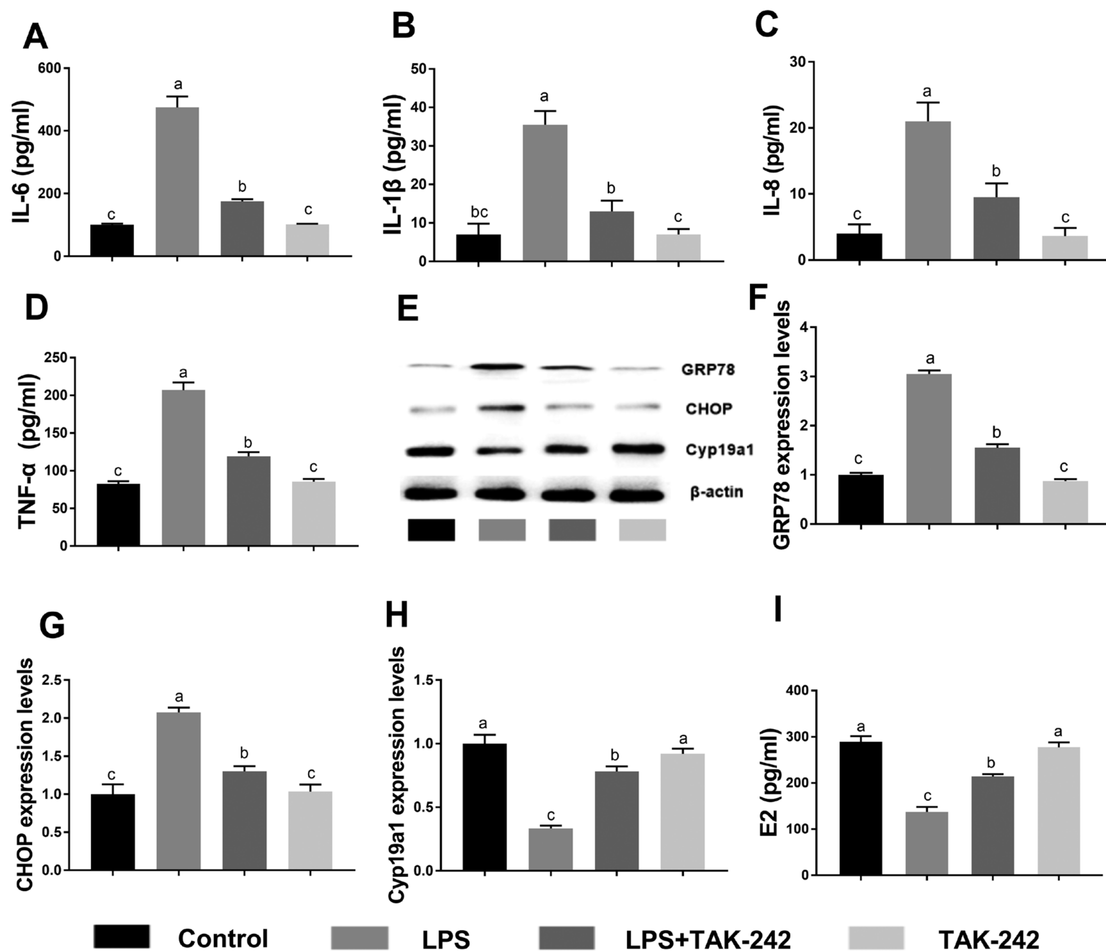

Fig. 5. TLR4 is involved in LPS-induced E_2 decrease and ER stress. (A–C) The concentration of IL-6, IL-1β, IL-8, and TNF-α in the culture media at 24 h; (E–H) The protein expression levels of GPR78, CHOP, Cyp19a1; (I) E_2 levels in the supernatant in different treatment groups. Different colors are used to represent different groups. Data are presented as the mean ± SEM of three independent experiments. Bars with different letters are significantly different ($P < 0.05$). β-actin acted as an internal control.

In overall, our study suggested that LPS treatment induces ER stress and an E_2 reduction in mouse granulosa cells. Inhibiting the inflammatory response by TAK-242 reduced the activation of ER stress and rescued the decrease of E_2. Conversely, inhibition of ER stress by 4-PBA also attenuated pro-inflammatory cytokines expression and the E_2 decrease. Therefore, ER stress plays a vital role in the E_2 reduction caused by LPS in murine granulosa cells, suggesting a cross-talk between the inflammatory response and ER stress during this process.

Conflict of Interest: The authors declare that they have no competing interests.

Acknowledgments

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